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Macromolecular and Dendrimer Based Magnetic Resonance Contrast Agents

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Abstract

Magnetic resonance imaging (MRI) is a powerful imaging modality that can provide an assessment of function or molecular expression in tandem with anatomic detail. Over the last 20–25 years, a number of gadolinium based MR contrast agents have been developed to enhance signal by altering proton relaxation properties. This review explores a range of these agents from small molecule chelates, such as Gd-DTPA and Gd-DOTA, to macromolecular structures composed of albumin, polylysine, polysaccharides (dextran, inulin, starch), poly(ethylene glycol), copolymers of cystamine and cystine with GD-DTPA, and various dendritic structures based on polyamidoamine and polylysine (Gadomers). The synthesis, structure, biodistribution and targeting of dendrimer-based MR contrast agents are also discussed.

Keywords

MR-Imaging; Contrast Agents – Intravenous; Chemistry; Molecular Imaging; Comparative Studies

Magnetic Resonance Basics

The advent of magnetic resonance imaging (MRI) has provided scientists and clinicians with a powerful tool to acquire *in vivo* images of anatomy and physiology in whole animals and humans. It is routinely used in the clinic for diagnostic imaging and has the advantage of providing exquisite anatomic resolution, routinely down to 0.5 to 1 mm. The signal-to-noise ratios in an MR image depend on the density of protons present in the region of interest and the degree of polarization of the nuclear spin states. When placed in a magnetic field, a slight majority of the magnetic moments of protons will orient in the direction of the magnetic field and precess at a resonance frequency, known as the Larmor frequency, related to the strength of the magnetic field. Relaxation is measured in two directions, longitudinal and transverse. Longitudinal or spin-lattice relaxation is defined by the time constant T1 and occurs in the direction of the main magnetic field. Signals related to T1 relaxation are obtained after excitation by an RF pulse at the Larmor frequency as the proton's dipole moment vector begins to realign or relax back to its ground state of alignment with the main magnetic field. Transverse or spin-spin relaxation corresponds to vector dephasing in the plane perpendicular to the main magnetic field and is characterized

by T_2 . T_1 represents the time required for the magnetization vector to be restored to 63% of its original magnitude and T_2 , a 37% decrease in net signal. T_2 is always shorter than T_1 . Inhomogeneity in the static magnetic field and spin-spin relaxation has an effect on the transverse magnetization and is characterized by:

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

where T_2' is a time constant arising from magnetic field inhomogeneity and T_2^* is the spin-spin time constant that takes into account these issues. T_2^* is always less than T_2 . Tissue types vary in their relaxation properties, and thus MRI is used to reconstruct images of structures such as organs and lesions and to evaluate perfusion and flow-related abnormalities.

MRI has proven to be extremely successful in the clinical environment. However, all technologies have their limits. In the case of MRI, signal intensity is dependent on several parameters such as proton density, T_1 , T_2 and T_2^* relaxation rates, magnetic field strength, and pulse sequence. While instrumentation and techniques continue to improve image quality, contrast agents (CAs) can dramatically highlight anatomic and pathologic features of interest. Paramagnetic ions decrease the relaxation time of water protons. Thus, MR contrast agents are not directly imaged, but rather, indirectly affect the surrounding water molecules that in turn influence signal. Relaxation is also a function of molecular tumbling, the fast exchange between CA-coordinated water molecules (inner shell) and water in the bulk solvent (outer shells). If exchange among protons in the shells is rapid, they all exhibit similar relaxation behavior. The overall signal is then a weighted average of relaxation rates from each local proton environment, with the principal contribution from within the hydration sphere of the ion.

This article focuses on macromolecular MR contrast agents, including proteins, polypeptides, polysaccharides, poly (ethylene glycols) and dendrimers. However, it is instructive to highlight the basic chemical structures and main paramagnetic behavior of small molecule CAs currently in clinical use, as they are used as the paramagnetic component of macromolecular CAs.

Small Molecule MR Contrast Agents

Paramagnetic ions including Mn^{2+} , Fe^{3+} and Gd^{3+} are potent class MRI contrast agents owing to their unpaired electrons, among which the lanthanide ion gadolinium with its 7 unpaired 4f electrons is by far the most commonly used agent. However, Gd ion is highly toxic in its free form, and thus a number of biocompatible chelating agents have been developed to render the metal ion nontoxic in its chelated form. These cages or chelates are designed with properties such as cavity size, geometry, donor character, and kinetic stability to have higher affinity for Gd^{3+} than for other metals commonly present *in vivo* such as Zn^{2+} , Ca^{2+} and Cu^{2+} , and thus the Gd chelates are highly stable *in vivo*.

One class of Gd complexes is macrocyclic chelates (Fig. 1), mostly derivatives of 1,4,7,10-tetraazacyclododecane (cyclen) (1). The tetraacetic acid derivative complex with gadolinium, Gd-DOTA (gadoterate, Dotarem®) is formulated as its *N*-methylglucamine salt, is highly water soluble, and thermodynamically stable. Two neutral macrocyclic derivatives of 1,4,7-tricarboxymethyl-1,4,7,10-tetraazacyclododecane (DO3A) are gadoteridol (ProHance®) and gadobutrol, (Gadovist®). They are characterized by the replacement of one carboxylate with a hydroxyl donor group.

