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REVIEW

Characterization of nanoparticle-based contrast agents for molecular magnetic resonance imaging

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Abstract The development of molecular imaging agents is currently undergoing a dramatic expansion. As of October 2011, ~4,800 newly developed agents have been synthesized and characterized in vitro and in animal models of human disease. Despite this rapid progress, the transfer of these agents to clinical practice is rather slow. To address this issue, the National Institutes of Health launched the Molecular Imaging and Contrast Agents Database (MICAD) in 2005 to provide freely accessible online information regarding molecular imaging probes and contrast agents for the imaging community. While compiling information regarding imaging agents published in peer-reviewed journals, the MICAD editors have observed that some important information regarding the characterization of a contrast agent is not consistently reported. This makes it difficult for investigators to evaluate and meta-analyze data generated from different studies of imaging agents, especially for the agents based on nanoparticles. This article is intended to serve as a guideline for new investigators for the characterization of preclinical studies performed with nanoparticle-based MRI contrast agents. The common characterization parameters are summarized into seven categories: contrast agent designation, physicochemical properties, magnetic properties, in vitro studies, animal studies, MRI studies, and toxicity. Although no single set of parameters is suitable to define the properties of the various types of contrast agents, it is essential to ensure that these agents meet certain quality control parameters at the preclinical stage, so that they can be used without delay for clinical studies.

Keywords Contrast agent · Nanoparticles · Characterization · Molecular imaging · MRI

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Introduction

Over the last decade, significant progress has been made in the development of molecular imaging agents for anatomical and biologic imaging (Massoud and Gambhir 2003; Mankoff et al. 2007). However, the translation of these agents from preclinical studies to clinical practice is significantly delayed (Weissleder 2006; Chopra et al. 2011). This delay can be attributed to several factors, including stringent regulations and economic consideration (Weissleder 2006). However, the requirement of complex multidisciplinary efforts

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to synthesize and characterize the imaging agents is undoubtedly impeding the progression of these agents from the laboratory to the clinic. To enhance the investigation in molecular imaging and to facilitate the translation of preclinical findings to clinical practice, the National Institutes of Health (NIH) launched the Molecular Imaging and Contrast Agents Database (MICAD; http://www.micad.nih.gov) in 2005 (Chopra et al. 2012; Cheng et al. 2007). This database aims to provide freely accessible online scientific information regarding imaging probes and contrast agents. For inclusion in MICAD, a molecular imaging agent must have both in vitro characterization and in vivo animal or human studies. A comprehensive literature search by the MICAD editors shows that >4,800 novel imaging agents have been published and characterized in vitro and in animal models of human disease between 1970 and October 2011, and this number increases by 300–500 new agents every year. Based on our analysis of the data in MICAD, majority of the agents have been developed for single-photon emission computed tomography (SPECT, ~36 %), positron emission tomography (PET, ~30 %), and magnetic resonance imaging (MRI, ~17 %). Compared with PET and SPECT, MRI is less sensitive in depicting contrast agents. The detection sensitivity of MRI for contrast agent (10^{-3} to 10^{-5} M) is a few orders of magnitude lower than that of the PET (10^{-11} to 10^{-12} M) and SPECT (10^{-10} to 10^{-11} M) for radiotracers. However, MRI offers a good depth penetration and high spatial resolution, and exhibits a superior ability to extract molecular and anatomic information simultaneously. Because of the marked advances in the design and chemistry of nanoparticle (NP)-based MRI contrast agents, low sensitivity is becoming less of an issue, and molecular imaging with MRI is attracting significant interest. NP contrast agents have large surface areas that hold functional groups for linking specific ligands such as antibodies, peptides, and small molecules. NP agents also have a large inner space for encapsulating a high payload of iron oxide (IO) or gadolinium (Gd) chelates. While compiling information on imaging agents from peer-reviewed published articles, the MICAD editors have observed that certain critical information about agent characterization is often inconsistently or incompletely reported in publications (Chopra et al. 2011; Leung et al. 2012). This makes the validation of an agent and the meta-analysis of data from different studies difficult. This issue is particularly problematic for MRI NP contrast agents because of the diversity in synthesis, the multidisciplinary nature of agent characterization, and the unique in vivo behavior of each NP agent.

