ACCOUNTS
of chemical research

Pushing the Sensitivity Envelope of Lanthanide-Based Magnetic Resonance Imaging (MRI) Contrast Agents for Molecular Imaging Applications

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RECEIVED ON SEPTEMBER 3, 2008

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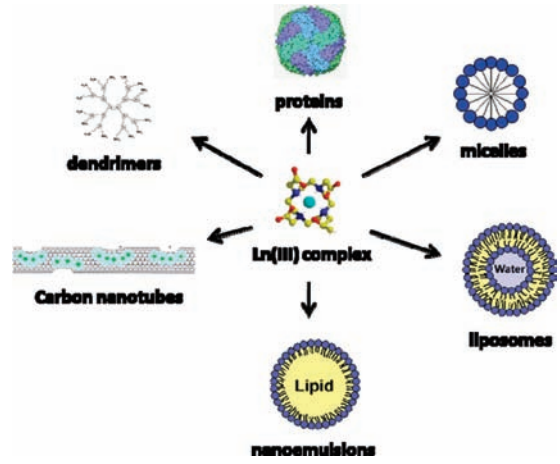


Contrast in magnetic resonance imaging (MRI) arises from changes in the intensity of the proton signal of water between voxels (essentially, the 3D counterpart of pixels). Differences in intervoxel intensity can be significantly enhanced with chemicals that alter the nuclear magnetic resonance (NMR) intensity of the imaged spins; this alteration can occur by various mechanisms. Paramagnetic lanthanide(III) complexes are used in two major classes of MRI contrast agent: the well-established class of Gd-based agents and the emerging class of chemical exchange saturation transfer (CEST) agents. A Gd-based complex increases water signal by enhancing the longitudinal relaxation rate of water protons, whereas CEST agents decrease water signal as a consequence of the transfer of saturated magnetization from the exchangeable protons of the agent. In this Account, we survey recent progress in both areas, focusing on how MRI is becoming a more competitive choice among the various molecular imaging methods.

Compared with other imaging modalities, MRI is set apart by its superb anatomical resolution; however, its success in molecular imaging suffers because of its intrinsic insensitivity. A relatively high concentration of molecular agents (0.01−0.1 mM) is necessary to produce a local alteration in the water signal intensity. Unfortunately, the most desirable molecules for visualization in molecular imaging are present at much lower concentrations, in the nano- or picomolar range. Therefore, augmenting the sensitivity of MRI agents is key to the development of MR-based molecular imaging applications. In principle, this task can be tackled either by increasing the sensitivity of the reporting units, through the optimization of their structural and dynamic properties, or by setting up proper amplification strategies that allow the accumulation of a huge number of imaging reporters at the site of interest.

For Gd-based agents, high sensitivities can be attained by exploiting a range of nanosized carriers (micelles, liposomes, microemulsions, and the like, as well as biological structures such as apoferritin and lipoproteins) properly loaded with Gd-based chelates. Furthermore, the sensitivity of Gd-based agents can be markedly affected either by their interactions with biological structures or by their cellular localization.

For CEST agents, a huge sensitivity enhancement has been obtained by using the water molecules contained in the inner cavity of liposomes as the exchangeable source of protons for magnetization transfer. Several "tricks" (for example, the use of multimeric lanthanide(III) shift reagents, changes in the shape of the liposome container, and so forth) have been devised to improve the chemical shift separation between the intraliposomal water and the "bulk" water resonances.

Overall, excellent sensitivity enhancements have been obtained for both classes of agents, enabling their use in MR molecular imaging applications.

Published on the Web 06/17/2009 www.pubs.acs.org/acr
10.1021/ar800192p CCC: $71.50

Introduction

To enhance the role of MR in molecular and cellular imaging investigations, it is necessary to improve the sensitivity of the involved probes. The sensitivity issue deals with either the intrinsic relaxivity of a given agent or specific features associated with the biological characteristics of the targeting experiments. As far as the intrinsic sensitivity is concerned, the chemist has to design systems endowed with structural and dynamic properties that lead to optimized values for those parameters that are relevant for a given contrast enhancing mechanism. Much work has been done in the last two decades to design structures with high relaxivity; however the theoretical maximum efficiency expected from the optimization of all the parameters involved in the paramagnetic relaxation has not yet been reached. In general, to attain the MR visualization of a given cellular target, one may rely on the exploitation of high-capacity carriers that can be loaded with a huge number of imaging reporters, thus allowing the internalization of a sufficient amount of contrast agent in the target cells. This approach has required the use of macromolecular and supramolecular systems that chemists have primarily developed for the setup of drug delivery experiments (Figure 1). Depending on their characteristics, the carriers may be loaded with commercial Gd agents or specifically tailored media may need to be prepared in order to be compatible with the structure of the carrier.

Interestingly, other than the molecular parameters, it has been envisaged that, for some types of nanocarriers, other contributions may be important for the creation of longitudinal relaxivity values that would be much higher than the ones that are currently available. The longitudinal relaxivity expresses the increase in water proton $1/T_1$ per millimolar concentration of paramagnetic agent. Representative examples are Gd-loaded carbon nanotubes, for which a relaxivity of ca. 200 mM_{Gd}^{-1} s^{-1} has been reported[1] or Gd-loaded apoferritin, for which a 20-fold enhancement has been observed upon the entrapment of Gd−HPDO3A (Acid 1,4,7-Tris(carboxymethyl)-10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecanoic) in the inner cavity of the protein.[2] An in-depth understanding of the origin of these enhancements may provide useful indications to help guide the design of even more sensitive Gd-based agents.