Another class of such agents is acyclic chelates (Fig. 2) which are comprised of derivatives of aminopolycarboxylic acids such as diethylenetriaminepentaacetic acid (DTPA). Gadolinium diethylenetriamine pentaacetic acid dimeglumine salt (Gd-DTPA), also known as Magnevist®, was approved for clinical use in adult patients in 1988 and has since become the most commonly used MR CA. The chelate has high thermodynamic stability and is water soluble (2). Two diamide derivatives of DTPA were approved for human use: Gd-DTPA-BMA (gadodiamide, Omniscan®) and Gd-DTPA-BMEA (gadoversetamide, OptiMARK®). By reacting the dianhydride of DTPA with the corresponding amine (methyl amine or methoxyethyl amine, respectively), two carboxylates were replaced with two amide oxygen donors, resulting in a neutral charge on the chelate. These neutral agents remain highly water soluble and were developed in part to lower the osmolality of aqueous solutions (3). However, in late 2006, the FDA issued a warning about exposure to gadolinium-containing CAs, increasing the risk for Nephrogenic Systemic Fibrosis in patients with severe kidney dysfunction (4,5).

While relaxivity is an important property, other factors also determine the efficacy of an agent in obtaining quality images, namely clearance rate. The amount of time it takes for a CA to be excreted, is dependent on a number of properties such as size, shape, surface charge and chemical makeup of the agent. With a molecular weight of 0.8 kDa, 90% of the injected dose of Gd-DTPA is cleared by renal filtration and vessel leakage in less than an hour (6), hampering the ability to complete time-dependent imaging studies or obtain highly resolved images. However, the rapid clearance improves the safety profile, at least in subjects with normal renal function.

Macromolecular MR Contrast Agents

Staudinger introduced the macromolecular concept in 1920s, describing how monomers react with each other to form large (high molecular weights) molecules, that he called “macromolecules” (7). This marked the beginning of macromolecular (polymer) science, and Staudinger was awarded the Nobel Prize in Chemistry for this work in 1953. The main types of natural and synthetic macromolecular or polymer architecture include linear, branched, graft (comb), multiarm (star), dendritic and crosslinked (or network). Most synthetic polymers are polydisperse, but a number of methods are also available for the synthesis of relatively monodisperse polymers. There are also a wide variety of functional polymers, both natural and synthetic, suitable for various biomedical applications including the delivery and targeting of drugs and contrast agents.

Macromolecular or polymeric metal-chelate complexes, also known as blood pool agents or macromolecular contrast media (MMCM), generally have a molecular weight greater than 30kDa and were originally designed for prolonged blood retention. Furthermore, they increase relaxivity due to slower molecular tumbling. The Solomon-Bloembergen-Morgan theory of paramagnetic relaxivity predicts that increasing the rotational correlation time of a paramagnetic ion with a relatively long electronic relaxation time, such as Gd^{3+} , will increase the ion's relaxivity at clinically used field strengths (8,9,10). The molecular dynamics of such paramagnetic ions dominate the dipole-dipole interactions between their unpaired electrons and the water protons in the inner shell (11,12). Increased steric hindrance due to bulkier physical structure slows the rotation of larger molecules, increasing the rotational correlation time, τ_r , and thus enhancing relaxivity and resulting in more enhancement per unit dose of the paramagnetic ion. Moreover, using a macromolecular structure potentially allows for the attachment of a large number of chelates and metal ions per agent further increasing enhancement. A lower local concentration of agent is thus required for satisfactory image acquisition.

In order to attach paramagnetic ions to macromolecular structures, a family of chelates known as bifunctional chelates (Fig. 3) were developed, including 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pentaacetic acid (1B4M-DTPA), *N*-[2-amino-3-(4-isothiocyanatobenzyl)propyl]-*cis*-cyclohexyl-1,2-diamine-*N,N',N'',N'''*-pentaacetic acid (CHX-A-DTPA), *N*-[2-amino-3-(4-isothiocyanatobenzyl)propyl]-*trans*-cyclohexyl-1,2-diamine-*N,N',N'',N'''*-pentaacetic acid (CHX-B-DTPA), and 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (p-SCN-Bz-DOTA) (13). These allow the chelates to bind the metal ion as well as to a macromolecule.

A number of MR macromolecular contrast agents have developed over the last 30 years, ranging from protein to polymer to dendrimer-based molecules. These agents typically have diameters greater than 1–2nm as renal excretion slows enough for the agent to be effective. By 8nm, the liver begins to predominate as the clearance mechanism and completely dominates by 10–12nm. Biodistribution differs significantly with long retention times and limited extravasation. While MR agents such as liposomes and gold and iron nanoparticles are beyond the scope of this article, Gd-chelate based structures are discussed herein.