This article seeks to summarize the common parameters required to characterize MRI contrast agents, especially those that are NP based, and proposes a general guideline for reporting of the characterization data. We also briefly discuss the limitations of the various techniques used to capture the data, and the rationale underlying different considerations that may be important for agent development. It is worth pointing out that each agent may have to be characterized differently because of its unique syntheses, composition, and application, although there are some common parameters that are important for the characterization of various contrast agents and also of the agents that are used with different imaging modalities. Furthermore, each parameter may have to be obtained with a different or a set of different techniques because of the diversity of the components constituting an NP agent. For clinical imaging studies, we recommend consulting with the experts of the Cancer Imaging Program at the National Cancer Institute (NCI) and following the imaging guidelines for clinical trials (http://imaging.cancer.gov/clinicaltrials/guidelines). In addition, this article has been prepared as a companion research paper to an earlier discussion about the characterization of radiolabeled agents developed for PET and SPECT (Chopra et al. 2011). For convenience, the parameters are grouped into seven categories: contrast agent designation, magnetic properties, physicochemical properties, in vitro studies, animal studies, MRI studies, and toxicity (Table 1).

Contrast agent designation: chemical name and abbreviated name

To date, most MRI contrast agents have been developed based on either Gd or IO (mainly Fe_3O_4) particles. The Gd-based agents are typically synthesized by chelating Gd to a chemical backbone through a chelating compound (Gd-chelate) or by incorporating Gd ions into inorganic NPs (Sherry et al. 2009). Synthesis of IO-based contrast agents is often complicated because the IO particles have to be further

Table 1 Critical parameters for characterization of nanoparticle-based MRI contrast agents

Parameters	Brief description
Agent designation	
Chemical name	Linking the standard chemical name of each component based on the conjugation relationships
Abbreviated name	Using a standard abbreviation for each component
Magnetic properties	
Relaxation time	T1, T2, T2*
Relaxivity	r_1, r_2
Physicochemical properties	
Chemical yield	Absolute or fractional yield
Chemical purity	Impurities are expected to be <5 % in the final preparation
Structure/composition	Even if difficult for precise structure, determine the relative or absolute amount of each component
Size and shape	Clarify techniques used to measure size
In vitro studies	
Agent-target binding	Binding affinity and specificity
Stability	Aggregation, protein absorption, degradation, zeta potential
Animal studies	
Biodistribution	Organ accumulation and in vivo specificity (blocking studies)
Elimination	Define elimination half-life and pathways
Control use	Animals, cell lines, and agents depending on study purpose
MRI studies	
Imaging protocol	Detailed scanning settings
Data analysis	Qualitative and quantitative (T1, T2, r_1, r_2, target/noise ratio)
Toxicity	
In vitro models	Cell viability, gene expression, reactive oxygen species
Animal studies	Acute toxicity and long-term toxicity

coated with inert organic or inorganic materials (Laurent et al. 2008; Gupta and Gupta 2005; Schladt et al. 2011). The nonmagnetic shell formed by the coating can be of 1–20 nm in thickness, and magnetization of the IO particles may be reduced by the nonmagnetic coating (Schladt et al. 2011; Alford et al. 2009). To achieve active targeting, Gd- and IO-based agents must be functionalized with target-specific ligands such as antibodies, peptides, or small molecules (Hermann et al. 2008; Makowski et al. 2009; Shan et al. 2007; Artemov et al. 2004). Success in the development of "smart" agents, signal of which depends on specific biochemical activities, has further increased the possibility to image molecular processes with MRI (Cipolla et al. 2011; Lowe 2004).

The complexity and diversity of agent's synthesis results in the development of diverse contrast agents, which possess physicochemical and biologic properties that are significantly different from their parent compounds and from each other. To ensure a valid comparison among agents, the first step is to clearly define each component of an agent and report its full chemical name. Excellent resources for the structures of chemical compounds are available in the NIH PubChem Compound Database (http://pubchem.ncbi.nlm.nih.gov/) and in the European Chemical Entities of Biological Interest (ChEBI; http://www.ebi.ac.uk/chebi/). Considering the complexity of contrast agents, we recommend designating the full chemical name based on the conjugation or the reaction relationships between components so that independent investigators can easily identify the composition of an agent and the potential role each component may contribute to the overall properties of an agent. For example, Tei et al. (2010) synthesized a contrast agent with the Gd-chelate [Gd-(1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid *t*-butyl ester)-10-acetic acid monoamide)] and a 16-amino-acid peptide (GKVLA KGGGGTVQQEL). A four-Gly spacer and a Lys residue were inserted into the peptide between the Lys

