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Engineering Gd-loaded nanoparticles to enhance MRI sensitivity via T_1 shortening

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Abstract

Magnetic resonance imaging (MRI) is a noninvasive imaging technique capable of obtaining high-resolution anatomical images of the body. Major drawbacks of MRI are the low contrast agent sensitivity and inability to distinguish healthy tissue from diseased tissue, making early detection challenging. To address this technological hurdle, paramagnetic contrast agents have been developed to increase the longitudinal relaxivity (R_1), leading to an increased signal-to-noise ratio. This review focuses on methods and principles that enabled the design and engineering of nanoparticles to deliver contrast agents with enhanced ionic relaxivities. Different engineering strategies and nanoparticle platforms will be compared in terms of their manufacturability, biocompatibility properties, and their overall potential to make an impact in clinical MR imaging.

1. Introduction

1.1 Magnetic resonance imaging and nanotechnology

Magnetic resonance imaging (MRI) is a medical imaging technique used to obtain high-resolution anatomical and physiological images of the body. MRI scanners are commonplace in hospitals around the world. It is a noninvasive diagnostic tool that uses non-ionizing radiation to measure the aligned nuclear magnetization of hydrogen atoms that are primarily hosted in water molecules, which will be referred to as water protons, or simply protons, in this review. This review focuses on how paramagnetic contrast agents affect the nuclear magnetization of water protons.

MR images frequently rely on the differences in tissue relaxation times, both longitudinal (T_1) and transverse (T_2), to generate image contrast (Caravan, 2006). After protons are excited with a radiofrequency (RF) pulse applied perpendicular to the magnetic field, the protons will realign themselves with the magnetic field, a process referred to as relaxation. MRI signals are modulated by the rates at which protons return to equilibrium after an RF pulse. The difference in T_1 and T_2 relaxation times allow differentiation between soft tissues, bone, air, and liquids in the body (Bottomley et al, 1987). Although proton MR spectroscopy also allows parametric mapping of the concentration of specific metabolites, these metabolites are usually at a concentration 4–5 orders of magnitude lower than that of water proton and therefore are much more difficult to quantify at high spatial resolution.

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Disease detection with MRI is often difficult because areas of disease have similar signal intensity compared to the surrounding healthy tissue; therefore requiring signal enhancement using contrast agents. Approximately 40–50% of the 7–10 million MRI examinations each year utilize image enhancing contrast agents as blood pool agents in angiograms, and to image inflammation and cancerous tissue (Shellock & Kanal, 1999). Contrast agents interact with water molecules, leading to altered T_1 or T_2 proton relaxation (Caravan et al, 2009). Paramagnetic lanthanide ions interact with water protons, leading to decreased longitudinal relaxation (T_1). Gadolinium (Gd) is the most popular paramagnetic imaging contrast agent used to produce a *positive* MR contrast (L Villaraza et al, 2010). Alternatively, superparamagnetic iron oxide effectively shortens the transverse relaxation time (T_2) and produces a *negative* intensity effect in MRI (Ho et al, 2011).

Nanotechnology has revolutionized the potentials of the MRI imaging modality. Nanoparticles, i.e. materials with one or more dimensions of 100 nm or less (although several materials with dimensions up to 500 nm are considered nanoparticles), can be loaded with large payloads of multiple different cargos, e.g. image contrast agents, therapeutics, and/or targeting ligands for direction to sites of disease (Parveen et al, 2012). The tissue/cell-specific delivery of nanoparticles carrying large payloads of image contrast agents opens the door for earlier and more accurate disease diagnosis, therefore allowing for subsequent therapeutic intervention. The application of nanoparticles loaded with high concentrations of Gd therefore holds the potential to overcome the current sensitivity disadvantage of MRI as a diagnosis tool.

Chemists, materials scientists, and engineers have designed, developed, and tested nanoparticles of varying size, shape, composition, and surface chemistries; a library of nanoparticles is available, each of which with advantages and disadvantages with regard to *in vivo* biocompatibility, toxicity, targeting, biodistribution, and clearance (see discussion in Section 2) (Li & Huang, 2008). Nanoparticles engineered as MRI contrast agents have been based on gold, silicon, carbon nanotubes and fullerenes, polymers and dendrimers, liposomes and micelles, and viral nanoparticles (VNPs) (Figure 1). Factors to consider when choosing a nanoparticle platform for MRI applications, are manufacturing costs, processability, i.e. reproducibility of size and shape and cargo loading, cargo loading efficiency, target-specificity, biocompatibility, toxicity, and overall MRI performance (signal enhancement). The overall goal lies in the engineering of non-toxic, contrast enhancing agents, with high tissue-specificity – a platform that fulfills these criteria may be successful on the long-winding road from the bench to the clinic.

In this article, we will review the art of engineering nanoparticles for applications as MRI T_1 contrast agents (for more information on the design of nanoparticles for T_2 enhancement, please refer to the following reviews (Colombo et al, 2012; Ho et al, 2011; Huang et al, 2012; Sosnovik et al, 2008)).

1.2 Magnetic resonance T_1 contrast enhancement mechanism

T_1 -weighted MRI generates image contrast based on the longitudinal relaxivity (R_1) of water protons, where $R_1=1/T_1$. Paramagnetic ions, such as Gd, interact with water protons to increase R_1 and therefore increase signal contrast. In general, the higher the concentration of the contrast agent, the bigger the change in tissue relaxation will be. At certain range, contrast agent-induced tissue relaxivity obeys the linear relationship, i.e.,

$$R_1=R_1^0+r_1[CA].$$

where R_1^0 is the tissue relaxivity in the absence of contrast agent ([CA]). The ionic relaxivity rate, r_1 (with units in $\text{mM}^{-1}\text{s}^{-1}$), of a contrast agent describes its capacity for contrast enhancement. R_1^0 varies with tissue type and applied field, but generally lies in the range of $0.5\text{--}2\text{ s}^{-1}$ (with 0.5 s^{-1} being the brightest) (Bottomley et al, 1987). The contrast enhancement detection threshold is around 10% of R_1^0 . For example, if imaging a tissue with R_1^0 of 0.5 s^{-1} , a minimum signal change to 0.45 s^{-1} is required. This would require accumulation of $10\text{ }\mu\text{M}$ of a contrast agent with an r_1 of $5\text{ mM}^{-1}\text{s}^{-1}$. It is clear that accumulation of free contrast agent to sites of disease at such high local concentrations is difficult (mostly unrealistic) to achieve without the assistance of a delivery vehicle.

There are four main factors that affect the ionic relaxivity of chelated Gd ions based on the Solomon-Bloembergen-Morgan theory of paramagnetic relaxation (Figure 2) (Bloembergen & Morgan, 1961; Solomon, 1955):

1. molecular diffusional (τ_D) and rotational (τ_R) correlation times,
2. number of coordinated water molecules (q),
3. water proton residency lifetime (τ_m), and
4. water exchange rate ($k_{\text{ex}} = 1/\tau_m$)

The Gd-chelator determines the water exchange rate (k_{ex}) and number of coordinated water molecules (q). Decreased Gd τ_D and τ_R generally yield increased ionic relaxivity rates at low magnetic fields ($<1.5\text{T}$). These are the parameters that need optimization to achieve the highest ionic relaxivity rate. The most common and clinically approved chelating complexes allow Gd ions to coordinate with a single water molecule ($q=1$). Novel chelators are being developed to increase the coordinated water molecules to $q=2$, and hence (almost) double the relaxivity rate (Figure 3) (Gugliotta et al, 2010). Conjugation of chelated Gd to a nanoparticle platform decreases its molecular tumbling rate, thus affecting diffusional (τ_D) and rotational (τ_R) correlation time, resulting in increased ionic relaxivity rate. For a more detailed discussion with regard to the physics behind ionic relaxivity enhancement of chelated Gd ions, the reader is referred to the following reviews (Caravan, 2006; Werner et al, 2008).

The impact of the nanoparticle platform—Nanoparticle contrast agents enhance ionic relaxivity (R_1) in three ways:

- *First*, the nanoparticle can be loaded with a large payload of contrast agent confined to a small volume.
- *Second*, the nanoparticle can be engineered with tissue-specific ligands promoting accumulation of the carriers in the target tissue, therefore increasing the local concentration of the contrast agent.
- *Third*, conjugation of a chelated Gd ion (similar effects are seen with other lanthanide ions) to a nanoparticle scaffold reduces the molecular tumbling rates of Gd ions leading to increased ionic relaxivity (r_1) (Caravan, 2006; Werner et al, 2008).