Albumin-Gadolinium Complexes

Covalently Bound

In 1987, Ogan and colleagues (14) published groundbreaking work on one of the first macromolecular agents, an albumin-(Gd-DTPA) complex. Human serum albumin (HSA) is the most abundant protein in the blood and its 58 lysine residues (15) can be used for covalent ligand conjugation. Approximately 19 DTPA groups were attached to each HSA molecule and the paramagnetic molecule showed an increase in blood retention time, while molecular relaxivity also increased significantly. T1 relaxivities of albumin-(Gd-DTPA) compared to native albumin at 0.23T were $273 \text{ mM}^{-1}\text{s}^{-1}$ and $0.40 \text{ mM}^{-1}\text{s}^{-1}$ relative to protein concentration, corresponding to $14.8 \text{ mM}^{-1}\text{s}^{-1}$ per gadolinium ion (14). The relaxivity of Gd-DTPA alone was $4.9 \text{ mM}^{-1}\text{s}^{-1}$ thus indicating that there was a three-fold increase in relaxivity. T2 relaxivity also increased from $5.8 \text{ mM}^{-1}\text{s}^{-1}$ for free albumin to $388 \text{ mM}^{-1}\text{s}^{-1}$ for albumin-(Gd-DTPA) (14).

One of the initial studies using albumin-(Gd-DTPA) as a MRI contrast agent was performed by Schmeidl and co-workers in 1987 (16). After intravenous injection of the agent in rats, T1 relaxation times were significantly reduced and were shown to remain low for 30 min in blood samples, as well as lung, heart, spleen, kidney, and brain tissue. *In vivo* MRI of rat and rabbit heart and lungs confirmed the prolonged contrast-enhancing effect of the labeled albumin. Since then Gd-albumin complexes have been used for blood-volume and perfusion-dependent contrast enhancement. Rat and primate studies have been conducted where albumin-(Gd-1B4M-DTPA)₅ was used to monitor real-time distribution of tumor-targeting cytotoxin interleukin 13 bound to *Pseudomonas* exotoxin (IL13-PE) (17). Gd-albumin acted as a surrogate tracer when co-injected with IL13-PE for brainstem perfusion by convection-enhanced delivery (Fig. 4). The agent has been particularly effective at characterizing microvasculature and has been used to study carcinomas such as breast (18) and prostate cancer (19). Quantitative measurements of albumin-(Gd-DTPA) leakage from intravascular space with inversion recovery echo planar imaging (IR-EPI) allow for noninvasive monitoring of microvascular injury (20).

Though it is possible to bind higher numbers of chelates, a typical synthesis yields 25–35 molecules of Gd-chelate per albumin producing a product with an average molecular weight of 92 kDa (21). Since albumin is partially responsible for osmotic pressure maintenance within blood vessels, it is no surprise that the volume of distribution of the macromolecule is

0.05 L/kg, close to the body's relative blood volume (21). However, the agent's ability to imitate native circulating albumin creates a potential problem for its use. Given that only 5% of albumin leaks from blood circulation each hour (22), the albumin complex also has prolonged retention in the intravascular space after injection leading to slow clearance and persistent non-specific pooling in the blood (3). Elimination is slow and incomplete, with retention times up to several weeks, particularly in the liver and bone. Although the covalently bound albumin-(Gd-DTPA) complex has been used extensively in preclinical assessment to establish its utility, it was not a viable clinical candidate because of practical issues with regards to difficulties in synthesis, instability during heat sterilization, as well as the slow clearance of Gd metabolites (3). This has led to the study of noncovalent complexes as discussed below. A more general problem is that albumin complexes may also be immunogenic, raising questions regarding its use for human imaging (23).

Non-Covalently Bound

To address the issue of substantial retention of albumin-(Gd-DTPA) agents, a new generation of Gd-chelates that would reversibly bind to albumin was developed. Non-covalent binding of gadolinium complexes to high molecular weight, endogenous proteins theoretically presented the perfect solution: initial intravascular retention due to reversible binding to serum proteins such as albumin followed by release of the discrete, and renally cleared low molecular weight Gd-chelate complex. In 1996, Lauffer (24,25) first investigated the reversible binding agent MS-325 (trisodium 2-(*R*)-[(4,4-diphenylcyclohexyl) phosphonooxymethyl]-diethylenetriaminepentaacetato *aquo* gadolinium), also known as Gadofosveset. An amphiphilic, monomeric chelate MS-325 consists of a lipophilic diphenylcyclohexyl group attached to a Gd-chelate by a phosphodiester linkage. The lipophilic group mediates reversible, non-covalent protein binding (Fig. 5). It is synthesized by forming hydroxymethyl-DTPA-penta-*tert*-butyl ester from L-serine methyl ester, followed by phosphoramidite chemistry to couple it to 4,4-diphenylcyclohexanol. The critically important phosphodiester linkage is stable under harsh conditions such as during chelation to Gd (26).

Following injection, MS-325 binds to serum albumin and other proteins and equilibrium is maintained between free and bound MS-325. As many as 30 MS-325 can bind to one albumin molecule (21), though at saturation of albumin binding sites, a greater concentration of free agent will also be found. Equilibrium between free and bound MS-325 depends on MS-325 concentration, plasma albumin level, and the albumin-binding affinity constant which is species dependent (27). MS-325-albumin complexes can achieve relaxivities nearly 10-fold higher than that of Gd-DTPA ($50.8 \text{ mM}^{-1}\text{s}^{-1}$ for MS325-HSA, $4.9 \text{ mM}^{-1}\text{s}^{-1}$ for Gd-DTPA) (28). The enhanced relaxivity is caused by a slower tumbling rate due to steric hindrance of the rotation of larger molecules, as described previously. These albumin complexes were excreted predominantly through the kidneys, rather than the liver (26).