and the Gln moieties for conjugation of the Gd-chelate. The full chemical name of this agent was thus reported as GKVLAK-Gd-(1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid *t*-butyl ester)-10-acetic acid monoamide)-GGGGTVQQEL. Because contrast agents are typically composed of multiple materials, an abbreviated name is desired for convenient recognition. In the literature, an abbreviated name is indeed provided for most agents, but some of the abbreviated names are confusing or less informative, such as "6a" and "liposome NPs." To create an informative abbreviated name, we recommend a format that uses the standard abbreviation of each component linked on the basis of the conjugation relationship between the components. For the agent described above, Tei et al. (2010) abbreviated the Gd-chelate as Gd-DOTAMA and designated the peptide as DCCP16; the resulting abbreviation for the intact agent is Gd-DOTAMA-DCCP16. It can be difficult to name some NP agents by defining all the components and the reaction relationships among them because of the complicated syntheses and a variety of nanomaterials used. The agent, RGD-conjugated Q-SiPaLCs, is an example of such an NP agent (Koole et al. 2008). This agent was first synthesized by coating CdSe quantum dot with seven monolayers of silica shells (2× CdS, 3× $Cd_{0.5}Zn_{0.5}S$, and 2× ZnS), and then coated with a dense monolayer of PEGylated paramagnetic lipids. Multiple cyclic RGD peptides were subsequently conjugated to the lipids to obtain a target-specific NP agent. In this situation, the NP agents may be named on the basis of the major components: for example, the full chemical name of RGD-conjugated Q-SiPaLCs may be described as RGD-conjugated, PEGylated paramagnetic lipid-coated silica NPs with a CdSe quantum dot core.

Magnetic properties: relaxation time and relaxivity

Magnetization recovery in the longitudinal direction and decay in the transverse plane form the basis of soft-tissue contrast in MRI (Blamire 2008; Delikatny and Poptani 2005). The relaxation characteristics of different tissues are thus represented with relaxation times T1 and T2 or relaxation rates R1 and R2, where R1 = 1/T1 and R2 = 1/T2 (Table 1). An inversion-recovery experiment is usually performed to measure T1 relaxation time, and the Carr–Purcell–Meiboom–Gill (CPMG) sequence is used to quantify T2 relaxation time (Shah et al. 2011). CPMG is a multi-echo spin echo sequence, and the signal in this sequence is dependent on T2. To evaluate the efficiency of a contrast agent, one important parameter is its relaxivity (r_1 or r_2), which reflects the ability of an agent to affect the relaxation rates of the surrounding water proton spins. The relaxation induced by paramagnetic agents is through the chemical exchange between water molecules coordinated to the paramagnetic complex and bulk water molecules, whereas the relaxation induced by iron oxide NPs is due to the diffusion of water molecules through the magnetic field inhomogeneities created by the magnetic NPs (de la Fuente and Grazu 2012). The relaxivity, expressed as $(\text{mM} \times \text{s})^{-1}$ or $\text{mM}^{-1}\ \text{s}^{-1}$, is the relaxation rate of the water protons per mM of Gd ion or IO. Relaxivity can also be calculated on a per particle basis (Cheng et al. 2010). The relaxivity of an agent is often first studied in vitro in aqueous solutions, and this value allows comparison among different contrast agents; however, this property may be different from that observed under in vivo conditions. Typically, the relaxivity of an agent is analyzed with NMR spectroscopy, and the r_1 and r_2 values are calculated by plotting the T1 and T2 over the Gd^{3+} or iron concentration (Shah et al. 2011). Alternatively, the fundamental biologic and MR-inducible tissue properties can be quantified with quantitative MRI (Shah et al. 2011). In contrast to clinical MRI, which employs differences in tissue properties to generate contrast for subsequent subjective image analysis, quantitative MRI makes use of MRI parameters for evaluation and determination of fundamental biologic properties of tissue, such as proton density, diffusion, T1 relaxation, T2 and T2* relaxation, and magnetization transfer (Shah et al. 2011).

The Gd-based contrast agents currently used in the clinic have typical r_1 and r_2 values in the range of 3–5 and 5–6 $(\text{mM} \times \text{s})^{-1}$, respectively (Makowski et al. 2009). The IO-based contrast agents have a much higher relaxivity than Gd-based agents and their r_1 is in the range of 20–25 $(\text{mM} \times \text{s})^{-1}$ and the r_2 has a range of 100–200 $(\text{mM} \times \text{s})^{-1}$ (Makowski et al. 2009). To date, most contrast agents approved by the United States Food and Drug Administration (FDA) are optimized for a magnetic field strength of 1.5 Tesla. Results obtained with T1 contrast agents (e.g., Gd-based agents) in a high-strength field cannot

be directly translated into clinical field strength because the r_1 may decrease with increased field strength. The field strength dependency of the r_1 is an essential parameter to consider while developing and optimizing T1 contrast agents. Conversely, r_2 is increased in higher field strengths, and IO-based contrast agents are better suited for 3.0 Tesla or higher (Haun et al. 2010). In addition, the medium and its temperature and pH also influence the values of r_1 and r_2; therefore, the conditions for relaxivity measurement should be reported clearly to avoid misleading comparison among different NP agents.