This Account surveys recent work on paramagnetic Ln(III)-based MRI agents and focuses on making MRI more competitive in the arena of molecular imaging modalities.

Sensitivity Issue for Gd(III)-Based Agents: General Considerations

The sensitivity of a given paramagnetic complex is closely related to its relaxivity (usually measured at 0.5 T and at 298 or 310 K). Relaxivity is the result of a complex interplay between the paramagnetic center's structural, dynamic, and electronic properties. Good estimates of the determinants of the relaxivity of a given paramagnetic complex can be

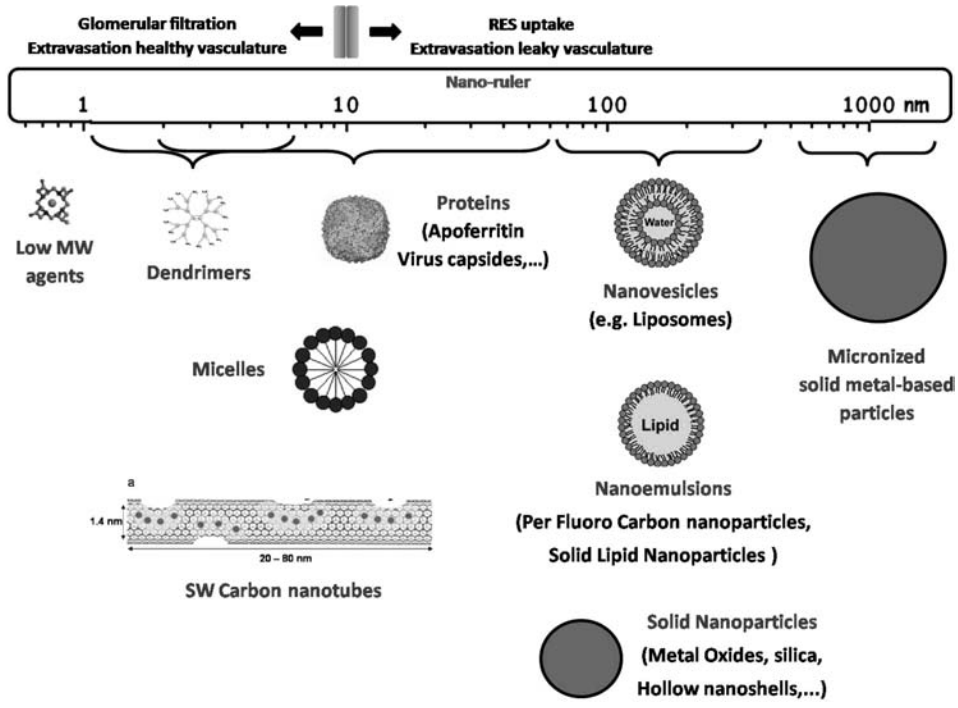


FIGURE 1. Average size of the nanocarriers considered so far for developing highly sensitive Gd(III)-based MRI agents.

obtained from the analysis of relaxivity data as a function of the applied magnetic field (NMRD profiles). This method gave rise to a prediction that high relaxivities could be attained, at the clinical field strength of 0.5−1.5 T, by lengthening the molecular reorientational time, τ_R.[3] Therefore, macromolecular systems were addressed by either the covalent or noncovalent binding of paramagnetic chelates to slowly moving substrates. Human serum albumin (HSA), the most abundant protein in serum, has been an important target for the development of a high-sensitivity agent for magnetic resonance angiography.[4] The relaxivities observed for HSA adducts were often lower than expected as a consequence of the increase in the residence lifetime (τ_M) of the water molecule at the metal site.[5] Moreover, it has been recently shown that, in addition to steric hindrance at the coordination metal site, τ_M is strongly modulated by the charge at the metal ion, thus reflecting changes that occur at the level of the donor atoms of the coordination cage.[6] Thus, the formation of hydrogen bonds between the carboxylic groups of the ligand and basic amino acids on the surface of the protein may result in a significant release of the residual electric charge on the complex that, in turn, yields a marked elongation of the exchange lifetime of the coordinated water molecule(s). Of course, the coordinated water itself may be involved in H-bond formation that directly affects its dissociation kinetics. Thus, upon binding to a protein (or more generally to a macromolecular substrate), the relaxivity of a given paramagnetic complex may vary as a consequence not only of τ_R but also of τ_M. Both parameters are site-dependent. In fact, the complex very seldomly assumes the molecular reorientational time of the macromolecule, but rather it experiences a faster reorientation motion superimposed onto the slower motion of the supramolecular adduct. Clearly, the "pincer attack" of the complex at two binding sites endows the system with the τ_R of the macromolecule as shown by Caravan and co-workers.[7]

Another issue related to the overall sensitivity of Gd(III)-based probes is the "clustering" effect. Some years ago, Merbach and co-workers showed that the dilution of Gd chelates with diamagnetic Y(III) complexes at the surface of micelles resulted in an enhanced relaxivity.[8] The observed behavior was ascribed to the occurrence of dipolar interactions among the Gd(III) ions able to enhance the electronic relaxation rate that, in turn, causes the decrease of the observed relaxation rate in neat Gd-containing micelles.