MS-325 protein binding varies amongst animal species due to small but significant differences in albumin composition among different species. For instance, elimination half-life is relatively long in primates and rabbits (2–3 h) and shorter in rats (25 min) (25). With such inconsistencies in animal models, it was necessary to characterize the agent in human subjects, making MS-325 the first Gd-based macromolecular agent used in human trials. Completed Phase II and III trials of MS-325 showed a good safety profile and that a dose of 0.03 mmol/kg was effective as an MR angiography agent for evaluation of aortoiliac occlusive disease (29,30).

A number of other non-covalently bound contrast agent derivatives have been developed. Moderate binding to HSA and extended blood half-life were demonstrated by *S*-4-(4-ethoxybenzyl)-3,6,9-tris(carboxymethyl)-3,6,9-triazaundecanedioic acid gadolinium

complex (Gd-EOB-DTPA; Eovist®)(31,32) and (9*R,S*)-2,5,8-tris(carboxymethyl)-12-phenyl-11-oxa-2,5,8-triazadodecane-1,9-dicarboxylic acid gadolinium complex (Gd-BOPTA; MultiHance®) (27,33). The blood pool agent 4-pentylbicyclo[2.2.2]octane-1-carboxyldi-L-aspartyllysine DTPA, also known as MP-2269 (34,35,36), has a non-aromatic side chain that allows it to reversibly bind to HSA. A contrast agent named B-22956/1 (gadocoletic acid trisodium salt) (37,38) was reported to have a high affinity for serum albumin. It is a trisodium salt of a derivative of gadopentetate linked to a deoxycholic acid moiety by means of a flexible spacer.

There are, however, a few disadvantages for using the class of agents that exhibit reversible protein binding, exemplified by MS-325. Due to the unpredictable mixture of relatively low molecular weight unbound chelate and the high molecular weight protein-bound chelate, the pharmacokinetics is complex. The protein affinity of these agents in the interstitial space is unknown and may differ from that in plasma. Though albumin-bound agent will have greater relaxivity, imaging instruments cannot differentiate amongst enhancement from the free and bound chelate forms, and thus, signal represents a dynamic average of the relative proportion of the two states of the contrast agent.

Polymer-Gadolinium Complexes

Polylysine Complexes

A number of polymer based structures were analyzed for use as platforms for attaching multiple units of chelated Gd^{3+} . Schuhmann-Giampieri et al. (39) synthesized polylysine-(Gd-DTPA)_n (MW ≈ 48.7 kDa, n=60 to 70) and characterized its pharmacokinetics in rats and rabbits by comparison with Gd-DTPA. Relaxivity increased three-fold and the half-life of distribution was significantly prolonged with elimination primarily through glomerular filtration. Only minimal Gd retention was found and no biodegradation of the polypeptide was observed. An issue noted in rabbits, however, was leakage of the compound into the extracellular interstitial space. Resolving vessels from surrounding tissue could therefore become a problem, hampering use of polylysine-(Gd-DTPA)_n as a strong blood-pool contrast agent. Chatal and co-workers (40) have targeted Gd chelated polylysine by attaching F(ab')₂ fragments of anti-carcinoembryonic antigen (CEA) mAb. The immunoreactivity of the Ab fragments remained high at 83–85% and tumor uptake was observed in mice with human colorectal carcinoma xenografts.

Polysaccharide Complexes

Polysaccharides are less immunogenic than proteins; they are also typically metabolized to smaller subunits that are excreted by glomerular filtration. Dextran, which has been used as a synthetic plasma expander for 50 years, was one of the initial polysaccharides to be complexed to Gd-chelates. A polymer of glucose molecules, dextran is highly water soluble, stable under mild acidic and basic conditions, inexpensive, and has a good safety profile in human subjects. Given that studies have shown that cells internalize dextran through passive fluid-phase endocytosis (41), it has potential not only as a blood-pool agent, but also as a means of drug delivery. Wang et al (42) attached 15 Gd-DTPA chelates per dextran molecule. The molecular weight was approximately 75 kDa and this agent remained intravascular for 1h after injection, showing enhancement of liver, spleen, kidneys, and myocardium. The typical dextran compound has a 43 min biological half-life and is broken down faster than albumin. However, dextrans do display a high degree of polydispersity, which, in turn, affects their distribution and elimination, making characterization difficult. Sirlin et al synthesized 165 kDa dextran conjugated with 187 gadolinium per polysaccharide and also noted prolonged intravascular half life of 58 hrs in rabbits (43). However, anaphylactic reactions with larger dextran complexes has been noted (44). Dextran-(Gd-

DTPA) has been used for MR angiography (45,46), acute myocardial infarction (47), and cardiac perfusion studies (48).

Lebduskova et al. (49) studied inulin and covalently conjugated approximately one Gd-chelate to each monosaccharide of the polysaccharide. The chelate was a derivative of H₅DTPA where the central pendant arm was replaced with a phosphinic acid functional group. The inulin-Gd-DTPA complex had an average of 24 Gd³⁺ ions per molecule, a molecular weight of 23,110 Da, and a relatively long rotational correlation time (866 ps at 298 K).