Physicochemical properties: chemical yield, chemical purity, structure/composition, size, and shape

A molecular imaging contrast agent is typically composed of an imaging moiety (label) for signaling the target distribution and density, a specific ligand for interacting with the target(s), and a carrier for label delivery. These components form the unique core–shell structure of most NP agents. The core determines the primary physicochemical properties of the NP, while the shell materials stabilize the core and affect the reactivity, solubility, and in vivo behavior of the NP agents. The core and shell together determine the usefulness of a NP as an imaging agent. Therefore, the intact contrast agent and, if possible, the intermediates, should be characterized during and after the final product has been obtained (Table 1).

Chemical yield and purity

The chemical yield is the amount of intact agent obtained from the chemical synthesis and is usually expressed as an absolute or a fractional yield. The absolute yield is presented as the weight in grams or, preferably, in moles (molar yield); the fractional yield, also known as the relative yield or percentage yield, is expressed as the percentage of the amount of product obtained in moles compared to the expected or theoretical yield (also in moles). The chemical yield measures the efficiency of the procedure(s) used for synthesis. Chemical purity represents the degree to which the intact agent is undiluted or unmixed with extraneous materials and is typically expressed as a percentage of the total final products. The chemical purity helps us evaluate the presence of the side products generated during synthesis and the toxic raw materials that were not removed during purification. Diverse methods have been applied to purify the intermediates and the final product. Affinity purification is often the method of choice to purify proteins, antibodies, peptides, and enzymes, whereas high-performance liquid chromatography is a sensitive technique to identify, quantify, and purify the individual components from a chemical mixture (Chopra et al. 2011). Purification of the NP agents can be achieved with dialysis, gel filtration, cross-flow microfiltration, evaporation under reduced pressure, or ultracentrifugation. Theoretically, all of the potentially toxic impurities, including the side products, organic solvents, stabilizers, residual monomers, free specific ligands, polymerization initiators, and large aggregates must be eliminated from the final purified preparation. Impurities can change the properties of an agent; for example, an impurity may interfere with the biologic activity of an agent or cause unexpected side effects in vivo. In general, the purity of a contrast agent is expected to be at least 95 % before it is used in any studies.

Structure/composition

Determination of the chemical structure of the contrast agents helps investigators determine the structure–pharmacokinetic relationship and predict the possible biologic properties of other chemical structures. Unfortunately, a robust structure–pharmacokinetic correlation is often absent for NP agents. Lack of this correlation may be due to the difficulty in precisely determining the structure and physicochemical properties of NP agents. Nevertheless, techniques such as high-resolution transmission electron microscopy (TEM) and X-ray diffraction (XRD) can provide useful information regarding the structure of a NP agent (Laurent et al. 2008; Lodhia et al. 2010). If the precise structure of a NP agent cannot be determined, then the agent's composition along with the relative amount of each component, especially for the conjugated ligands and label payload, should be determined. This information is necessary to optimize the properties of a contrast agent. The proportion of ligand and label in an agent can influence the agent's behavior in vivo, such as receptor binding, internalization, and relaxation effect. For example, the agent PEG-P(Lys-

DOTA-Gd) was synthesized by the conjugation of Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) to the lysine residues of poly(ethylene glycol)-*b*-poly(L-lysine) block copolymer (Shiraishi et al. 2010, 2009). The amounts of ethylene glycol, lysine residues, conjugated DOTA, and Gd ions all influence the relaxivity of this agent. The PEG-P(Lys-DOTA-Gd) micelle has been reported to exhibit optimal in vivo behavior only when it has 118 ethylene glycol units, 17 lysine units, 17 DOTA units, and 7 Gd ions. The absolute amount of Gd^{3+} or iron in an agent and in a sample can be obtained with atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICPMS). The AAS is relatively easy to use, and ICPMS is rapid and sensitive, but both are subject to interference from trace contaminants (Lodhia et al. 2010). Alternatively, the amount of Gd^{3+} can be quantified by determining the magnetic susceptibility of an agent with high-resolution nuclear magnetic resonance (Corsi et al. 2001).