The link between relaxivity and local concentration has also been recently tackled by Sherry and co-workers, who used a Gd−DO3A−peptide chimera able to bind anti-FLAG monoclonal antibodies.[9] The relaxivity of the supramolecular Gd−DO3A−antibody adduct was only ca. 17 mM^{-1} s^{-1} (likely due to a long τ_M), and the system was MR silent at concentrations below ~9 μM. However, when the antibodies were clustered together to form microdomains of high local concentration at the surface of agarose beads, the MR detection limit of the agent improved down to 4 μM. On the basis of simulated data, the authors concluded that the detection limit of a single highly efficient low molecular weight Gd(III) agent (relaxivity of 100 mM^{-1} s^{-1}) targeted in a protein microdomain is ca. 0.7 μM at 9.4 T, thus suggesting that small sized agents could still have a future in MR molecular imaging applications.

Gd-Loaded Nanocarriers

Several systems containing from tens to hundreds of thousands of Gd chelates have been reported in recent years. These systems have been designed to deliver a high payload of Gd complexes to targeting sites. This involves the use of a number of nanocarriers that chemistry has made available, such as dendrimers, micelles, liposomes, solid lipid nanoparticles, nanoemulsions, carbon nanotubes, and silica-like particles, as well as biologically derived systems such as apoferritin, lipoproteins, and viral capsids (Figure 1).[10,11]

A major property to be considered in the choice of the most suitable nanocarrier is its size, because this can strongly affect the biodistribution of the system and, consequently, whether the probe can arrive at the target site. Size is very important for the relaxometric properties of the system, both because size and payload are usually directly proportional and because the size and the typology of the carrier can significantly modify the structural and dynamic parameters that control the relaxivity of the Gd complexes. In most of the nanocarriers, the Gd(III) agent needs to be suitably functionalized in order to be loaded onto the outer surface of the system through proper covalent linkages (e.g., dendrimers or silica particles) or through strong noncovalent self-assembling or hydrophobic aggregations (e.g., micelles, solid lipid nanoparticles, nanoemulsions, lipoproteins, or carbon nanotubes). On the other hand nanovesicular carriers, like apoferritin and liposomes, can be loaded with hydrophilic Gd agents without any specific chemical derivatization. The binding of the paramagnetic chelate to the nanocarrier yields a relaxivity enhancement that is primarily due to the restricted rotational mobility (τ_R elongation) of the complex. In principle, one aims at endowing the Gd chelate with the same τ_R value as the carrier particle, but often, this is not the possible because internal motions overlap with the slow tumbling motion of the carrier. This task is more easily accomplished in case of non-

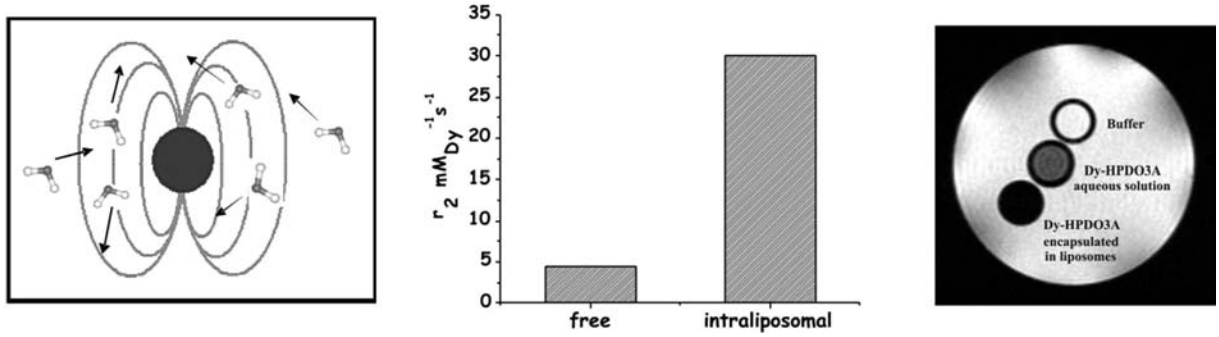


FIGURE 2. Schematic view (left) of the interactions between the local magnetic fields generated by a a paramagnetic nanoparticle and the water molecules diffusing around it, effect (middle) of the liposome encapsulation of a paramagnetic complex (Dy−HPDO3A) on the transverse relaxation rate at 7 T and 312 K, and *in vitro* RARE $T_{2,\text{weighted}}$ MR image (right) obtained at 7 T and 312 K.[16]

covalent supramolecular adducts where the binding of the paramagnetic agent to the carrier can occur without the presence of the spacer, which is usually responsible for the fast motion of the chelate. For nanovesicular carriers, the relaxivity of the encapsulated Gd complex can increase when specific contributions to the relaxivity take place (e.g., for apoferritin thanks to the contribution arising from the mobile protons of the protein) or even decrease, as typically occurs in case of liposomes, where the relaxivity of the entrapped complex can be "quenched" by the low water permeability of the liposome membrane.[12] Such apparent disadvantages have been successfully exploited in the development of MRI nanoprobes whose contrast is made sensitive to (i) changes in the water permeability of the liposomal bilayer, such as occurs upon heating (development of MRI thermometers),[13] or (ii) the integrity of the vesicle (visualization of drug delivery/release processes).[14]