The impact of magnetic field strength—The common MRI instrument found in the clinic has a magnetic strength of 1.5T or 3.0T with many pre-clinical (small animal) MRI scanners being 7.0T or higher (Pautler, 2004). The increased magnetic field strength provides higher resolution images with shorter acquisition times. For this reason, clinical trials have started utilizing MRI scanners with magnetic field strength as high as 9.4T (Vaughan et al, 2006). However, the relaxivity of macromolecular Gd chelated contrast agents decreases with increasing magnetic field strength: Nanoparticle contrast agents

primarily enhance ionic relaxivity at low magnetic strengths (0.5–1.5 T) by reducing the molecular tumbling rate. At high magnetic field strength (> 3.0T) this effect needs to be optimized. At high field strength, free chelated Gd ions ($\tau_R \sim 0.1$ ns) and nanoparticle immobilized Gd ions ($\tau_R \sim 10.0$ ns) have similar relaxivities. Rotational correlation times (τ_R) between 0.5–4 ns will yield the highest relaxivities at high magnetic strengths. Based on optimized parameters, ionic relaxivity rates of 80, 45, and 18 mM⁻¹s⁻¹ can be achieved at 1.5, 3, and 9.4 T, respectively (Caravan et al, 2009). With the recent movement toward translating high magnetic field strength MR scanners into clinic use, engineering protocols for contrast agents must be adapted to accommodate increased relaxivities at high magnetic field strength.

The impact of the chelator—Shown in Figure 4 is the most common Gd chelating molecules that are safe for *in vivo* use. The most influential parameter is the number of inner sphere coordinated water molecules (q). Increasing from $q=1$ to $q=2$ will, in theory, double the relaxivity (Caravan et al, 2009). Recently, several $q=2$ complexes with kinetically stable Gd chelation were designed. These complexes do not coordinate with other anions (Supkowski & Horrocks, 1999), therefore reducing the risk of displacement of coordinated water molecules (see also discussion in section 3.2) (Aime et al, 2004; Aime et al, 2006; Werner et al, 2008). Interestingly, the proton residency lifetime (τ_m) has negligible effects at higher magnetic strengths and should not require further optimization. Increasing the outer sphere hydration (number of water molecules coordinated with the complex, but not coordinated directly with the Gd in the inner sphere), however, will increase ionic relaxivity at high fields.

2. A library of nanoparticle platform technologies available for development of MRI contrast agents

There are many important factors to consider when engineering a nanoparticle-based MRI contrast agent. The primary goal is to design a nanoparticle that can deliver a high payload of paramagnetic ions to diseased tissue without negatively affecting the body. Many laboratories are investigating nanoparticle design rules to improve disease targeting by varying size, shape, and composition. We would like to refer the reader to our recent review on design rules for engineering nanoparticles to more efficiently diagnose and treat diseases (Wen et al, 2013). Beyond targeting efficiency, the biocompatibility and toxicity issues related to nanoparticles have effectively halted their clinical translation with a few exceptions (Yildirim et al, 2011). Here, we will make some general comparisons highlighting the biocompatibility advantages and/or disadvantages of each nanomaterial (VNPs, nanoemulsions, dendrimers, silica, gold, and carbon structures) discussed in this review. Additionally, we will be focusing primarily on intravenous injection of nanoparticles; alternative administration routes have different biocompatibility problems (Yildirim et al, 2011).

We are defining biocompatibility here as the body's overall reaction to contact with nanoparticles. Ideal nanoparticles would elicit little to no response from the body other than their timely excretion. This entails generating no immune response, quick tissue clearance (kidney, liver, spleen...), and no additional toxic effects. This nanoparticle, however, does not yet exist.

Surface charge, chemical make-up, and hydrophobicity play a central role in predicting the body's response to nanoparticles. For example, non-charged, hydrophilic materials are less likely to activate an immune response (Caldorera-Moore et al, 2010; Daum et al, 2012). Therefore, liposomes and micelles generate less of an immune response (Peer, 2012). In contrast, the repetitive coat protein assemblies formed by VNPs are known to elicit an

immune response. However, shielding strategies such as PEGylation can be employed to overcome their immunogenicity (Basu et al, 2006; O’Riordan et al, 1999; Park et al, 2010; Raja et al, 2003; Yang et al, 2004; Zhou & Chen, 2011).

Based on the abundance of nanoparticles of all size-regimes, the effects of size are rather well-understood: spheres less than 3 nm in diameter extravasate from tissues nonspecifically, those 3–8 nm in diameter undergo renal clearance, those 30–80 nm in diameter are more likely to be sequestered in the lungs (leaky vasculature), liver and spleen, and finally particles greater than 80 nm in diameter are generally trapped by the liver and spleen (Lunov et al, 2011; Maldiney et al, 2011). Nanomaterials in the size-regime 50–200 nm have also been shown to home to solid tumors based on the enhanced permeability and retention (EPR) effect, a result of leaky vasculature and poor lymphatic drainage (Fang et al, 2011). Size has an effect on clearance as well as disease targeting.

More recent research has turned toward dissecting the impact of nanoparticle shape on *in vivo* behavior. Data indicate distinct biodistribution and disease homing properties of spherical *versus* non-spherical objects. Although more research will be required to comprehensively understand how geometry plays a role in cellular uptake and organ distribution, some general trends have been observed: elongated materials have a lower propensity to be taken up and sequestered in macrophages thus increasing blood pool presence (Champion & Mitragotri, 2006; Geng et al, 2007). Further research indicated that based on distinct flow properties, non-spherical materials have enhanced interaction with the vascular bed and thus a higher propensity to “see” a disease site, resulting in enhanced extravasation into tumor tissue or accumulation of nanoparticles at cardiovascular events (Gentile et al, 2008; Lee et al, 2009). It is clear that besides chemical make up, size and shape of the carrier are engineering handles that must be considered as they impact clearance as well as disease targeting.

2.1 Toxicity and clearance of Gd-based contrast agents

Clearance of nanoparticles from the body is important, especially for Gd-based nanoparticle contrast agents. The potential toxicity of Gd in the body stems largely from its long-term residence in specific tissues, for example, the kidneys leading to nephrogenic systemic fibrosis (NSF) (Grobner, 2005).

It was established in the late 90s, that free Gd ions cause toxicity in liver, spleen, bone marrow, and the lymphatic system with symptoms such as nausea, emesis, hives, and headache (Bellin, 2006; Shellock & Kanal, 1999; Tweedle et al, 1995). In 2006, toxic effects of FDA-approved MRI contrast agents were observed in patients with severe kidney disease; these group of patients developed nephrogenic systemic fibrosis (NSF) (Grobner, 2005). It was found that patients with stage 5 (and rarely stage 4) chronic kidney disease do not clear small molecule MRI contrast agents from the body; the circulation half-life ($t_{1/2}$) increased from 1.5 hours for healthy subjects to 10+ hours for patients with chronic kidney disease; the increased half-life also extends the half-life and exposure in the kidneys, leading to NSF (Prince et al, 2008; Wertman BA et al, 2008; Yerram et al, 2007).

This challenge can be addressed by using the appropriate chelators: data indicate that macrocyclic chelated Gd contrast agents, such as FDA-approved derivatives of DOTA and DO3A are DOTAREM®, Prohance®, Gadovist® are less likely to release Gd ions and therefore less likely to induce NSF compared to linear chelators; examples include FDA-approved derivatives of DTPA, such as Magnevist®, Omniscan®, Eovist®, Multihance®, and Vasovist® (Martin et al, 2010). Chelating molecules with a low equilibrium constants are susceptible to trans-metallization with endogenous metals – such as zinc, copper, and calcium – leading to obstructed ion passage through muscle cells, and block the flow of

these ions in bone epiphyses and nerve tissue cells causing the arrest of neuromuscular transmission (Sharma, 2012; Shellock & Kanal, 1999).

The development of new *in vitro* and preclinical models to assess biocompatibility and toxicity is critical to drive the development of novel nanoparticle-based contrast agents. The recent development of a pre-clinical rat model to evaluate the side effects and the development of NSF due to administration of Gd-based contrast agents is an important stepping stone (Sharma, 2012; Sieber et al, 2008).

The complexation of Gd to a nanocarrier adds a new level of complexity. On the one hand the nanoparticle provides a handle to engineer a desired half-life; size, charge, and surface chemistry can be utilized as handles to control pharmacokinetics (Albanese et al, 2012). On the other hand, although the nanocarrier itself may not induce any side effects (it should be noted that this also needs to be carefully evaluated for a number of nanoparticle platforms), the combination of molecules may induce toxic effects. Once conjugated to the nanoparticle carrier, the biodistribution and clearance of the contrast agent differs. While larger nanoparticles may provide higher Gd-loading and therefore result in increased relaxivities; smaller nanoparticles may be cleared faster through renal clearance and may thus be safer. Nanoparticles (>10 nm) are typically cleared through the macrophagocytotic system (MPS) and not via renal clearance. For some nanoparticles, such as gold, silica, and carbon nanostructures, clearance from the MPS and body can be slow (Jaganathan & Godin, 2012; Liu et al, 2012; Semmler-Behnke et al, 2008; Tarn et al, 2013), which may result in long-term adverse effects in liver and spleen. Initial formulations of dendrimers shared this fate, but synthetic strategies have been devised improving their biodegradability leading to quicker body clearance times (Cheng et al, 2011).