Size and shape

The NP size and shape are important considerations because of their effects on the magnetization, biodistribution, circulation time, extravasation, target binding, and cellular internalization (Debbage and Jaschke 2008; Gaumet et al. 2008). IO-Based NP agents should also have a narrow size distribution to achieve a superior T2 effect. Uniform IO particles are usually prepared via homogeneous precipitation reactions by controlling the ratio of ions, types of salts, pH, and ionic strengths of solutions (Laurent et al. 2008; Lodhia et al. 2010). In practice, the synthesis of NP agents with a defined size is often difficult to control (Laurent et al. 2008; Rosenblum et al. 2010). Smaller particles are easily taken up by targeted cells via endocytosis and are eliminated mainly through filtration by the renal glomeruli. NP agents above 50 nm are prone to be taken up by the reticuloendothelial system (RES) in the liver, spleen, and, to a lesser extent, the bone marrow. It becomes difficult for NP agents >150 nm to enter cells via endocytosis (Gaumet et al. 2008). NP agents also have diverse shapes, such as spherical, rod-shaped, tubular, and disk-like (Tao et al. 2011). Compared to a spherical shape, a nonspherical shape appears to increase the NP adhesion to the cell surface receptors. Cylindrical filomicelles have been shown to effectively escape the phagocytosis of RES (Geng et al. 2007). Precise control of the size and shape helps one to determine the size- and shape-dependent behaviors of an agent in a more reliable manner.

When determining the size of a NP agent, we are in fact measuring the sizes of different parts of the NP agent, including the core, the shell, and the intact particle (Laurent et al. 2008; Lodhia et al. 2010). Dynamic light scattering (DLS), TEM, and XRD are the three techniques frequently used for size determination (Di Marco et al. 2007b). TEM gives the size of each part as well as the size distribution and the shape of an NP agent in solid condition. In addition, the lattice structure of the NP agents can be determined within a few nanometers with TEM. However, agent preparation for TEM, such as drying samples, may induce artificial changes in NP characteristics. Information about hydrodynamic size and size distribution in solutions can be obtained with DLS, generally at a lower cost and less time. The hydrodynamic size is usually larger than the diameter obtained with TEM and is related more to the NP stability. However, the hydrodynamic size of the same agent can change with the suspension conditions. XRD is also a useful technique for the size characterization and provides the crystal size and crystalline structure information of a NP agent even in suspension. In some reports, the magnetic size has also been measured with the Langevin function. The magnetic diameter is generally smaller than the diameter obtained with TEM, and the presence of interparticle interactions and size distribution can cause deviation from the Langevin function (Laurent et al. 2008).

In vitro studies: agent–target binding and stability

Agent–target binding (binding affinity and specificity)

Specific binding to target biomarkers is achieved by the attachment of a ligand that specifically targets the NP agents to the biomarker. Given the inherent complexities in the interaction of NP surfaces with proteins and the structural diversity of NP scaffolds and ligands, the binding affinity of an intact agent and its receptor-mediated internalization can change significantly compared to the native ligand. Measuring

the rate and extent of binding to a target provides information regarding the number of binding sites, and the affinity and accessibility of a biomarker to an agent. The in vitro binding affinity of an agent is often evaluated with competition assays using either a cell-free system (e.g., isolated cell membrane, recombinant antigen, or receptor fragment) or cultured cells (Chopra et al. 2011; Eckelman et al. 2009). The binding affinity of an agent depends on the contact between the target molecule or receptor and the ligand, and the concentration of each reagent in the solution. Therefore, the binding parameters of an NP agent are expressed as the association constant (K_a), dissociation constant (K_d), available target density (B_{max}), and binding potential (BP) (Eckelman et al. 2009). In general, when the K_d is low (e.g., pM or nM), only a low concentration of the agent is required to occupy the receptors. Considering the influence of cell growth on the binding affinity, optimization of the cell growth conditions is necessary to obtain optimal expression of the receptor or the target molecule and reproducible assessment of the binding affinity.

The binding specificity of an agent to the biomarker is especially important when the agent is used to quantify the biomarker. In vitro specificity can be evaluated in the presence of a saturating amount of an unlabeled blocking compound that binds to the same target as the agent under investigation (Chopra et al. 2011). The blocking agent can be the native ligand or the unlabeled contrast agent. However, a compound chemically distinct from the native ligand is more preferable for the determination of the binding specificity because only then this parameter can be established with certainty. Nonspecific binding largely depends on the charge and hydrophobicity of an NP agent, and less on its exact structure. Nonspecific binding is often proportional to the concentration of an NP agent that is available for binding. In general, the nonspecific binding of an agent should be <10–20 % of the total binding.