Paramagnetic Liposomes as MRI Agents

Liposomes are a very versatile class of nanocarriers that are widely used in the pharmaceutical field. When an imaging probe is incorporated into the phospholipid bilayer approximately half of its metal centers are exposed to the exterior of the vesicle, whereas the remaining ones point inward toward the cavity. On the basis of the water exchange rate across the liposome membrane, it is expected that these two portions may contribute differently to the overall relaxivity of the nanoprobe. Recently, Muller and co-workers proposed a smart method of separating the two contributions by comparing two liposome preparations: one fully loaded with an amphiphilic Gd(III) complex and the other containing the paramagnetic complex only on the outer side.[15] The preparation of the latter system used liposomes fully loaded with the corresponding La(III) complex in which the external ions were successively replaced by paramagnetic Gd(III) ions by exploiting the higher thermodynamic stability of the latter system. The reported results supported the view that the relaxivity contribution deriving from the complexes in the inner portion of the vesicle is dependent on the water permeability and, in turn, on the formulation of the liposome membrane.

Liposomes also allow a simultaneous loading of the imaging probe in the cavity and into the membrane, thus (i) considerably increasing the overall payload of imaging probes and (ii) providing the nanoprobes with unique relaxometric properties. Although this approach has not yet been exploited in the field of T_1-agents, it has been demonstrated as being advantageous for the development of highly sensitive T_2-susceptibility agents as a possible alternative to the well-established class of iron oxide nanoparticles.[16] In fact, the compartmentalization of paramagnetic Ln(III) complexes in the nanovesicle can generate a strong T_2-specific relaxivity contribution arising from the local inhomogeneous magnetic fields created by the nanoparticle. This compartmentalization effect, which is proportional to the magnetic field strength, is clearly illustrated in Figure 2.

The sensitivity of such systems is dependent on the intrinsic paramagnetism (described by the effective magnetic moment, μ_{eff}) of the Ln ion (Dy(III) is the most efficient) and on the overall concentration of the paramagnetic centers compartmentalized in the vesicle. Hence, the incorporation of amphiphilic Dy(III) complexes in the liposome bilayer, in addition to the encapsulation of a high amount of a hydrophilic Dy(III) agent, yields a marked sensitivity enhancement that allowed the MRI visualization of cellular epitopes, such as membrane transporters, present at very low concentrations.[17]

Sensitivity Issues in Cellular Systems

Because molecular imaging is mainly devoted to the visualization of molecules or events that occur at cellular level, it is

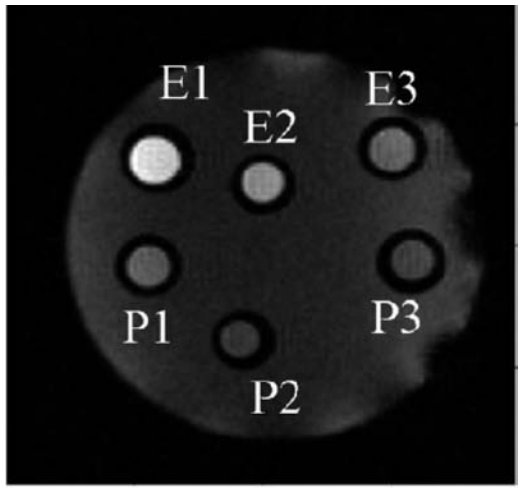


FIGURE 3. T_{1w} spin echo image (TR/TE/NEX, 100/3.2/24) of a phantom containing HTC (rat hepatoma tumor cells) cells dispersed in agar and labeled with Gd−HPDO3A internalized by pinocytosis (P) or electroporation (E). Cell densities (cells/μL): (1) 10 000, (2) 5000, and (3) 1000.

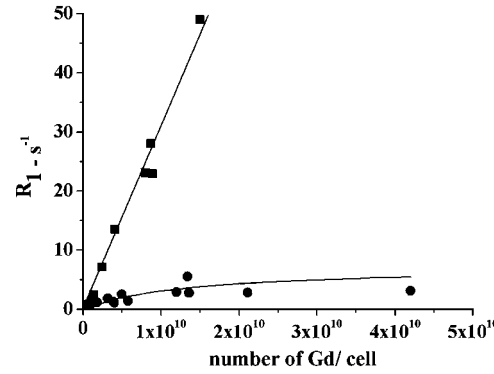


FIGURE 4. Water proton longitudinal relaxation rates (20 MHz, 25 °C) for HTC cells labeled with Gd−HPDO3A by pinocytosis (●) or by electroporation (■). The solid curves represent the best data fitting to the hyperbolic (pinocytosis) and linear (electroporation) behavior.

of paramount importance to understand the effects on the attainable relaxivity that are associated with cellular uptake.