The development pipeline has been moving rapidly, and many new nanoparticle-based MR contrast agents have been designed and synthesized; there is an increasing need to turn toward toxicity studies, to develop methods to decrease Gd toxicity (especially in patients with renal dysfunction) as well as to develop potential alternatives that replace toxic Gd with non-toxic agents.

3. Chemical conjugation strategies available for designing nanoparticle-based MRI contrast agents

3.1 Conjugation chemistry

Based on the inherent toxicity of free Gd ions, coordination chemistry must be used for potential FDA approval and clinical use. Chemical conjugation is the simplest method to immobilize chelated Gd ions to any nanoparticle platform.

The most common functional handles utilized in nanoparticle functionalization are amines and carboxylic acids. Briefly, primary amines are easily susceptible to modification by using *N*-hydroxysuccinimidyl ester (Figure 3E) or isothiocyanate (Figure 3F) derivatized bifunctional chelators to form amide or thiourea bonds, respectively. Carboxylic acids are easily functionalized by carbodiimide activation of amine groups (Figure 3H), and subsequent coupling to form an amide bond. Thiol functional groups react with maleimides (Figure 3C) or other thiols for generate a thioether or dithiol bonds (not shown), respectively. These three reactions can be performed under aqueous conditions and room temperature, therefore forgoing the need to use organic solvents. Nevertheless, these “standard coupling procedures” have slow reaction kinetics and require large excesses of reagents for efficient labeling (Hermanson, 2008). Orthogonal chemistries such as Cu(I)-catalyzed azide-alkyne cycloaddition (click chemistry) (Figure 3G), diazonium coupling (Figure 3D), and oxime ligation (Figure 3A and 3B) are highly efficient alternatives. This is

helpful when reagents are scarce, expensive or if water solubility is a problem. To enable these chemistries ligation handles can be introduced through metabolic labeling (e.g. when working with a nanoparticle of biological origin) or chemical labeling. Finally, material-specific conjugation strategies apply; for example silica and gold nanoparticles are loaded with bifunctional chelators containing trimethoxy (Figure 3I) and thiol groups (not shown), respectively.

3.2 Chelating agents

The most popular chelators used are based on diethylenetriaminepentacetate (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (Figure 4) (Caravan, 2006). These molecules and similar derivatives have been approved for clinical use (Bellin, 2006). Based on the utility and necessity of the chelating agent, bifunctional derivatives of DOTA and DTPA containing *N*-hydroxysuccinimide/isothiocyanates, amine, maleimide, and azide chemical handles for conjugation to amines, carboxylic acids, thiols, and alkynes are commercially available (e.g. Macrocyclics).

Recently, novel Gd chelators with enhanced relaxivities have been synthesized for MRI. Researchers designed molecules capable of strongly chelating Gd ions with two coordinated water molecules ($q=2$). The three core structures that have received significant attention are 6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid (AAZTA), 4-carboxyamido-3,2-hydroxypyridinone (HOPO), and 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid (DO3A) (Figure 4) (Botta & Tei, 2012). The HOPO and AAZTA ligands efficiently coordinate two water molecules ($q=2$) while maintaining stable chelation with Gd ions suitable for *in vivo* application. Furthermore, synthetic analogs of these molecules have been synthesized to optimize their water exchange rates for promising use as high field MRI contrast agents (Werner et al, 2008). Chemical derivations of HOPO and AAZTA should be sought after for engineering the next-generation of nanoparticle MRI contrast agents. Sadly, the DO3A molecule has been proven insufficient for *in vivo* use due to the potential for alternative coordination with bidentate anions *in vivo* therefore reducing the relaxivity of Gd chelated molecules, and also introducing a potential safety concern (Botta et al, 2003; Terreno et al, 2005).

Nanoparticles are loaded with Gd ions using two methods: conjugation of the bifunctional chelator to the nanoparticle platform followed by binding of the Gd ions (in the form of a salt) to the chelator. Alternatively, Gd ions are bound to the chelator prior to conjugation to the nanoparticle. In each case, care should be taken to ensure that no free Gd remains nonspecifically bound to the nanoparticle platform. A common method to remove free Gd is extensive dialysis (2–4 days). Prior to *in vivo* experimentation, quality checks for free Gd ions should be performed using colorimetric chemicals, such as Arsenazo III (Sigma Aldrich).

It should be noted that although complexation of Gd with a chelator reduces safety concerns, side effects sometimes still occur. While chelated Gd is generally well tolerated at high doses, adverse effects, such as nausea, headache, and vomiting have been reported (Bellin, 2006). A primary safety concern is for patients with compromised kidney functions is nephrogenic systemic fibrosis as a result of MRI contrast agent administration (Grobner, 2005). It is hypothesized that extended retention of chelated Gd causes dissociation from the agent resulting in an inflammatory response (Agarwal et al, 2008). For more information regarding the toxicity of clinical MRI contrast agents, please refer to the following reports (Agarwal et al, 2008; Cowper & Boyer, 2006; Grobner, 2005; Knopp & Cowper, 2008; Peak & Sheller, 2007; Shellock & Kanal, 1999; Thomsen et al, 2006).

4. Nanoparticle-based MR contrast agents in development

Here, we will discuss a variety of nanoparticle platforms currently undergoing development and testing as MRI contrast agents. Primary features discussed are biocompatibility, chemical modification, manufacturing, and Gd payload per unit volume as it pertains to improving the relaxivity [$\text{mM}^{-1}\text{s}^{-1}$ per nm^3] of the nanoagent. The relaxivity of the nanoparticle contrast agent is dependent on the structural features of the agent (e.g. rigid vs. flexible) and the degree of Gd-loading. For some materials Gd-loading per nanoparticle is easily quantified e.g. VNPs. However, for other materials, e.g. liposomes, methods to determine the relative Gd amount per nanoparticle formulation are unreliable or not available at the present time. The technological challenge is to define methods that allow reliable quantification of the nanocarrier in solution; VNPs are protein-based materials and standard biochemical assays can be used to quantify the amounts of protein, based on the well-defined structural properties, the nanoparticle concentration is readily determined. In those cases where the degree of Gd-loading per nanoparticle is given, we report the *ionic relaxivity* [$\text{mM}^{-1}\text{s}^{-1}$], the *nanoparticle relaxivity* [$\text{mM}^{-1}\text{s}^{-1}$], as well as the *volumetric relaxivity* [$\text{mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$], in short the **I-N-V relaxivity**. Comparisons can then be made between different nanoconjugates in terms of the efficiency of the formulation (see Table 1 and Figure 9).

4.1 Viral Nanoparticles

VNPs are nature's nanomaterials; their highly monodisperse structures consist of many identical coat protein subunits self-assembled into well-defined, stable structures encapsulating their genomic material (Soto & Ratna, 2010). A wide variety of VNP structures and species are currently under evaluation for biomedical applications (Ma et al, 2012). Structures include icosahedrons (27 – 64 nm in diameter) and rod-shaped or filamentous structures with aspect ratio ranging between 16 – 100 (Figure 5) (Yildiz et al, 2011). Functionalization of these protein-based nanoparticles is easily achieved using bioconjugation reactions targeting select solvent-exposed reactive amino acids (Hermanson, 2008; Pokorski & Steinmetz, 2011). While each VNP displays a unique set of reactive amino acids, the most common targets include the amine groups of lysines, thiol groups of cysteines, carboxylic acid groups of glutamic and aspartic acids, and the phenol group of tyrosines. The interior and exterior surfaces are often accessible for modification based on the VNP's flexible and sometimes porous structure. Furthermore, because VNPs are of biological origin, their surface properties can also be tailored using genetic engineering.

The first chemists to turn toward the study and application of a viral scaffold as a candidate for complexation of Gd were Douglas et al. at Montana State University in 2005. In their studies, the 28 nm-sized cowpea chlorotic mottle virus (CCMV) was utilized: CCMV is comprised of 180 copies of an identical coat protein unit; the structure offers 180 intrinsic metal binding sites at the three-fold axes of the icosahedral capsid structure; under physiological conditions Ca^{2+} ions are bound to these sites. The team showed Ca^{2+} cations could be removed and replaced with Gd^{3+} or Tb^{3+} cations; up to 140 lanthanides could be bound (Allen et al, 2005; Basu et al, 2003). The measured I-N-V relaxivities were $202 \text{ mM}^{-1}\text{s}^{-1}$ – $28,280 \text{ mM}^{-1}\text{s}^{-1}$ – $2.46 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 64 MHz. Genetic engineering was used to incorporate metal binding peptides to further enhance Gd loading and affinity, yielding VNPs loaded with 172 Gd per CCMV with I-N-V relaxivities of $210 \text{ mM}^{-1}\text{s}^{-1}$ – $36,120 \text{ mM}^{-1}\text{s}^{-1}$ – $3.14 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 64 MHz (Liepold et al, 2007). This work presents a nice example highlighting the unique opportunities in structure-based VNP engineering. However, we must note that while the ionic relaxivity is impressively high, in fact the highest ionic relaxivity reported to date, the particles cannot be used in vivo due to the potential toxicity of releasing Gd. To overcome this hurdle, next steps were to covalently

attached chelators (DOTA) for safe complexation of the paramagnetic ions; 60 chelated Gd per CCMV were introduced resulting in I-N-V relaxivities of $46 \text{ mM}^{-1}\text{s}^{-1} - 2,806 \text{ mM}^{-1}\text{s}^{-1} - 0.24 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 64 MHz (Liepold et al, 2007).