Stability (aggregation, protein absorption, degradation, and zeta potential)

Stability is a significant concern for NP contrast agents, and several relevant factors that influence this parameter should be considered carefully (Table 1) (Di Marco et al. 2007a, b). First is the aggregation of an NP agent, which affects its biodistribution and poses an immediate potential risk to human health because the aggregates can obstruct small blood vessels in important organs such as the brain and lungs (Shiraishi et al. 2009). This issue is particularly important for IO-based agents because the aggregation of ultrasmall IO particles starts rapidly. The second factor is the absorption of serum proteins on the surface of NPs (Karmali and Simberg 2011). Attachment of serum proteins on the particle surface can initiate phagocytosis and evoke an immune response in the host. The third factor is agent degradation. The stability of Gd-chelates and Gd-containing NPs should be particularly considered because release of free Gd ion and its subsequent retention within the body is a major concern of safety (Morcos 2008). Most chelates used currently are either linear or macrocyclic, and they differ in thermodynamic and kinetic stabilities. The colloidal stability of NPs is typically studied with DLS on the basis of the time evolution of the hydrodynamic size (aggregation kinetics) as a function of the ionic strength (Di Marco et al. 2007a). The interaction with serum proteins and degradation of an agent can be evaluated by keeping the agent in a physiological buffer and by exposing it to animal or human serum at room temperature and at 37 °C. Analysis of an agent after storage under different conditions for various time periods helps one estimate the degree to which the agent remains intact, the amount and types of serum components the agent absorbs, and the types of products the agent will degrade into at different time intervals (Chopra et al. 2011).

Zeta potential is a term for the electrokinetic potential of particles in colloidal systems and refers to the potential difference between the dispersion medium and the stationary layer of medium that attaches to the dispersed particles (Di Marco et al. 2007a). The significance of measuring zeta potential is its relationship to the stability of colloidal dispersions. The zeta potential of colloidal NP agents is often measured with electrophoretic techniques, but the measurements are indirect and the values obtained for the same charged interface may differ significantly, depending on the techniques or theoretical approaches. The zeta potential value obtained with electrophoretic mobility measurement is usually higher than that obtained with the streaming potential method. Therefore, any comparison of the zeta potential among

studies should ensure comparable conditions of measurements and calculations. We have observed that few investigators determine and report the zeta potential of colloidal contrast agents.

Animal studies: biodistribution, elimination, and use of proper controls

Study of contrast agents in animals is a vital step to determine the biodistribution, elimination, and other biologic properties of an agent before it is used in humans (Eckelman et al. 2007). As in other research fields, issues arise in molecular imaging regarding the validity of animal models for the investigation of human diseases and whether the study results in animals can be directly translated to humans (Pakzad et al. 2005). Apparently, the biodistribution of some contrast agents is not only different among animal species, but also between animals and humans (Maina et al. 2005). An understanding of inter-species differences and the careful design of animal studies are essential to generate meaningful data and provide a more realistic interpretation of the in vivo results (Table 1).

Biodistribution (organ distribution and specificity)

Several factors need to be considered when designing biodistribution studies. First, the optimal dose of a NP agent has to be determined. Most nonspecific Gd-based contrast agents currently used in the clinic are approved at a dose of 0.1 mmol Gd/kg. More recently developed Gd-agents, such as gadofosveset and gadoxetic acids, are approved at lower doses (0.03 and 0.025 mmol/kg, respectively). The safety margin for these Gd-chelates is twenty times higher than that of $GdCl_3$. The doses of these agents can be used as a reference for the dose of newly developed contrast agents in biodistribution studies. The Gd-based NP agents are expected to be used at lower doses than Gd-chelates. Second, to optimize the target/background signal ratio, the time to measure accumulation of the agent in the various organs and blood has to be determined, often with a time-course study. The absolute amount of Gd or iron in an organ can be quantified ex vivo with AAS and ICPMS techniques. The distribution in vital organs can then be expressed as mmol, μmol, or μg for Gd or iron per gram of tissue and is reported as an average with standard deviation for the animal group. Alternatively, MRI can be used to calculate the agent distribution, although it is not a fully quantitative method. In addition, the number of animals used, the time course of studies, and the route of administration should be reported (Eckelman et al. 2007). Because anesthesia often affects biodistribution, details of this procedure should be reported as well. This background information helps other researchers evaluate the validity of the experimental design and the interpretation of the data obtained from the study.