Other polysaccharides are also being evaluated as potential MR contrast agents. Helbich et al. (50) used a polysaccharide backbone of hydroxyethyl starch (HES). Carboxymethyl HES-(Gd-DO3A)₃₅ [CMHES-(Gd-DO3A)₃₅] consists of a polysaccharide covalently derivatized with 35 macrocyclic chelating groups to have a molecular weight of 72 kD. T1 relaxivity in terms of per gadolinium ion was measured to be more than 4 times that of Gd-DTPA. CMHES-(Gd-DO3A)₃₅ is of sufficient size to prevent kidney filtration of the unmetabolized agent and extravasation through healthy vessels into the extravascular interstitial space. The study showed a half-time of 8.4 h with slow and incomplete elimination (50% persistence after 7 days). The compound was well tolerated in dosages required for diagnostic efficacy by all experimental animals. Following injection of CMHES-(Gd-DO3A)₃₅ in rats bearing MAT-LyLu prostate cancer tumors, dynamic enhancement patterns of experimental tumors were successfully analyzed by quantitative estimates of tumor plasma volume and microvessel permeability.

PEG and Other Polymer Complexes

Polyethylene glycol (PEG) is a nontoxic, biocompatible, hydrophilic polymer that has a large hydrodynamic radius rendering itself ideal for biomedical applications. The advantage of using a polymer such as PEG resides in the ability to manipulate size to analyze appropriate molecular weight for blood pool retention. Ladd et al. (51) used a series of Gd-DTPA-PEG diamines ranging from 10.8 to 83.4 kDa in a rabbit model. Linear relationships were found between molecular weight and both overall blood clearance half-lives and volumes of distribution. Linear Gd-DTPA-PEG polymers of 20 kDa and higher exhibited significant blood pool retention over a 60 min time frame (51). This group further designed polymers with a globular shape to compare to linear polymers. The more globular shape decreased solution viscosity. Cross-linking within the globular polymer structure (13.6 kDa) also increased rigidity, resulting in 68% higher relaxivities than for the linear polymer (20.2kDa).

A new type of macromolecular MR contrast agent has been reported incorporating disulfide bonds that are cleavable by thiol-disulfide exchange reactions with endogenous or exogenous thiols such as cysteine and glutathione to release low molecular weight Gd-DTPA chelates. The human plasma concentration of free thiols is approximately 15 μ M (52,53). However, it is important to note that *in vivo* metabolic degradation of polydisulfides is also complicated by enzymatic and oxidative reactions as a result of high blood oxygen concentration (54). Lu and coworkers developed the first such biodegradable contrast agent, a Gd-DTPA cystamine copolymer (GDCC) synthesized by a copolymerization of cystamine and DTPA dianhydride in dimethyl sulfoxide (DMSO) (55). *In vitro* and *in vivo* verification of degradation also demonstrated dependence on chemical structure surrounding the disulfide bond. Modification of GDCC to Gd-DTPA cystine copolymers (GDCP) (56) or PEGylated GDCP (57,58) decreased degradation. MALDI-TOF mass spectroscopy of urine samples collected from rats that were systemically administered GDCC and GCCP confirmed degradation by identification of low molecular weight and oligomeric Gd complexes (55,56). In rats, the agent had initial higher blood concentration than clinically

approved Gd-DTPA-BMA (Omniscan) in a timeframe relevant to a clinical MR scan with accumulation comparable to the control after ten days (59,60). These biodegradable agents have also been used for MR angiography in a porcine model where they provided a clearer delineation of vessels when compared to MS-325 (61).

Dendrimer-Based MR Agents

Macromolecular CAs discussed above have broadly linear structures, but dendrimers (from the Greek word *dendron* meaning tree) have a globular architecture comprising a central core, an interior and a surface (or periphery). Most, if not all, dendrimer end groups are located in the outer surface layer, and can thus be functionalized for use in various applications.

Synthesis and Structure

There are two basic strategies of dendrimer synthesis, divergent and convergent (Fig. 6). The divergent method was disclosed by Tomalia (62) and Newkome (63) and is characterized by molecular growth of the dendron, or molecular tree, which originates from a root or core site and propagates outward. Monomeric modules are assembled in an “arborol” or branch-to-branch approach, with each additional layer increasing dendrimer size and generation (G). This method is the current preferred approach of companies such as Dendrimax and Dendritech. The convergent method was initiated by Fréchet (64) and Vögtle (65). In this method, the dendron is synthesized from the “leaves” back to the focal “root”. Branches with a preset number of generations are connected to the central core. Convergent building of dendrimers allows for the synthesis of nonsymmetrical dendrimers and for specific incorporation of function into the dendrimer interior (66).