In principle, one may expect the MR imaging reporter to be located (a) at the outer cellular membrane, (b) in endosomal vesicles (upon pinocytosis, receptor-mediated endocytosis, or phagocytosis), and (c) in the cytoplasm, either free or associated with organelles or even at the nucleus. As far as the imaging result is concerned, it is important to recall that the typical size of a MRI voxel includes thousands of cells and the MRI signal receives contributions from water protons from a variety of biological compartments (vascular, extracellular, intracellular, and intraorganelles). Thus, the longitudinal relaxation process of the ^{1}H signal is expected to be monoexponential only in the presence of fast exchange across the membranes that separate the biological compartments or, in the case of similar T_1 values, among the different environments. The heterogeneous distribution of contrast agent molecules in the compartments may cause profound effects on the overall MR response arising from a single voxel. As an illustrative example, Figure 3 compares T_{1w}-MR images of cellular pellets labeled with Gd−HPDO3A by pinocytosis or electroporation.[18]

In the former case, the paramagnetic complex is entrapped in cellular endosomes (small vesicles that arise from the invagination of the cellular membrane, thus encapsulating the portion of extracellular fluid containing the labeling agent) whose volume fraction may account for ca. 1% of the overall cellular volume. As far as the MR signal is concerned, the very short T_1 of the endosomal water protons can be transferred to the other voxel compartments only if two barriers, the endosomal and the cellular membranes, are crossed. In qualitative terms, if the relaxation time of the endosomal protons is much shorter than their lifetime in the vesicle, the presence of the labeling agent can hardly, or cannot at all, be "felt" by the water molecules in the other compartments because the relaxation enhancement is largely lost during the time required for the membrane crossing. On the other hand, when the paramagnetic agent is distributed in the cytosol, a condition that occurs when the electroporation route is used, its presence can directly affect most of the water protons in the voxel, and in addition, the intracytosolic concentration of the labeling agent cannot be high enough to prevent the transfer of the relaxation enhancement to the extracellular compartment. Thus, upon comparison of the relaxation rates obtained from cellular pellets labeled by electroporation and pinocytosis as function of the amount of internalized Gd (Figure 4), a characteristic "quenching" of the attainable relaxation rate is observed, above ca. 10^{10} Gd/cell, for the cells labeled by pinocytosis.

Work on promoting the endosomal escape of the contrast agent molecules in order to overcome this "quenching" is currently underway. A procedure that appears to work particularly well consists of inserting a photoactivable molecule into the endosome membrane. Upon UV exposure, porphyrins are known to generate singlet oxygen, which can locally react with the components of the endosomal membrane, thus destabilizing the phospholipid bilayer.[19] As shown in Figure 5, cells that entrap different amounts of Gd−HPDO3A in their endosomes clearly show the endosomal escape of their payload when subjected to UV irradiation. This results in a marked enhancement in the relaxation rate. Other approaches, based on the use of membrane-penetrating peptides that may be suitably activated by the acidic pH of late endosomes, are currently in progress.

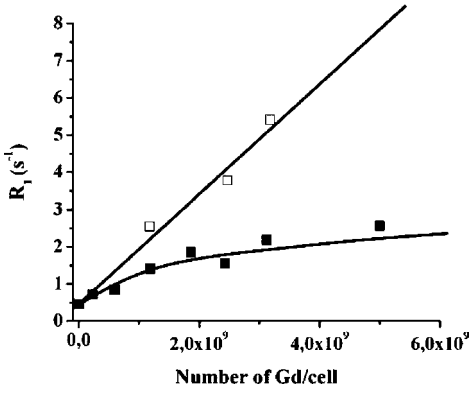


FIGURE 5. Water proton longitudinal relaxation rates, at 300 MHz and 25 °C, of HTC cells labeled with Gd−HPDO3A. The cells have (□) or have not (■) been irradiated by UV light. The solid curves represent the best data fitting to the hyperbolic (pinocytosis) and linear (electroporation) behavior.

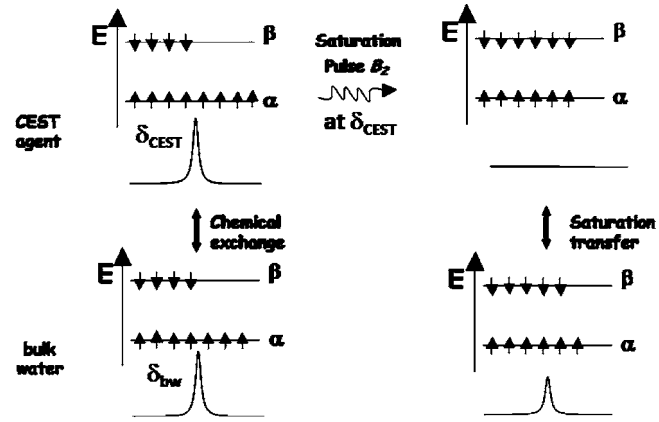


FIGURE 6. Schematic picture illustrating the saturation transfer process mediated by chemical exchange. The application of the saturation field B_2 at the resonance frequency of the spins of the CEST agent equals their populations. The chemical exchange perturbs the Boltzmann equilibrium of the bulk water protons due to the transfer of an excess of CEST spins from the high energy level. The CEST contrast arises from the consequent decrease in the spin population difference between α and β states.

Sensitivity Issues in the CEST Approach

The novel landscape of molecular imaging prompted the search for new paradigms in the design of MR-imaging reporters. Whereas the currently used agents only act on the relaxation properties of water protons, much attention has been addressed to the exploitation of frequency, the key parameter in the NMR phenomenon. One feasible approach is the development of agents containing naturally highly sensitive (e.g., ^{19}F) or hyperpolarized (noble gases or ^{13}C-based molecules) heteronuclei. In this way, MR images with no background signal can be obtained, but issues like the attainment of sufficiently high contrast-to-noise ratio or the setup of experiments with the proper temporal resolution could somehow limit the great potential of this approach. Hence, the possibility of designing ^{1}H-MRI protocols based on a "frequency-dependent" contrast mechanism remains a very important task.