As discussed above for in vitro specificity, it is also important to determine the in vivo specificity of an agent. For this purpose, a competition experiment (also called a blocking study) is performed in the presence of a saturating amount of an unlabeled blocking compound (Chopra et al. 2011; Eckelman et al. 2009). The animals are treated before or simultaneously with a high dose of the blocking agent (100–1,000-fold excess concentration of the contrast agent). The accumulation of a contrast agent at the site of interest is then measured and compared between animals treated with and without the blocking agent. When the native ligands (e.g., transferrin) are used as the blocking agents, they often have different pharmacokinetics compared to the intact NP agent (e.g., transferrin-conjugated NPs) and may not efficiently block the specific binding of the NP agent. Another indirect method to evaluate the specificity is the use of tumor xenografts generated in animals with cultured cells that overexpress the target biomarker and the results are compared with those obtained from tumors generated with cells that lack or have very low expression of the target biomarker. Regardless of the methods used, nonspecific binding and the environmental difference among tumors should be considered when interpreting the results. In vivo blocking studies provide the information on the specificity as well as the clues as to whether the structure, formulation, dosage, or the method of administration of an imaging agent has to be modified to obtain the preferred results; however, many investigators did not perform blocking studies for the targeted contrast agents.

Elimination half-life and pathways

The elimination half-life ($t_{1/2}$) is the time it takes for the plasma concentration of a contrast agent to be

reduced by half after its administration to the animals (also called plasma or blood half-life). The relationship between the biologic and elimination half-lives of a targeted contrast agent can be complicated because of tissue accumulation, active metabolites, ligand–receptor interactions, etc. In most cases, a short but sufficiently long $t_{1/2}$ is favorable for NP agents to penetrate into deep pathologic tissues and actively bind with biomarkers. A longer $t_{1/2}$ may increase the toxicity potential of the NP and decrease the target/background signal ratio. A standard two-compartment distribution model is typically used to determine the $t_{1/2}$, whereas a three-compartment pharmacokinetic model is used to describe the in vivo behavior of an agent in both the blood pool and the target tissue (Shiraishi et al. 2010).

MRI contrast agents are eliminated through different pathways, depending on the properties of the agents (Aime and Caravan 2009; Benet 2010). In general, NP agents with a small size are excreted primarily through renal filtration, whereas larger agents are captured and degraded by the RES. Hydrophilic agents tend to be cleared through the kidneys, whereas lipophilic agents are usually excreted through the hepatobiliary system. Receptor-targeted agents are taken up by cells via receptor-mediated endocytosis and then metabolized by the lysosomal enzymes. IO-Based agents are generally degraded rapidly, and the Fe can be incorporated into hemoglobin as early as 1–2 days after administration (Weissleder et al. 1989). However, there is concern about the Gd deposition in normal tissues, especially for Gd-chelated NP agents that can release free Gd into circulation. In animals, the bone, liver, and spleen are the main target organs for Gd deposition. The clearance of Gd from organs takes from several days to weeks and is highly dependent on the formulation of Gd-complexes. Prolonged residence of Gd-based agents increases the probability of Gd deposition in these organs. The elimination pathway of NP agents at the cellular and subcellular levels should also be studied in detail because of their potential long-term toxicity when used in humans (Aime and Caravan 2009).

Use of proper controls

Careful use of a control is necessary to determine the contrast effect and specificity of an agent. Controls used in studies vary greatly depending on the nature of the study. In some studies, tumor xenografts generated with a cell line that has little or no expression of the target are used as the control, but the permeability of the tumors produced with such cell lines must be equivalent to that of the test tumors. In other studies, clinical contrast agents are used as controls to compare and demonstrate the contrast-generating efficiency of the test agent. In terms of the imaging specificity and sensitivity, even if contrast enhancement with a targeted agent is observed clearly, the results must be interpreted with caution because nontargeted contrast agents may also accumulate in the area of interest because of enhanced permeability and retention effects in tumors and potentially nonspecific binding.

In vivo MRI studies: imaging protocol and data analysis

Imaging protocol

Different settings used for imaging may result in the generation of different results, even with the same agent. Two MRI sequences are often used, including the spin echo and gradient echo. Several subtypes of the two sequences have also been developed to obtain optimal contrast, depending on the nature of the contrast agent, location, motion, and other imaging requirements. Because of the strong influence of equipment settings on the acquisition of high-quality images and accurate data, all the relevant parameters for an imaging study should be optimized and reported clearly (Delikatny and Poptani 2005). These parameters include the sequence, field strength, repetition time, echo time, inversion time for inversion recovery, flip angle for gradient echo sequences, field of view, slice thickness and number, and scan time.