Consequently, several other VNP icosahedrons have been developed as potential MRI contrast agents. For example, the 30 nm-sized cowpea mosaic virus (CPMV) and bacteriophage Q β , underwent a two-step bioconjugation protocol to covalently link Gd(DOTA) to their exterior surfaces (Prasuhn et al, 2007). CPMV and Q β were first coated with azido groups by reacting lysine residues with *N*-hydroxysuccinimide azide followed by a click reaction with a custom synthesized DOTA alkyne chelated with Gd. CPMV and Q β had a I-N-V relaxivities of $12.8 \text{ mM}^{-1}\text{s}^{-1} - 2,850 \text{ mM}^{-1}\text{s}^{-1} - 0.20 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ and $11.9 \text{ mM}^{-1}\text{s}^{-1} - 1,820 \text{ mM}^{-1}\text{s}^{-1} - 0.13 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 64 MHz, respectively. In addition, the affinity of lanthanides for RNA was investigated using the RNA-containing CPMV nanoparticles. Approximately 80 Gd³⁺ ions could be loaded per CPMV, with similar values observed for Tb³⁺ ions. Dual-labeled CPMV particles were also generated by covalent conjugation of Gd-DOTA followed by internal binding of Gd³⁺ (Prasuhn et al, 2007). The CPMV formulation is the only formulation evaluated in preclinical mouse studies: CPMV loaded with Gd-DOTA on the exterior and Gd³⁺ loaded in the interior showed similar plasma clearance kinetics, with very low levels of particles detected in the bloodstream after 30 minutes and 90% of the injected dose deposited in the liver (Singh et al, 2007). Histological examination was also conducted and showed no evidence of pathology, indicating that the CPMV constructs are safely cleared from the system with no toxic effects (Singh et al, 2007).

With the desire to increase payload, a recent strategy reported also by the Finn Lab at The Scripps Research Institute focused on the synthesis of VNP-polymer hybrids. Polymers were grafted from the surface of Q β phage using atom transfer radical polymerization; the incorporation of azide functional groups into the polymer side chains facilitated subsequent labeling with Gd(DOTA) alkyne to afford a VNP containing 610 Gd moieties per Q β nanoparticle, resulting in I-N-V relaxivities of $11.6 \text{ mM}^{-1}\text{s}^{-1} - 7,094 \text{ mM}^{-1}\text{s}^{-1} - 0.50 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 60 MHz (Pokorski et al, 2011). Similarly, Douglas and coworkers engineered a dendrimer network on the interior of the genetically engineered (K118C) 64 nm-sized wiffleball structure of bacteriophage P22 (Qazi et al, 2012). This was achieved by anchoring an alkyne group to interior cysteine residues using a bromoacetamide-alkyne followed by successive click reactions of di-azido-Gd(DTPA) complex and tripropargyl amine to achieve up to 7 Gd ions on each of the 420 capsid proteins, a total of 1900 Gd(DTPA) per P22 nanoparticle. The measured I-N-V relaxivities were $14.0 \text{ mM}^{-1}\text{s}^{-1} - 26,600 \text{ mM}^{-1}\text{s}^{-1} - 0.19 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 60 MHz.

The Francis Lab at UC Berkeley has focused their efforts toward engineering the 27 nm-sized bacteriophage MS2. Specifically, MS2 phage was used as a model system to address how the local environment and stiffness of the linker would impact the ionic relaxivity (Datta et al, 2008; Garimella et al, 2011; Hooker et al, 2007). In brief, a derivative of aforementioned HOPO ligand (TREN-bis-HOPO-TAM) was synthesized with appropriate ligation handles for coupling to tyrosine residues (on the interior surface) or lysine residues (on the exterior surface). Display of the contrast agent at the stiffer tyrosine residues (93 Gd per MS2) resulted in higher ionic relaxivity compared to conjugation to the more flexible lysine side chains (81 Gd per MS2) with I-N-V relaxivities of $30.9 \text{ mM}^{-1}\text{s}^{-1} - 2,883 \text{ mM}^{-1}\text{s}^{-1} - 0.20 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ versus $23.2 \text{ mM}^{-1}\text{s}^{-1} - 1,880 \text{ mM}^{-1}\text{s}^{-1} - 0.13 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 60 MHz (Datta et al, 2008). Further chemical modification of the HOPO ligand was carried out to further increase stiffness of the chelating agent; these efforts resulted in further enhancement of ionic relaxivities and I-N-V relaxivities of $41.2 \text{ mM}^{-1}\text{s}^{-1}$

$-7,416 \text{ mM}^{-1}\text{s}^{-1} - 0.52 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 60 MHz with display of 180 Gd per MS2 (the target site was a genetically introduced cysteine side chain) (Garimella et al, 2011).

Finally, our lab at Case Western Reserve University recently developed the first rod-shaped VNP-based MRI contrast agent. Specifically, the rod-shaped tobacco mosaic virus (TMV) measuring $300 \times 18 \text{ nm}$ in its native form was used as a starting material. The interior (glutamic acids) and exterior (tyrosine) surfaces were selectively labeled with Gd(DOTA) molecules using a two-step bioconjugation process. The high labeling efficiency of these reactions yielded TMV rods loaded with 3417 Gd(DOTA) at interior sites or 1712 Gd(DOTA) at the exterior surface. The measured I-N-V relaxivities of the interior and exterior labeled rods were $10.7 \text{ mM}^{-1}\text{s}^{-1} - 36,562 \text{ mM}^{-1}\text{s}^{-1} - 0.48 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ and $18.4 \text{ mM}^{-1}\text{s}^{-1} - 31,501 \text{ mM}^{-1}\text{s}^{-1} - 0.41 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 64 MHz, respectively. Finally, we applied a recently established heating protocol to induce thermal transition of TMV rods into TMV spherical nanoparticles (SNPs) measuring 170 nm in diameter; these TMV SNPs contain several TMV rods and thus have an increased Gd content measured on a per particle basis. SNPs contained over 25,000 Gd ions per SNP with I-N-V relaxivities of $15.2 \text{ mM}^{-1}\text{s}^{-1} - 380,000 \text{ mM}^{-1}\text{s}^{-1} - 0.15 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$; this formulation has the highest per particle relaxivity among all VNP contrast agents (Bruckman et al, 2013).

In summary, several VNP-based materials are under development as MRI contrast agents; an *in vivo* MRI performance of these agents, however, has not been reported to date.

4.2 Micelles and Liposomes (Nanoemulsions)

Nanoemulsions, such as liposomes and micelles, are the most widely studied nanometer-scale platforms for MRI contrast agent development. The ease of preparation, scalability, the use of simple and (mostly) inexpensive reagents, and the ability to control their size and membrane permeability explain their popularity. Nanoemulsions are self-assembled from small molecules made of a hydrophilic domain with a hydrophobic tail, referred to as amphiphiles or surfactants (Figure 6). The size, morphology, and surface properties of nanoemulsions are controlled by the composition of hydrophobic tail, size and charge of the hydrophilic head, and assembly conditions, such as concentration of amphiphiles, pH, and temperature (Israelachvili et al, 1976). Micelles are core-shell structures in the size range of $5\text{--}50 \text{ nm}$ with the interior containing the hydrophobic chains (Jones & Leroux, 1999). The most important parameter of micelle formation is the critical micellar concentration (cmc), which is the amphiphile concentration required for micelle formation. While micelles form stable structures at the cmc for extended periods of time, they often disassemble upon dilution in blood. This has been problematic for *in vivo* experimentation and potential translation. Liposomes are nanoemulsions characterized by their self-assembled lipid bilayers (which can be unilamellar or multilamellar) entrapping an aqueous core measuring between $50\text{--}1000 \text{ nm}$ (Torchilin, 2005). Unilamellar vesicles (Figure 6) are the most common liposomes used in biomedicine.

Nanoemulsions are modified using one of three general protocols (Figure 6); (Figure 6 – A) incorporating desired molecules into the nanoemulsions during the self-assembly process, (Figure 6 – D) hydrophobic/hydrophilic molecules associating with the hydrophobic tail sections or encapsulation in the aqueous core of liposomes and special amphiphilic molecules, respectively. Alternatively, (Figure 6 – B) maleimide capped (reactive with thiols) and (Figure 6 – C) biotin capped (for biotin-avidin binding) amphiphiles can be used for post-assembly modification (other functionalities have been explored with success).