A convenient way of generating a "frequency-encoding" contrast is to exploit the well-known magnetization/saturation transfer (ST) phenomena. The basic rationale is to deal with a system containing at least one set of protons resonating at a different resonance frequency with respect to bulk water, whose exchange with the latter pool, k_{ex}, is slow on the NMR/MRI time scale. In other words, k_{ex} has to be smaller than the difference in the resonance frequency between CEST and bulk water pools ($k_{ex} < \Delta\omega$, $\Delta\omega = 2\pi(\delta_{CEST} - \delta_{bw})$). Upon irradiation of the CEST probe's mobile protons by a proper radiofrequency field (of intensity B_2), saturated magnetization is transferred, via chemical exchange, to the bulk water resonance, thus decreasing the MRI signal and generating the so-called CEST contrast. The basic CEST experiment is sketched in Figure 6.

With respect to conventional MRI agents, CEST probes allow the design of MRI protocols in which the contrast can be generated "at will" only when the proper frequency corresponding to the exchangeable protons of the CEST agent is saturated. As a consequence, the precontrast image can be recorded almost simultaneously to the postcontrast ones as their acquisition simply differs from the on/off switch of the irradiation field. Even more importantly, this new approach offers the intriguing possibility of visualizing more than one agent in the same region, thus opening new exciting perspectives for the MRI applications in the biomedical field. In addition, CEST agents were also very soon identified as candidates for designing concentration-independent responsive probes, primarily by exploiting a ratiometric approach, based on the detection of CEST contrast from two pools of magnetically nonequivalent mobile protons belonging to the same agent or to different agents with similar biodistributions.[20,21]

Since CEST agents were first proposed in 2000,[22] sensitivity has been recognized as one of the major issues for *in vivo* applications of such systems. Among the many variables that control the efficiency of the saturation transfer process, the exchange rate of the mobile proton of the agent, k_{ex}, has received much attention. This is because k_{ex} can be modulated by changing the chemical nature of the proton site and, in addition, is dependent on physicochemical variables, like temperature or pH, thus making CEST agents potential responsive probes.

In principle, ST efficiency is directly correlated to k_{ex}, but when the exchange rate approaches the $\Delta\omega$ value, the CEST effect decreases and eventually disappears at coalescence

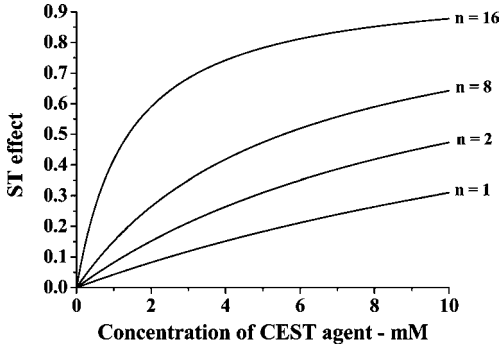


FIGURE 7. Simulated concentration dependence of the ST efficiency as a function of the number of equivalent mobile protons per CEST molecule.

($k_{ex} \geq \Delta\omega$). Furthermore, the complete saturation of fast-exchanging spins requires high-intensity B_2 fields (the optimal B_2 value can be calculated from $B_2 = k_{ex}/(2\pi)$). This condition may yield undesirable results, either because it can reduce the ST efficiency due to spillover effects on the bulk water signal or because it could exceed the maximum energy allowed to be deposited on tissues.

A ST enhancement can also be obtained by increasing the number of magnetically equivalent or pseudoequivalent (narrow range of $\Delta\omega$ values) mobile protons per molecule of CEST agent. This concept is graphically illustrated in Figure 7 where the reported curves were simulated by using the model developed in the case of weak B_2 fields (no spillover effects on bulk water).[23]

On this basis, the sensitivity, herein expressed as the concentration of the CEST system necessary to detect a given ST effect (usually 0.05 or 0.1 are considered sufficient), is inversely related to the number of saturated mobile protons.

The data reported in Figure 7 indicate that the minimum concentration of mobile protons needed to detect a CEST contrast is on the order of a few millimolar (i.e., ca. 10^5 times smaller than the concentration of bulk water), but the minimum concentration of the CEST system will be dependent on the number of "CEST-active" protons per single molecule of agent. Actually, the detection limit for small sized CEST systems (amino acids, heterocyclic compounds, sugars, paramagnetic metal complexes) that contain up to 10 protons/molecule lies in the millimolar range, regardless of the dia- or paramagnetic nature of the system. A considerable sensitivity gain was achieved by using macromolecular CEST systems, either diamagnetic, like poly(amino acid)s, dendrimers,[23] or RNA-like polymers,[24] or paramagnetic.[25,26] As expected, the presence of thousands of mobile protons per macromolecule allowed enhancement of the sensitivity by ca. 3 orders of magnitude from the milli- to the micromolar scale. Following the same line of reasoning, nanosystems should provide a further significant sensitivity gain. This would be proportional to the increase of the number of mobile protons that are associated with a given system.