The first dendrimer family to be characterized and commercialized was polyamidoamine (PAMAM) dendrimers. They are synthesized through the divergent method by Michael addition of methyl acrylate to an amine core. The ester is directly reacted with an excess of diamine, such as ethylenediamine. Aminolysis for two complete cycles produces a generation 2 (G2) dendrimer. Controlled growth of the dendrimer exponentially increases the number of terminal groups from the core. PAMAM dendrimers can also be synthesized from an ammonia core. Table 1 describes the properties of some available PAMAM dendrimers. Other dendrimers include diaminobutane (DAB) dendrimers which unlike PAMAM dendrimers that are comprised of a scaffold of repeating amine and amide units, contain only amines on a propyleneamine scaffold linked through a diaminobutane core. Over fifty other dendrimer families have been characterized, each possessing compositionally different interiors, i.e. carbon, nitrogen, silicon, sulfur, phosphorus or metals (67). Fig. 7 is a schematic of ammonia core (AC) and ethylenediamine (EDA) PAMAM and diaminobutane (DAB) dendrimers.

Though terminal groups are typically amines or carboxylic acid, they can be functionalized to other end groups, thus allowing them to be tailored for a variety of applications. Larger dendrimers have more terminal groups and thus more chelates can be attached. G5 and G10 PAMAM-EDA dendrimers can theoretically bind up to 96 and 1860 Gd^{3+} ions, respectively, and demonstrate r_1 relaxivities per gadolinium ion of 30 and 36 $\text{mM}^{-1}\text{s}^{-1}$ (20mHz, 0.47T) (68). Although such numbers are rarely actually achieved, the net effect of the large numbers of metal ions on a single molecule is to greatly improve MR signal. Fig. 8 is an example of an EDA core PAMAM dendrimer used to visualize the vascular system.

Polylysine dendrimers, also known as Gadomers, are also spherical molecules that possess the advantage of consistent and reproducible control of molecule physical size. Gadomer-17 (Bayer Schering Pharma, Berlin, Germany) is a fully synthetic dendritic Gd complex

chelating 24 gadolinium atoms and with a molecular weight of 17kD (69). It is built from a trisubstituted aromatic central core where the first generation consists of three diethylenetriamine building blocks. Two generations of 6 and 12 lysine amino acid residues then yield a 24-mer polyamine intermediate. Each of the 24 amino groups of the outmost 12 lysine residues are covalently linked to a gadolinium chelate, resulting in a high Gd density per molecule (Fig. 9). The agent's higher molecular weight increases the rotational correlation time and, thus, also the T1 and T2 relaxivities. Gadomer internal structures that incorporate aromatic rings are more compact and result in smaller sizes than polyamine dendrimer-based agents of the same generation (70).

Biodistribution of Untargeted Dendrimer

The key factors in biological behavior of contrast agents include their chemical structure, size, topography and surface charge. Macromolecular MR contrast agents, including dendrimers, were initially developed for imaging the lumen of blood vessels because of their prolonged vascular retention and enhanced relaxivities. Thus, vascular retention and biodistribution of various dendrimers have been studied extensively, as outlined below.

Gd-1B4M modified PAMAM dendrimers of generations 6 through 8 provide optimal retention as G10 aggregates and precipitate at physiological pH (71). Smaller PAMAM dendrimers are rapidly cleared by glomerular filtration (68,72,73) but they have been proven useful for visualizing renal structural and functional damage and the lymphatic system when injected intradermally (74). Typically, smaller dendrimers are rapidly filtered by the kidneys, but 100% excretion is not reported because some reabsorption in the proximal tubules does occur. Kobayashi and co-workers have shown that renal excretion can be increased 5.4-fold by co-injection of lysine (75). The concern with larger modified dendrimers is that they will be sequestered in the liver, however this can be mitigated by PEGylation. For example, PAMAM-EDA-G4-1B4M with one or two PEG groups prolonged blood retention and increased excretion while decreasing organ uptake (76).

However, dendrimer biodistribution is also dependent on molecular topography and charge. High-generation dendrimers have typically been accepted as spheroidal molecules with the inner core completely shielded by the densely-packed branched scaffolding surrounding it. However, a number of studies have demonstrated that the initiator core of a dendrimer greatly affects its biological distribution. Preliminary studies comparing PAMAM and DAB dendrimers revealed distinct differences in pharmacokinetics. DAB-G2-(Gd-1B4M)₁₂ demonstrated a half-life of 3 h, similar to the 3.3 h half-life of the larger PAMAM-AC-G6-(Gd-1B4M)₁₇₀, rather than the 40 min of its generational counterpart PAMAM-AC-G2-(Gd-1B4M)₁₁ (77). Another study showed a 13 h half-life for DAB-G2-(Gd-1B4M)₁₆ (78). Thus, minor changes in the molecular design can have significant implications for pharmacokinetics. DAB based dendrimers also accumulated in the liver significantly more than their generational PAMAM counterparts. The opposite was true for kidney accumulation, however, DAB agents exhibited higher signal intensity. This may indicate that a large portion of DAB agents remained in areas with free water, i.e. urine, whereas PAMAM agents localized in lipid vesicles, perhaps in tubular cells. These differences in biological behavior have been attributed to differences in hydrophobicity between the two dendrimer interior architectures (78).