Nanoemulsions used for MRI applications are formed primarily from amphiphilic Gd chelators using the general rules of nanoemulsion assembly. The first use of Gd loaded nanoemulsions as MRI contrast agents was published around 25 years ago when researchers

synthesized and assembled an amphiphilic DTPA derivative into micelles (Devoisselle et al, 1988; Fossheim et al, 1999; Tilcock et al, 1989; Unger et al, 1988). Over the years, a wide variety of amphiphilic chelators have been synthesized for MRI applications based on DTPA, DOTA, DO3A, and AAZTA chelator molecules (Accardo et al, 2009). Micelle and liposome MRI contrast agents have demonstrated increased ionic relaxivities based on reduced molecular rotation (or tumbling) effects (Botta & Tei, 2012). Unfortunately, because nanoemulsions are soft nanoparticles, the ionic relaxivity (per Gd) often is only marginally increased compared to free Gd-DOTA (Accardo et al, 2009; Caravan, 2006; Carniato et al, 2010).

Several properties of nanoemulsions can lower relaxivities:

- **Packing:** The close packing of Gd ions upon micelle formation keeps relaxivity low: when two paramagnetic centers get in close proximity, the transverse electronic relaxivity (τ_e) of the ions increases, which in turn decreases the proton relaxivity (Nicolle et al, 2003). Increased ionic relaxivities have been achieved through reduction of the Gd-load per micelle by substituting amphiphilic Gd-chelates with amphiphilic Y-chelates (Gianolio et al, 2007).
- **Water permeability:** Formulation of Gd-containing liposomes experiences a different challenge. When liposomes are formed with Gd amphiphiles, some of the Gd ions will reside on the interior surface. Interior located Gd ions will not interact with the bulk water, and therefore do not contribute to the overall relaxivity. Engineering increased lipid bilayer water permeability has therefore become important (Terreno et al, 2008). Increased relaxivities were found for liposomes formed with bilayers with high water flux (Koenig et al, 1992). The water permeability of the lipid bilayer depends primarily on the chemical make-up of the hydrophobic portion of the amphiphilic molecules. Specifically, permeability increases with shorter alkyl chains in addition to the presence of one or more unsaturated bonds (Drummond et al, 1999).
- **Size:** Increasing the size of the liposome can lead to reduced relaxivity possibly due to formation of multilamellar bilayers with lower permeability and reduced surface area to volume ratio (Tilcock et al, 1989; Unger et al, 1991). Consequently, smaller liposomal assemblies with short alkyl chains and unsaturated bonds have higher water permeability and therefore higher relaxivities. The highest ionic relaxivities are observed by placing Gd ions on only the exterior surfaces liposomes (Laurent et al, 2008).

In the following sections, we will highlight several interesting examples with ionic relaxivities up to $40 \text{ mM}^{-1}\text{s}^{-1}$. It is difficult to determine how many amphiphiles, specifically Gd-labeled amphiphiles, are incorporated into a micelle or liposome of specific size and composition. Therefore, there are relatively few reports that include an estimate of Gd loading per nanoemulsion and accurate I-N-V relaxivities are only partially reported.

Several studies were conducted dissecting the parameters to generate Gd-loaded micelles and/or liposomes with high relaxivities: the length and chemical structure of the hydrophobic component were analyzed (Andre et al, 1999; Nicolle et al, 2002). Using the Solomon-Bloembergen-Morgan model it was extrapolated that increasing the hydrophobic tail length would decrease the molecular tumbling rate, resulting in increased relaxivity. For example, DOTA with a 14-carbon tail (Gd(DOTAC14)) assembled into micelles has an ionic relaxivity of $22.0 \text{ mM}^{-1}\text{s}^{-1}$ at 60 MHz while DOTA with a 10-carbon tail (Gd(DOTAC10)) micelles have an ionic relaxivity of $10.8 \text{ mM}^{-1}\text{s}^{-1}$ at 60 MHz (Andre et al, 1999; Nicolle et al, 2002)

Additional efforts to reduce molecular tumbling rates were focused on the study of different chemical structure and composition of the chains: GdDOTA(GAC₂)₂, which is a DOTA derivative with two C₁₄ chains instead of one, was studied and developed (Kielar et al, 2010). It was shown that these bis-substituted Gd-DOTA chains assembled into micelles (16 nm) and liposomes (45–60 nm) with remarkably high ionic relaxivity values of 34.8 mM⁻¹s⁻¹ and 40.0 mM⁻¹s⁻¹ per Gd at 20 MHz, respectively. By comparison, the mono-substituted C₁₄ Gd-DOTA micelles and liposomes had ionic relaxivities of 15.4 mM⁻¹s⁻¹ and 17.0 mM⁻¹s⁻¹ per Gd at 20 MHz.

Fine-tuning of the hydrophilic head group can further enhance ionic relaxivities. In general, more rigid head group structures enhance ionic relaxivity. For example, substitution of DOTA chelator with 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetate (PCTA) (a stiffer DOTA derivative) resulted an increased ionic relaxivity from 18 to 29 mM⁻¹s⁻¹ at 20 MHz (Hovland et al, 2003).

To further improve ionic relaxivity, chelator amphiphiles that coordinate with two water molecules can be synthesized ($q=2$). Synthesis and assembly of Gd-AAZTAC₁₇ into 5.5 nm micelles (95 Gd per micelle) yielded I-N-V relaxivities of ~30 mM⁻¹s⁻¹ – 2,850 mM⁻¹s⁻¹ – 32.71 mM⁻¹s⁻¹nm⁻³ at 60 MHz (Gianolio et al, 2007). The authors replaced 98% of Gd(III) with Y(III) obtaining an I-N-V of ~40 mM⁻¹s⁻¹ – 80 mM⁻¹s⁻¹ – 0.92 mM⁻¹s⁻¹nm⁻³ at 60 MHz. It should be noted, that while the ionic relaxivity is increased, the particle and volumetric relaxivities decrease based on the reduced Gd-loading. This study confirmed that decreased transverse electronic relaxivity (as a result of less concentrated or dense Gd packing), enhances the water proton relaxivity of the system (Nicolle et al, 2003).

In subsequent studies, co-assembly of micelles with lipoproteins also improved the ionic relaxivities of the Gd-AAZTAC₁₇. Co-assembly with high-density lipoprotein (HDL) increased the I-N-V relaxivities to 35.5 mM⁻¹s⁻¹ – 2,840 mM⁻¹s⁻¹ – 4.31 mM⁻¹s⁻¹nm⁻³ at 60 MHz for a 10.8 nm-sized nanoparticle with 80 Gd ions per particle (Briley-Saebo et al, 2009). These nanoparticles were used for imaging cardiovascular inflammation by natural uptake of HDL to inflammatory sites.

In summary, intensive research and development of Gd-based micelles and liposomes has paved the way for disease diagnosis by MRI using nanoparticles. (We would like to refer the reader for the following reviews for more information on micelle-based and liposomal MRI contrast agents (Accardo et al, 2009; Mulder et al, 2006)).

4.3 Dendrimers

Dendrimers are well-defined, monodisperse hyperbranched nanoparticles widely used in biomedical research (Menjoge et al, 2010). The most popular families of dendrimers used in biomedicine include polyamidoamines (PAMAM) (most popular), polypropyleneimines (PPI), and polylysines (PLL) (Grayson & Frechet, 2001). These dendrimer structures present amine functional groups on their periphery amenable to modification via amine reactive chemistries (see section 3.1). Whereas nearly all dendrimer-based MRI contrast agents are based on PAMAM, PPI or PLL, new strategies are continuously being developed to improve biocompatibility by incorporating cleavable bonds into the dendrimer backbone (Menjoge et al, 2010).

Dendrimer structures are characterized by the number of tree branches, referred to as generations (G), with the number of functional groups growing exponentially with each generation (Figure 7). Each additional generation typically increases the diameter by approximately 1 nm (Venditto et al, 2005). In contrast to VNPs and micelles/liposomes, dendrimer structures useful for biomedical application are limited to sizes below 15 nm

(G1–G10). Although larger dendrimers can be constructed, G10 and higher are not stable in aqueous buffer conditions therefore limiting their applicability for *in vivo* imaging (Kobayashi et al, 2001a). The biocompatibility and toxicity, specifically of amine-based dendrimers is a concern: based on the high number of positive charges, cellular cytotoxicity increases as a function of dendrimer generation using PAMAM and PPI based structures (Malik et al, 2000; Zinselmeyer et al, 2002). However, data indicate that PAMAM and PLL dendrimers up to G5 are biocompatible (Roberts et al, 1996; Sadler & Tam, 2002).