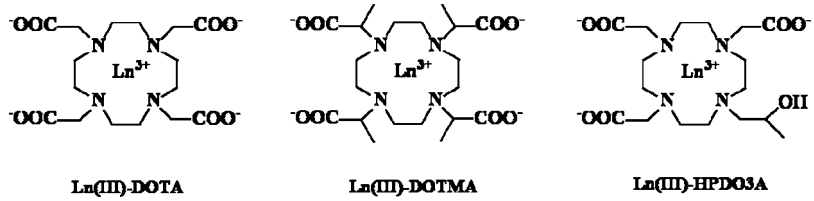
LipoCEST Agents

Nanovesicles that are able to entrap solvents in their cavity, such as liposomes, represent an ideal system for developing highly sensitive CEST agents. In fact, the number of water protons inside the liposome cavity is several orders of magnitude larger (10^6-10^9 depending on the size of the vesicle) than macromolecular systems. In addition, liposome membranes are water-permeable and the exchange rate of the intraliposomal water protons (i.e., the k_{ex} of the CEST pool) can be properly modulated by changing the lipid membrane composition. Furthermore, the exchange rate of the water protons in the cavity is not very fast (10^2-10^3 s^{-1}), and therefore, a high ST efficiency can be attained without using high intensity B_2 fields. Nevertheless, in order to act as a CEST agent, the resonance frequency of the intraliposomal water protons must be sufficiently different from the bulk water protons in which the liposomes are suspended, and this task was successfully accomplished by encapsulating a paramagnetic lanthanide(III)-based shift reagent (SR) in the liposome cavity.

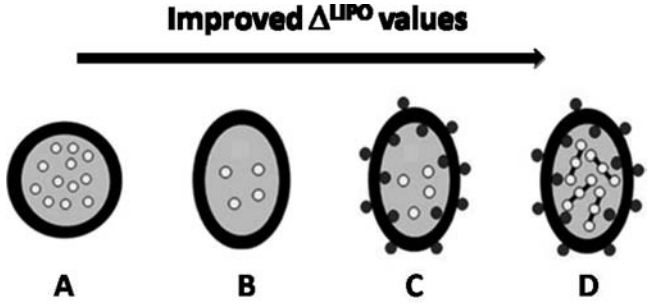
Paramagnetic lanthanide(III) complexes are recognized as the best class of shift reagents, especially those with a high-symmetry macrocyclic structure, like $[LnDOTMA]^-$, $[LnDOTA]^-$, or [LnHPDO3A] (Chart 1), where one fast-exchanging water molecule is axially coordinated to the metal center, thus maximizing the induced paramagnetic shift that is dominated by the dipolar contribution.

Tm(III) and Dy(III) are the most used Ln(III) ions by virtue of their high values (although positive for one and negative for the other) of the dipolar constant, C_D, that directly affects the dipolar contribution. Interestingly, these ions, when they are complexed by the same ligand, shift in opposite directions, and this behavior expands the range of the saturation frequency offset values that are accessible for these nanoprobes. The liposome encapsulation of a high amount (ca. 200 mM) of the SRs reported in Chart 1 induces paramagnetic shift offset of about ± 4 ppm (Δ^{LIPO}). The sensitivity of these nanosystems, dubbed LipoCEST, is very high: subnanomolar amounts of vesicles are sufficient to generate a ST of ca. 10% *in vitro*.[27] Since the efficiency of the saturation transfer can be limited by the occurrence of spillover effects that are to be expected for saturation frequency offsets close to the resonance frequency of bulk water (associated with the use of high-intensity B_2 fields), the development of LipoCEST agents with highly

CHART 1. Macrocyclic Ln(III)-Based Complexes Typically Used as Shift Reagents for Liposome-Based CEST Agents



SCHEME 1. Different families of liposome-based CEST agents[a]



[a] A, spherical liposomes; B, C, and D, nonspherical liposomes; white circles, mononuclear hydrophilic SR (in D polynuclear); dark filled circles, amphiphilic SR.

shifted intraliposomal water protons is very beneficial to the improvement of the potential of such systems.

Two approaches have been adopted to achieve this task: (i) exploiting the additional shift contribution that arises from bulk magnetic susceptibility (BMS) effects and (ii) increasing the concentration of paramagnetic centers encapsulated in the liposome cavity. The former route requires the compartmentalization of the paramagnetic agent in nonspherical vesicles (Scheme 1B,C,D).

Liposomes are flexible objects, and due to the water permeability of their membrane, they are sensitive to osmotic forces. When suspended in a hyperosmotic medium, liposomes react by shrinking themselves, leaking water, and changing shape. Hence, a liposome encapsulating a hypotonic solution of a SR (which is better when neutral, as in the case of [LnHPDO3A]) and dialyzed against an isotonic buffer (a step necessary for the separation of the nonencapsulated SR) will not be spherical anymore (Scheme 1B). As a consequence, the BMS contribution will be operative and the Δ^{LIPO} offset will increase. In addition, the osmotic shrinkage concentrates the SR in the liposome aqueous core, thus also enhancing the extent of the dipolar contribution. A nice demonstration of this effect was obtained by encapsulating a Gd(III) complex ([GdHPDO3A]), for which the dipolar contribution is null (C_D for Gd is zero). The observation of the signal of the intraliposomal water protons at ca. 7 ppm from the bulk and the detection of a CEST effect upon saturation, clearly highlights the potential of this approach.[28] Of course, when the BMS contribution is added to the dipolar one (for instance, by encapsulating [TmHPDO3A]), a further increase in the $\Delta_{intralipo}$ values is obtained.[29]