DAB-1B4M has been found useful for liver imaging based on its biodistribution and clearance (13), and a similar approach was used to identify the best candidate for renal imaging. AC and EDA core G6 PAMAM dendrimers have comparable diameters, 6.7 and 6.9nm, respectively, yet the glomerular filtration and renal accumulation for PAMAM-AC-G6-1B4M is higher than PAMAM-EDA-G6-1B4M (79). This discrepancy may be due to differences in molecular charge given the number of metal ions complexed to each

dendrimer, ~180 Gd³⁺ on PAMAM-AC-G6-1B4M and ~242 Gd³⁺ on PAMAM-EDA-G6-1B4M. In another study comparing two samples of ¹¹¹In-labeled PAMAM-EDA-G2-1B4M one of which was fully loaded with stable In³⁺, the unloaded dendrimer had slower blood clearance and higher accumulation in liver, kidney, spleen and bone (80). Thus, more studies on the effect of macromolecular charge are needed.

Gadomers are typically smaller in diameter when compared to same generation (G3) PAMAM dendrimers, and are excreted through glomerular filtration. The pharmacokinetics, elimination, and biodistribution of Gadomer-17, the largest of the series, was investigated in different species (rat, rabbit, dog, monkey) for up to 7 days after single intravenous injection and was found to distribute almost exclusively within the intravascular space without significant diffusion into the interstitial space. Excretion occurred through glomerular filtration and there was no long-term accumulation or retention of the nonmetabolized agent detected in organs or tissues (69). These qualities make Gadomer-17 promising for use as an MRA contrast agent, similar to the known linear Gd-dtpa-polylysine, but with a superior elimination rate, presumably as a result of the globular nature of the dendrimer derivative (81). While Gadomers have been extensively characterized preclinically in rats, rabbits, dogs, and monkeys (69), they are not yet clinically available and are under investigation for use in humans (82).

Targeting Dendrimer

The macromolecular CAs discussed above possess increased relaxivity and blood retention time, but they remain untargeted. Attachment of high numbers of chelate directly to targeting agents such as antibodies is problematic because the immunoreactivity of the antibody may be compromised. For instance, Shreve et al demonstrated that when conjugated to 20, 100, and 250 Gd³⁺ DTPA complexes, mAb immunoreactivity decreased to 56%, 36% and 17%, respectively (83). In principle, this limitation may be overcome by tailoring suitably functional polymers and dendrimers that can be conjugated to both metal chelated and targeting moieties, examples of which are those of polylysine and of dendrimers discussed below.

One of the first such constructs to be reported was 2E4 mAb conjugated to a PAMAM-AC-G2-DOTA dendrimer. Positive 5.1.2 control cells showed 76.6% binding as compared to 6.0% binding to negative control RAJI cells (84). In other studies, PAMAM-EDA-1B4M was conjugated to humanized anti-Tac IgG to yield 73% binding (80) and to mAb OST7 for 91% immunoreactivity (85).

Aside from antibodies, a number of other targeting agents have been attached to dendrimers. Endothelial carcinomas, including breast and ovarian cancers, are known to express high levels of human folate receptor (hFR) (86,87). Wiener et al. showed that by targeting hFR expressing cells with folate-conjugated PAMAM-DTPA dendrimer, the relaxivity of the cells doubled allowing for significant signal enhancement (88). Twenty-four hours after folate-conjugated dendrimers were injected into mice bearing OVCA 432 human ovarian tumors that express hFR, MRI contrast enhancement was observed (89). Fluorescein was also conjugated to the same folate-conjugated dendrimer and when hFR-expressing cells were incubated with the agent for 30 min, fluorescence increased 650% (88). This demonstrates the potential of dendrimers as polyvalent targeted multi-modality imaging agents.

Avidin is a tetrameric glycoprotein known to have an affinity to surface lectins on tumor cells and to have high uptake by liver cells (90,91). Thus, direct conjugation of avidin to PAMAM-EDA-G6-(1B4M-Gd)₂₅₄ results in a 366-fold higher uptake compared to Gd-DTPA, and a 108-fold increase compared to untargeted control dendrimer, in SHIN3 tumor

cells one day post-injection (92). The tumor-to-normal tissue ratio for the targeted conjugate increased from 17:1 to 638:1 at 24 h post-injection (92). A second indirect use of avidin with dendrimer exploits the well understood biotin-avidin interaction (93,94). Biotin-conjugated dendrimers have similar pharmacokinetics to untargeted dendrimer (95). An injection of avidin 4 min after the biotin-dendrimer results in clearance of the blood pool as avidin rapidly binds to the biotin-dendrimer in circulation and the complex is quickly absorbed by the liver. In theory, the “avidin chase” can be applied for cancer diagnostics. Tumors are known to have leaky vasculature and the biotin-PAMAM-chelate agent accumulates in the tumor by enhanced permeation and retention (EPR). An avidin chase removes the circulating dendrimer from the blood pool, leaving only the dendrimer that is retained in the tumor (95). Xu et al. (96) further explored the avidin-biotin-dendrimer targeting system by the use of a Gd-labeled cystamine core dendrimer. Cystamine PAMAM dendrimers have a unique disulfide bond in the core. By reducing and splitting this bond, the dendron was attached to a maleimide-functionalized biotin. Up to four copies of the biotinylated dendrimer were then immobilized on fluorescently labeled avidin, yielding a dual-modality avidin-biotin-dendrimer complex. It should be noted that avidin is highly immunogenic, thus has limited clinical translation. The construct was injected in mice bearing ovarian cancer tumors that were visualized post-injection by both optical and MR imaging. Dual labeling of dendrimers was also reported by Talanov and co-workers using a PAMAM dendrimer-based nanoprobe for dual magnetic resonance and fluorescence imaging of sentinel lymph nodes (Fig. 10) by injection into the mammary fat pad of normal nu/nu mice (97).