As with other nanoparticles, clearance and *in vivo* fate are determined by size. Here, G1–G5 dendrimers measuring 1 to 5 nm are cleared primarily via the kidneys, G6 and higher, 6 nm and larger, clearance is mainly through the lymph nodes, lungs, liver, and spleen (Figure 7) (Kobayashi et al, 2004; Kobayashi et al, 2001b; Kobayashi et al, 2003; Kobayashi et al, 2001c; Menjoge et al, 2010). Biocompatibility of dendrimers has been improved through PEGylation, however this typically results in lowered relaxivities (Kojima et al, 2011). For a more detailed overview on dendrimer pharmacokinetics and biodistribution, the reader is referred to the following recent reviews (Menjoge et al, 2010; Sadekar & Ghandehari, 2012; Wijagkanalan et al, 2011).

The development and application of dendrimers as MRI contrast agents began nearly two decades ago with pioneering studies covalently attaching Gd-DTPA molecules to PAMAM dendrimers (Adam et al, 1994; Wiener et al, 1994). As with other nanoparticles, increased ionic relaxivity of Gd chelates upon conjugation to dendrimers is attributed to reduced molecular rotational correlation times (Langereis et al, 2004). The ionic relaxivities of most dendrimer-based contrast agents fall in the range from 10 to 40 mM⁻¹s⁻¹ at 20 MHz using the common chelators of DOTA, DTPA, and DO3A (Boswell et al, 2008; Han et al, 2011; Kaneshiro et al, 2008; Langereis et al, 2004; Langereis et al, 2006; Margerum et al, 1997; Rudovský et al, 2006; Rudovský et al, 2005; Swanson et al, 2008). As the size of the dendrimers increases, so does the ionic relaxivity. However, the differences become less significant in higher generations. For example, PAMAM dendrimers loaded with GdDOTA behaved as follows: G5 loaded with 96 Gd ions, G7 loaded with 380 Gd ions, G9 loaded with 1320 Gd ions, and G10 loaded with 1860 Gd ions yielded contrast agents with I-N-V relaxivities (dendrimer sizes based on Figure 7) of 30.0 mM⁻¹s⁻¹ – 2,880 mM⁻¹s⁻¹ – 69.17 mM⁻¹s⁻¹nm⁻³ (G5), 35.0 mM⁻¹s⁻¹ – 13,300 mM⁻¹s⁻¹ – 49.61 mM⁻¹s⁻¹nm⁻³ (G7), 36.0 mM⁻¹s⁻¹ – 47,520 mM⁻¹s⁻¹ – 47.59 mM⁻¹s⁻¹nm⁻³ (G9), and 36.0 mM⁻¹s⁻¹ – 66,960 mM⁻¹s⁻¹ – 40.25 mM⁻¹s⁻¹nm⁻³ (G10) at 20 MHz, respectively (Bryant et al, 1999).

Attempts to further increase relaxivity have been made using various bifunctional chelators: for example, a HOPO-based, TREN-capped Gd chelate (*q*=2) was attached to the biocompatible esteramide (EA) and PLL dendrimers (Floyd et al, 2011). The resulting G3 dendrimers (estimated to have a diameter of 3 nm) provided 8 reactive sites, of which 6 (EA) and 4.5 (PLL) Gd ions were bound resulting in I-N-V relaxivities of 38.1 mM⁻¹s⁻¹ – 228 mM⁻¹s⁻¹ – 16.19 mM⁻¹s⁻¹nm⁻³ and 21.0 mM⁻¹s⁻¹ – 94 mM⁻¹s⁻¹ – 6.68 mM⁻¹s⁻¹nm⁻³ at 60 MHz, respectively (Kojima et al, 2011).

In summary, based on the ease of synthesis and high ionic relaxivities, dendrimer-based MR contrast agents provide an interesting class of candidate materials; and as new materials and chemistries emerge, there is good potential for the engineering of biocompatible agents for future translation into the clinic. Dendrimer based MRI contrast agents have the highest relaxivity per volume reaching up to 70 mM⁻¹s⁻¹nm⁻³ due to their small size and compactness.

4.4. Silica-based nanoparticles

Silica-based materials are used as carrier materials for delivery of cargo (imaging or therapeutics) to sites of disease because they are easily synthesized and functionalized, have a high surface area and are biocompatible (Anglin et al, 2008; Lin et al, 2012; Vallet-Regí et al, 2007). Silica nanoparticles are readily manufactured in a variety of well-controlled sizes (20 nm to micron-sized) and geometries (rods and spheres); silica nanoparticles are porous materials with tunable pore diameters (1 to 10 nm) (see Figure 8) (Chiappini et al, 2010; Jaganathan & Godin, 2012; Tang et al, 2012). Although generally accepted as biocompatible materials, the *in vivo* behavior of silica-based materials depends on the particle size, porosity (surface area), surface charge, and shape (Jaganathan & Godin, 2012). Modification strategies, such as PEGylation, have also been developed to further improve *in vivo* properties such as pharmacokinetics (Vivero-Escoto et al, 2011).

Porous, silica-based nanoparticles can be selectively modified at their three surfaces; the silica framework, internal pores, and exterior surface (Figure 8). Modification is accomplished using two methods (Brühwiler, 2010; Jin et al, 2009): first, co-condensation, or one-pot synthesis, in which the desired functionality (here chelated Gd molecules) are modified with a silane functional group and added to the silane precursor reaction prior to the sol-gel condensation incorporating the desired functionality into the silica framework. The second method is based on post-functionalization of the silica-based nanoparticles on either the exterior surface or inside their pores. This is accomplished by reacting a functional triethoxysilane (TEOS) molecule with the surface silanol groups present on the silica nanoparticles. If a TEOS derivative of a molecule is difficult to synthesize, amine (and other functional groups) can also be incorporated onto the silica surface through reaction with aminopropyltriethoxysilane (or similar compounds).

Here, we can provide a more comprehensive review of the literature, because development of silica-based nanoparticles as MRI contrast agents is still a relatively young field with only a few literature reports. Initially, Gd ions were incorporated into 100 nm mesoporous silica nanoparticles at variable concentrations of 1.6%, 2.3%, 3.1%, and 6.8% (w/v). Ionic relaxivity decreased with increasing Gd concentration; indicating that at high loading concentration, transverse electronic interactions reduced the ionic relaxivity, as observed for nanoemulsion loaded with high amounts of Gd ions (see section 4.2). Nevertheless, rather high ionic relaxivities of $23.6 \text{ mM}^{-1}\text{s}^{-1}$ (1.6%), $20.8 \text{ mM}^{-1}\text{s}^{-1}$ (2.3%), $7.2 \text{ mM}^{-1}\text{s}^{-1}$ (3.1%), and $4.4 \text{ mM}^{-1}\text{s}^{-1}$ (6.8%) at 400 MHz were achieved (Lin et al, 2004). While this particular formulation is not suitable for *in vivo* medical applications, based on weakly-bound Gd ions, which may dissociate *in vivo*, the reported high relaxivity at *high magnetic field strength* (400 MHz) is interesting and supporting the further development of silica-based materials for MRI.

Following these studies, various silica-based MRI candidate formulations have emerged, all of which employ covalent conjugation of chelated Gd. For example, 40 nm-sized mesoporous silica nanoparticles were post-functionalized using trimethoxysilyl-derivatized Gd-DTTA molecules (Gd-Si-DTTA) and bis(silylated)-Gd-DTPA (Gd-Si-DTPA) (Rieter et al, 2007). The Gd-Si-DTTA (10,200 Gd per nanoparticle) and Gd-Si-DTPA (63,200 Gd per nanoparticle) nanoparticles had I-N-V relaxivities of $19.7 \text{ mM}^{-1}\text{s}^{-1} - 200,940 \text{ mM}^{-1}\text{s}^{-1} - 7.58 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ and $7.8 \text{ mM}^{-1}\text{s}^{-1} - 492,960 \text{ mM}^{-1}\text{s}^{-1} - 14.71 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 128 MHz, respectively. Subsequently, larger 75 nm-sized mesoporous silica nanoparticles functionalized with Gd-DTTA were applied in *in vitro* cell studies and *in vivo* biodistribution MR imaging studies (Taylor et al, 2008). The measured I-N-V relaxivities for those materials were $28.8 \text{ mM}^{-1}\text{s}^{-1} - 699,840 \text{ mM}^{-1}\text{s}^{-1} - 3.17 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 128 MHz and $10.2 \text{ mM}^{-1}\text{s}^{-1} - 247,860 \text{ mM}^{-1}\text{s}^{-1} - 1.12 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 400 MHz. Again, we would like to point out the high ionic relaxivities at high magnetic field strength. So far,

these are the highest ionic relaxivities measured at high magnetic strength reported using biologically safe formulations.