A further increase in Δ^{LIPO} can be achieved by incorporating an amphiphilic SR in the bilayer. This approach (Scheme 1C) has two main advantages: (i) it increases the overall amount of paramagnetic centers in the liposome cavity with the consequent increase in both dipolar and BMS contributions, and (ii) it strongly affects the magnetic anisotropy of the liposome membrane, thus favoring the perpendicular alignment of the vesicles within the static magnetic field of the spectrometer. Since the orientation of the vesicles can modulate the sign and the magnitude of the paramagnetic shift, Δ^{LIPO} values for these systems are strongly dependent on the magnetic properties of the incorporated SR.[30]

The number of paramagnetic centers encapsulated in the aqueous core of a liposome can be further increased by using neutral polynuclear hydrophilic SRs (Scheme 1D). In fact, because the maximum concentration allowed is limited by osmotic rules, neutral multimers increase the overall payload of paramagnets inside the vesicle.[31]

Actually, the spectra reported in Figure 8 indicate that the use of bi- or trinuclear SRs increases the $\Delta_{intralipo}$ values with respect to a mononuclear (here [TmHPDO3A]) agent.

By use of all the strategies mentioned above, it is now possible to prepare liposome-based CEST agents with Δ^{LIPO} values in the interval $\pm$60 ppm. This result is relevant for the sensitivity issue, even if for osmotically shrunken liposomes the advantage of saturating far from the bulk water resonance is partly counterbalanced by the decreased number of intraliposomal water protons that can be saturated due to the osmotic shrinkage. Nevertheless, the current range of available Δ^{LIPO} values makes these nanosystems promising candidates for the multiple detection of CEST agents, as has already been demonstrated on an *ex vivo* model.[32]

Conclusions

To overcome the intrinsic insensitivity of MRI with respect to other competitive imaging modalities, it is necessary to implement suitable amplification procedures that lead to the accu-

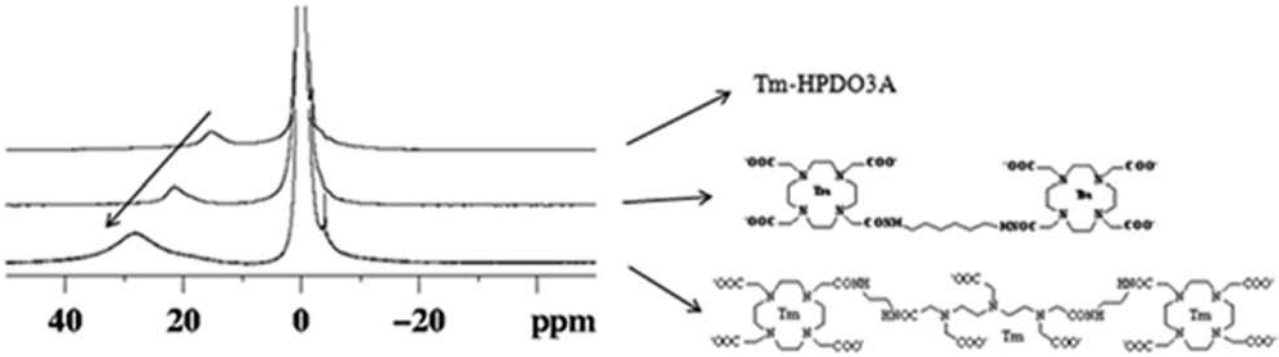


FIGURE 8. ^{1}H NMR spectra (14.1 T, 25 °C) of aqueous suspensions of osmotically shrunken liposomes incorporating the same Tm-based amphiphilic SR and encapsulating the same amount of the hydrophilic SRs shown on the right. The broad peaks correspond to the intraliposomal water protons. For more details, see ref 31.

mulation of the imaging reporters at the site of interest. It has been calculated that the MRI visualization of cells labeled with Gd(III) complexes requires 10^7-10^9 metal chelates per cell. This task can be tackled either by using nanosized carriers or by exploiting high-capacity transporting systems that allow the cellular uptake of suitably functionalized complexes. The understanding of the relationships between the attainable relaxivity and the characteristics of the probe and its interferences with the biological environment is of outstanding importance for designing and assessing molecular imaging protocols. Some of the achievements made in the field of Gd(III) chelates are translatable to the emerging class of CEST agents, although their particular characteristics often necessitate the search for novel approaches to the sensitivity issues. LipoCEST agents display excellent sensitivities because they use the water molecules contained in the inner cavity of liposomes as a source of mobile protons. The frequencies of these entrapped protons are then properly shifted by the addition of a shift reagent. Moreover, for *in vivo* work, it is particularly important to deal with LipoCEST agents displaying a very large separation between the resonances of intraliposomal water and the bulk signal. This task has been successfully tackled by generating nonspherical liposomes in which the intraliposomal water resonance receives a substantial contribution from the magnetic susceptibility effects.

In summary, on the basis of the progress made in recent years in the sensitivity issue, both Gd- and CEST-agent-based protocols may be considered part of the overall armory in the molecular imaging tool set.

Economic and scientific support from MIUR (FIRB RBNE-03PX83_006, FIRB RBIP06293N, and PRIN 2005039914 projects), EU-6-projects DiMI (LSHB-CT-2005-512146), EMIL (LSHC-CT-2004-503569), MEDITRANS (Targeted Delivery of Nanomedicine, NMP4-CT-2006-026668), ENCITE (201842), and EU-COST D38 Action is gratefully acknowledged. We are grateful to Dr. Dale Lawson for his valuable contribution to the writing of the manuscript.

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FOOTNOTES

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