Though only a few targeting agents have been mentioned here, an entire field of dendrimer-targeting exists encompassing a range of molecules. Dendrimer structure not only allows for functionalization with targeting and chelating molecules, but also other imaging contrast and therapeutic agents.

Conclusions

The use of macromolecular MR agents to study and modulate biological events is an exciting and dynamic field of research for both fundamental knowledge and clinical applications. More than a thousand papers on dendrimers alone have been published in the last three years. Architectural control, well-defined structure, modifiable functionality, tailored bioelimination, and consistent clinical grade synthesis are desirable properties for an ideal macromolecular vector. The agents described in this review have been characterized for these properties. Already these constructs have proven their value as magnetic-resonance contrast agents, but their future may lie in the ability to combine them with other agents for diagnostics and therapeutics. The remarkable biomimicry of structure and kinetics of various biological species also opens doors for combinational use in proteomics, immunodiagnostics, pathogen pacification, gene transfection, and drug delivery. It is expected that these macromolecular and dendrimer based MR agents will play a significant role in the development of new biomedical devices, contrast agents, and strategies for the treatment of human disease.

Acknowledgments

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Fig. 1.
Chemical structures of cyclen (1,4,7,10-tetraazacyclododecane) and commercially available MRI contrast agents based on Gd chelates of cyclen derivatives.

Fig. 2. Chemical structures of acyclic commercially available MRI contrast agents based on Gd chelates.

Fig. 3. Bifunctional chelates synthesized to attach to a ligand while also trapping a metal ion such as Gd^{3+} . Diastereoisomers of CHX-DTPA chelates are shown.

Fig. 4. T1-weighted (a) sagittal, (b) coronal, and (c) axial MR images of primate after perfusion of the brainstem with a co-infusion of Gd-albumin and IL13-PE. Acting as an IL13-PE tracer, Gd-albumin distinctly highlights the pons relative to its surrounding tissue (refer to (17)).

Fig. 5. Chemical structure of MS-325, a reversible binding chelate, composed of a Gd-DTPA chelate, a phosphodiester linker, and a lipophilic residue (diphenylcyclohexyl) for noncovalent attachment to proteins.

Fig. 6.

Two alternative methods for dendrimer synthesis. Divergent synthesis begins from a polyfunctional core and grows outwards through a stepwise procedure of coupling (a) and deprotection (b). In the convergent method the branches (dendrons) are constructed separately followed by attachment to a central a multifunctional core. PG, protective group (refer to (98)).

PAMAM Ethylenediamine Core

DAB Diaminobutane Core

PAMAM Ammonia Core

Fig. 7.

Schematic drawing of second generation ammonia core (AC) and ethylenediamine (EDA) PAMAM and diaminobutane (DAB) dendrimers.

Fig. 8. Subtraction maximum intensity projection (MIP) MR image of a mouse given an EDA core PAMAM-G6-(1B4M-Gd)₁₉₂ injection at 0.033 mmolGd/kg. Note the exquisite depiction of the vascular system including the heart chambers with moderate uptake in both the liver and kidneys.

Fig. 9.
Schematic drawing of Gadomer-17.

Fig. 10.

Mouse sentinel lymph node images obtained by injection of $G6-(Cy5.5)_{1.25}(1B4M-Gd)_{145}$, as a dual modality imaging agent where Cy5.5 is a near-infrared fluorophore (refer to (97)). (a) Maximum intensity projection calculated from a 3D spoiled gradient echo MRI that shows enhanced signal at the mammary fat pad injection site, draining lymphatics, and axillary lymph node which is also visible by (b) the optical image obtained under simultaneous white light and filtered (615–665 nm) excitation light with a 720nm emission filter.

Table 1

Properties of ammonia core (AC) and ethylenediamine core (EDA) PAMAM dendrimers (13).

Core	Generation	MW (kDa)	No. of terminal amines	Diameter (nm)
AC	0	0.36	3	1.1 ^a
AC	1	1.04	6	1.6 ^a
AC	2	2.41	12	2.2 ^a
AC	3	5.15	24	3.1 ^a
AC	4	10.62	48	4.0 ^a
AC	5	21.56	96	5.3 ^a
AC	6	43.45	192	6.7 ^a
AC	7	87.23	384	8.0 ^a
AC	8	174.78	768	9.2 ^a
AC	9	349.88	1536	10.5 ^a
AC	10	700.09	3072	12.4 ^a
EDA	0	0.52	4	
EDA	1	1.43	8	
EDA	2	3.26	16	
EDA	3	6.91	32	
EDA	4	14.21	64	
EDA	5	28.83	128	4.3 ^b
EDA	6	58.05	256	6.9 ^b
EDA	7	116.49	512	8.0 ^b
EDA	8	233.38	1024	10.2 ^b
EDA	9	467.15	2048	12.4 ^b
EDA	10	934.70	4096	14.7 ^b

^a diameter measured by size exclusion chromatography^b diameter measured by transmission electron micrograph (TEM)