To study the effect of pore shape and size, Gd-loaded DOTA was conjugated to spherical 20–50 nm-sized MCM-41 silica nanoparticle with pore size of 2.5 nm, and 650 nm SBA-15 silica nanoparticles with rod-shaped pores 8.5 nm in diameter (MCM-41 and SBA-15 are the most common types of mesoporous silica materials) (Carniato et al, 2009). The I-N-V relaxivities of Gd-labeled MCM-41 (360 Gd per nanoparticle) and SBA-15 (2.98×10^7 Gd per nanoparticle) were measured at $27.0 \text{ mM}^{-1}\text{s}^{-1} - 9,720 \text{ mM}^{-1}\text{s}^{-1} - 0.43 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ and $6.7 \text{ mM}^{-1}\text{s}^{-1} - 199,660,000 \text{ mM}^{-1}\text{s}^{-1} - 1.39 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 20 MHz, respectively. The lower relaxivity for Gd-SBA-15 *versus* MCM-41 was explained by the varying pore sizes: the paramagnetic centers likely diffused into the larger pores of the nanorods but not inside the smaller pores of the spheres, therefore the Gd ions conjugated to SBA-15 were less solvent accessible compared to the centers presented on the surface of the spheres. On the other hand, per particle relaxivities are extremely high for the SB-15 material, based on the high loading with Gd ions.

The impact of chelator on ionic relaxivity was evaluated (Carniato et al, 2010): specifically, DOTA molecules with two linker lengths were tested as well as a $q=2$ DO3A derivative. Results indicate that use of DOTA molecules resulted in higher relaxivity compared to incorporation of DO3A derivative; further the shorter linker yielded the highest ionic relaxivity with I-N-V relaxivities of $37.8 \text{ mM}^{-1}\text{s}^{-1} - 29,484 \text{ mM}^{-1}\text{s}^{-1} - 1.31 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 20 MHz. The increased ionic relaxivity is a result of increased rigidity of the chelator (see discussion above).

Finally, ionic relaxivities can be maximized by isolating the Gd-DOTA molecules to the entryway of the pores in mesoporous silica nanoparticles as shown in Figure 8 (Davis et al, 2012). In brief, 62 nm-sized mesoporous silica nanoparticles were loaded with 14,700 Gd molecules at the entryway of the pores achieving I-N-V relaxivities values of $39.3 \text{ mM}^{-1}\text{s}^{-1} - 577,120 \text{ mM}^{-1}\text{s}^{-1} - 4.62 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ and $33.5 \text{ mM}^{-1}\text{s}^{-1} - 492,450 \text{ mM}^{-1}\text{s}^{-1} - 2.99 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 128 and 300 MHz, respectively. We want to point out once more the high ionic relaxivities at high magnetic field strength; this has not yet been accomplished with any other material. A mechanism for this observation has not yet been reported.

In conclusion, based on their biocompatibility, ease of synthesis and modification, silica-based nanomaterials present an interesting and potentially promising class of MRI contrast agents.

4.5 Gold nanoparticles

Gold nanoparticles are used in a wide variety of nanotechnology-based applications (Sperling et al, 2008; Wilson, 2008). Their use for *in vivo* applications, such as imaging, is somewhat controversial because several studies have indicated slow tissue clearance leading to increased toxicity effects. Synthesis and functionalization of gold spherical and rod-shaped nanoparticles is well understood (Dreaden et al, 2012). Gold nanoparticles are also excellent candidates for X-ray computed tomography imaging (CT) enhancement because of their high electron density (Dreaden et al, 2012). Recent efforts labeling gold with Gd lead to the development of bimodal MRI-CT contrast agents (Beija et al, 2012).

The first Gd-labeled gold nanoparticles was functionalized with dithiol-DTPA derivative. Here, 2.4 nm-sized gold nanoparticles yielding approximately 150 Gd per nanoparticle (the ionic relaxivity matched that of free Gd) with I-N-V relaxivities of $3.9 \text{ mM}^{-1}\text{s}^{-1} - 585.39 \text{ mM}^{-1}\text{s}^{-1} - 28.42 \text{ mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 300 MHz (Debouttière et al, 2006). Bimodal imaging was fully realized by tracking gold nanoparticle biodistribution in mice using CT

and MRI (Alric et al, 2008). Gold nanoparticles of increasing size were functionalized with Gd-DTPA-SH and the results demonstrated that the ionic relaxivities increased with size. Gold nanoparticles of 2, 4, and 6 nm had ionic relaxivities of 4.8, 8.4, and 11.0 $\text{mM}^{-1}\text{s}^{-1}$ at 300 MHz (Warsi & Chechik, 2011). The size-dependent increase of the ionic relaxivity can be explained by reduction of molecular rotation with increased size. Further efforts include the application of alternative chelators. For example, immobilization of 70 Gd-DO3A molecules on a 4.8 nm-sized gold nanoparticles led to I-N-V relaxivities of 20.0 $\text{mM}^{-1}\text{s}^{-1}$ – 1,400 $\text{mM}^{-1}\text{s}^{-1}$ – 24.17 $\text{mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 30 MHz (Ferreira et al, 2012). The increased water exchange rate of the DO3A chelator led to an increase in ionic relaxivity in this case.

By far the most impressive result for a gold-based MRI contrast agents was achieved through conjugation of 56 Gd-DTTA-SH molecules to the surface of 2.3 nm gold nanoparticle, yielding a hydrodynamic diameter of 4.8 nm. The I-N-V relaxivities were estimated at $\sim 60.0 \text{ mM}^{-1}\text{s}^{-1}$ – 3,360 $\text{mM}^{-1}\text{s}^{-1}$ – 58.02 $\text{mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$ at 30 MHz (Moriggi et al, 2009).

In summary, gold has played a significant role in biomedical nanoparticle research for decades and it continues to be an excellent candidate material, also as a candidate for MRI contrast agents. However, toxicity and biocompatibility concerns are evident here.

4.6 Carbon-based nanoparticles and tubes

The recent excitement of carbon nanostructures for biomedical applications stem from their unique mechanical properties, high surface areas, and available chemistries for functionalization (Anilkumar et al, 2011; Liu et al, 2012; Mochalin et al, 2011; Pantarotto et al, 2003). Carbon-based nanoparticles can be synthesized as 1 nm-sized spherical fullerenes, 5 nm-sized fluorescent nanodiamonds, or carbon nanotubes with controllable diameters and lengths. Engineering carbon nanoparticles with functional groups is achieved by activation of the carbon structure using an oxidation process to impart carboxylic acid functionality. From there, the nanoparticles can be functionalized using carbodiimide-coupling techniques with primary amines (see section 3.1). One of the major concerns with carbon nanostructures is the potential toxicity affects due to persistence in tissue and cells (Liu et al, 2012; Mochalin et al, 2011).

With regards to MRI contrast agent development, the most effort has been focused on loading the interior of fullerenes with Gd ions (referred to as gadofullerenes) (Zheng et al, 2012). Essentially, during synthesis of the fullerenes, one to three Gd ions can be trapped inside the fullerene cage. Since the entrapped Gd ions are not coordinated with a chelating agent, they coordinate with more water protons and have a fast water exchange rate. This strategy has yielded MR candidate materials with ionic relaxivities as high as 20 to 80 $\text{mM}^{-1}\text{s}^{-1}$. These relaxivities are generally maintained in high magnetic field strengths (Zheng et al, 2012). However, the biological safety of these compounds has yet to be demonstrated.

Similarly, carbon nanotubes have been chelated directly with Gd ions (referred to as gadonanotubes). These structures have ionic relaxivities up to 170 $\text{mM}^{-1}\text{s}^{-1}$ at 60 MHz (Ananta et al, 2010; Hartman et al, 2008; Sitharaman et al, 2005). Another example of carbon-based materials are nanodiamonds, which are generated by exploding diamonds. Nanodiamonds have been covalently modified with Gd-DO3A-amine to afford an ionic relaxivity of 58.8 $\text{mM}^{-1}\text{s}^{-1}$ at 64 MHz (Manus et al, 2010).

While the ionic relaxivities of these carbon-based nanostructures are incredibly high, careful safety considerations must be evaluated. Persistence in tissues followed by release of the

non-chelated Gd ions (in case of gadofullerenes/nanotubes) could induce severe health problems.

5. Conclusions and future perspectives

In conclusion, research has demonstrated the promise of nanoparticle-based MRI contrast agents; nanoparticles are promising candidates to deliver a high payload of lanthanide ions, such as Gd, to enhance longitudinal relaxivity for improved MRI. Each platform has its advantages and disadvantages in terms of chemistry, availability, production costs, and biocompatibility properties. The field is too young to draw conclusions in terms of which platform is best suited for clinical use. Important factors to consider when choosing a specific nanoparticle are the biocompatibility, size, shape, and the payload or relaxivity per volume. Since the local environment impacts the ionic relaxivity, the choice of ligation strategy and bifunctional chelator is almost as important as the nanoparticle platform itself. Since clinical MRI is moving toward the implementation of MRI systems with high field strengths, bifunctional chelators that maintain high relaxivity enhancement in high magnetic fields (3.0 and higher) need to be pursued. Future studies must turn toward the evaluation of *in vivo* safety of the large library of agents already at our disposal.

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Figure 1.
Summary of nanoparticle formulations discussed in this review article and their size ranges.

Figure 2.
Schematic of the contributing factors for ionic relaxivity enhancement.

Figure 3.
Chemical conjugation methods used to functionalize nanoparticles.

Figure 4.
Chemical structures of common Gd chelating molecules.

Functional group	Chemistry	VNP (Location - #/VNP)
Amine	NHS or isothiocyanate	CPMV (e - 180), Q β (e - 720), MS2 (e - 540), CCMV (e - 540)
Thiol	Maleimide	MS2 (i(ge) - 180), P22 (i(ge) - 420)
Phenol	Diazonium + click	TMV (e - 2130), MS2 (i - 180)
Acid	Amine + click	TMV (i - 4260)
Azide/Alkyne	Azide/Alkyne	TMV, CPMV, Q β

Figure 5.

Crystal structures of viral nanoparticles (VNPs) currently explored as MRI contrast agents. The table shows the available functional groups for modification and their location: exterior (e) versus interior (i). Attachment sites can either be found in native VNP shells or, if a desired functional group is unavailable, it can be introduced via genetically engineering (ge). The number of reactive sites is indicated, e.g. CPMV has 180 amines on its exterior surface.

Figure 6.

Amphiphiles of varying compositions are assembled into micelles or liposomes. Micelles and liposomes can be modified by incorporating amphiphiles with (A) Gd chelates, (B) functional groups for further chemical conjugation, or (C) biotin for biotin-avidin binding. Encapsulation can be accomplished with liposomal assembly.

Generation	Terminal AC core Groups	Terminal Groups EDA core	Approximate diameter (nm)	Excretion Route
0	3	4	1	Kidney
1	6	8	1-2	Kidney
2	12	16	2-3	Kidney
3	24	32	3-5	Kidney
4	48	64	4-6	Kidney
5	96	128	4-7	Mostly Kidney
6	192	256	6-9	Kidney and Liver
7	384	512	7-11	Mostly Liver
8	768	1024	9-13	Liver
9	1536	2048	10-14	Liver
10	3072	4096	12-15	Liver

Figure 7. Common dendrimer cores and their modification strategies. A chart showing dendrimer generation number with estimated size and clearance mechanism for PAMAM dendrimers.

Figure 8. Silica based MRI contrast agents are loaded with chelated gadolinium molecules in several different locations; (A) embedded in the silica network during the sol-gel process, (B) chemical modification to load the entire pore, (C) or localized to the the pore entrance, (D) or on the nanoparticle exterior.

Figure 9. Graph comparing the total nanoparticle relaxivities and relaxivity per volume for nanoparticle MRI contrast agents.

Table 1

Summary of nanoparticle I-N-V relaxivities discussed in this review.

Nanoparticle platform	Formulation	Ionic relaxivity [$\text{mM}^{-1}\text{s}^{-1}$]	I-N-V relaxivity Nanoparticle relaxivity [$\text{mM}^{-1}\text{s}^{-1}$]	Volumetric relaxivity [$\text{mM}^{-1}\text{s}^{-1}\text{nm}^{-3}$]	Magnetic field strength [mHz]	Reference
VNPs	CCMV with Gd^{3+} bound at 3-fold axes	202–210	28,280–36,120	2.46–3.14	64	(Allen et al, 2005; Basuet al, 2003)
	CCMV-Gd(DOTA)	46	2,806	0.24	64	(Liepold et al, 2007)
	CPMV-Gd(DOTA)	12.8	2,850	0.20	64	(Prasuhn et al, 2007)
	Q β -Gd(DOTA)	11.9	1,820	0.13	64	(Prasuhn et al, 2007)
	Q β -polymer hybrids with Gd(DOTA)	11.6	7,094	0.50	60	(Pokorski et al, 2011)
	P22 with internal Gd(DTPA) network	14.0	26,600	0.19	60	(Qazi et al, 2012)
	MS2 with Gd(DTPA) at external Tyr side chains	30.9	2,883	0.20	60	(Datta et al, 2008)
	MS2 with Gd(DTPA) at internal Lys side chains	23.2	1,880	0.13	60	(Datta et al, 2008)
	MS2 with Gd(HOPO) at external Cys side chain	41.2	7,416	0.52	60	(Garimella et al, 2011)
	TMV with Gd(DOTA) at external Tyr side chain	18.4	31,501	0.41	64	(Bruckman et al, 2013)
	TMV with Gd(DOTA) at internal Glu side chains	10.7	36,562	0.48	64	(Bruckman et al, 2013)
	TMVSNP-Gd(DOTA)	15.2	380,000	0.15	64	(Bruckman et al, 2013)
Nanoemulsions	Gd(DOTAC ₁₄) micelles	22.0	NA	NA	60	(Andre et al, 1999; Nicolle et al, 2002)
	Gd(DOTAC ₁₀) micelles	10.8	NA	NA	60	(Andre et al, 1999; Nicolle et al, 2002)
	Gd(DOTA)(GAC ₂) ₂ micelles	34.8	NA	NA	20	(Kielar et al, 2010).
	Gd(DOTA)(GAC ₂) ₂ liposomes	40.0	NA	NA	20	(Kielar et al, 2010).
	Gd(DOTAC ₁₂) micelles	18.0	NA	NA	20	(Hovland et al, 2003)

Nanoparticle platform	Formulation	Ionic relaxivity [mM ⁻¹ s ⁻¹]	1-N-V nanoparticle relaxivity [mM ⁻¹ s ⁻¹]	Volumetric relaxivity [mM ⁻¹ s ⁻¹ nm ⁻³]	Magnetic field strength [mHz]	Reference
	Gd(PCTAC ₁₂) micelles	29.0	NA	NA	20	(Hovland et al, 2003)
	Gd(III) replaced with Y(III)					
	Gd(AAZTAC ₁₇) micelles co-assembled with lipoproteins	35.5	2,840	4.31	60	(Briley-Saebo et al, 2009)
Dendrimer	G5 PAMAM-GdDOTA	30	2,880	69.17	20	(Bryant et al, 1999)
	G7 PAMAM-GdDOTA	35	13,300	49.61	20	(Bryant et al, 1999)
	G9 PAMAM-GdDOTA	36.0	47,520	47.59	20	(Bryant et al, 1999)
	G10 PAMAM-GdDOTA	36.0	66,960	40.25	20	(Bryant et al, 1999)
	PLL G3 dendrimer with HOPO-based, TREN-capped Gd chelate	38.1	228	16.19	60	(Kojima et al, 2011) (Floyd et al, 2011).
	EA G3 dendrimer with HOPO-based, TREN-capped Gd chelate	21.0	94	6.68	60	(Kojima et al, 2011) (Floyd et al, 2011).
Silica	100 nm mesoporous silica nanoparticles at variable concentrations of Gd: 1.6%, 2.3%, 3.1%, and 6.8% (w/v).	23.6, 20.8, 7.2, and 4.4	NA	NA	400	(Lin et al, 2004)
	Gd-Si-DTPA (40 nm)	7.8	492,960	14.71	128	(Rieter et al, 2007)
	Gd-Si-DTTA (40 nm)	19.7	200,940	7.58	128	(Rieter et al, 2007)
	Gd-Si-DTTA (75 nm)	28.8	699,840	3.17	128	(Taylor et al, 2008)
	Gd-labeled MCM-41	27.0	9,720	0.43	20	(Carniato et al, 2009)
	Gd-labeled SB-15	6.7	199,660,000	1.39	20	(Carniato et al, 2009)
	Gd-DOTA molecules at the entryway of 62-nm-sized mesoporous silica nanoparticles	39.3	577,120	4.62	128	(Carniato et al, 2010)
Gold	2.4 nm Gold Gd-DTPA-SH	3.9	585	28.42	300	(Debouttière et al, 2006)
	Gold Gd-DTPA-SH: 2, 4, and 6 nm	4.8, 8.4, and 11.0	NA	NA	300	(Warsi & Chedhik, 2011)

Nanoparticle platform	Formulation	Ionic relaxivity [mM ⁻¹ s ⁻¹]	I-N-V relaxivity Nanoparticle relaxivity [mM ⁻¹ s ⁻¹]	Volumetric relaxivity [mM ⁻¹ s ⁻¹ nm ⁻³]	Magnetic field strength [mHz]	Reference
	70 Gd-DO3A molecules on a 4.8 nm-sized gold nanoparticles	20	1,400	24.17	30	(Ferreira et al, 2012)
	56 Gd-DTTA-SH molecules to the surface of 2.3 nm gold nanoparticle	60	3,360	58.02	30	(Moriggi et al, 2009)
Carbon	gadofullerenes	20–80	NA	NA	60	(Zheng et al, 2012)
	gadonnanotubes	170	NA	NA	60	(Ananta et al, 2010; Hartman et al, 2008; Sitharaman et al, 2005)
	Gd-DO3A-amine nanodiamonds	58.8	NA	NA	64	(Manus et al, 2010).