Image data analysis

Interpretation of the acquired images usually requires the aid of computer software. Most software products are designed to perform two functions: feature extraction and image analysis (qualitative and quantitative). The fundamental parameters that are commonly measured include relaxation times (T1, T2, and T2*) and target/background signal ratio (Table 1)

(Shah et al. 2011). Analyzing a series of images over time is especially important to extract functional information (e.g., the dynamic behavior of signal intensity) after the administration of a contrast agent. A few quantitative pharmacokinetic parameters can be obtained from the signal intensity–time curves, such as the start of enhancement, time to peak, peak enhancement, and washout (Koh et al. 2011). In the case of quantification of the biomarker expression or agent accumulation at the site of interest, it may be necessary to wait for the plasma concentration of an agent to decrease sufficiently to distinguish specific from non-specific signals. Because signal intensity in MRI depends primarily on the local values of the longitudinal and transverse relaxation rates of water protons, Gd- and IO-based contrast agents are not detectable themselves, but are detected by their effect on surrounding water protons (Langley et al. 2011). Therefore, careful use of controls and competition experiments are necessary to help establish the basis of the contrast effect.

Toxicity: in vitro, short-term and long-term toxicities

NP contrast agents are synthesized by means of a variety of nanomaterials using different strategies. Each component of an NP agent can pose individual toxicity risks, and an intact agent might have toxic risks that differ from the toxicity of each component (Schrand et al. 2010; Sayes and Warheit 2009; Brown et al. 2010). It is well known that Gd-based agents can cause nephrogenic systemic fibrosis in patients with renal failure or insufficient renal flow (Hellman 2011). However, few studies are available regarding the toxicity of NP agents that encapsulate Gd atoms or Gd-chelates. In addition, toxicity studies are largely performed to investigate the acute side effects of NP agents within a short duration. The long-term effects of NP agents on the biologic systems and tissues of the host are poorly understood. IO-based contrast agents appear to be safe for human use because Fe is an essential mineral in the human body with $\sim$50 mg/kg body weight (Fadeel and Garcia-Bennett 2010). In the case of FDA-approved ferumoxides, the recommended dosage for human use to perform MRI is 0.56 mg Fe/kg body weight. Studies on chronic Fe toxicity have shown that hepatic cirrhosis and hepatocellular carcinoma develop only after the Fe concentration in the liver exceeds 4 g/kg wet weight of the organ (Bulte 2009). Nevertheless, the high variability in composition, structure, and properties of NP contrast agents makes the generalization on the toxicity of NP agents extremely difficult. Delayed excretion due to encapsulation and surface modification poses risks of long-term toxicity as well as a significant barrier to the approval of NP agents for clinical use by the FDA.

The toxicity of a contrast agent can be evaluated at the in vitro and in vivo levels. In vitro studies (i.e., cultured cells) are generally used as a prescreening tool by assessing cell viability, morphology, changes in gene expression, reactive oxygen species, etc. It is important to understand that a contrast agent may produce toxicity in some cell lines, but not in others, and the results obtained from in vitro cell culture models may not correlate with those from in vivo measurements. Thus, the in vitro toxicity of a contrast agent should be investigated with several cell types using multiple doses of the contrast agent (Fadeel and Garcia-Bennett 2010). In vivo animal studies are necessary to characterize the potential adverse effects of contrast agents. Several factors such as dose, time, and route of agent administration should be considered when designing animal experiments (Schrand et al. 2010; Sayes and Warheit 2009). Toxicity assessment with a wide range of doses and exposure times provides threshold values for toxicity with an agent. Some parameters indicating the in vivo toxicity of an agent include the median lethal dose, the ability to penetrate through the blood–brain barrier and blood–testis barrier, and the effect on the physiological processes at the organ, cellular, mRNA, and protein levels. However, there are no standard parameters and techniques accepted for all NP contrast agents. Several agencies and societies have developed nanotechnology programs and are beginning to establish guidelines for the physicochemical characterization relevant to toxicology of nanomaterials. Some of these programs include the NIH NanoHealth and Safety Enterprise Initiative, the NCI Nanotechnology Database and the Nanotechnology Characterization Laboratory, the FDA Nanotechnology Core Facility, and the European Nano Health Environment Commented Database project (Sayes and Warheit 2009; Tinkle 2010; Ptak et al. 2010). These integrated efforts will enhance the understanding of the potential risks of NP agents and facilitate their clinical use.

Summary

The emergence of molecular imaging is based on the concept that targeted delivery of imaging agents can specifically increase the signal/noise ratio in the area of interest by targeting the difference in molecular profiles between diseased and normal tissues. Progress in molecular imaging is yielding important clinical benefits. It is safe to predict that molecular imaging will be routinely applied in many steps of clinical practice in the foreseeable future (Debbage and Jaschke 2008; Fox et al. 2009). To facilitate this progress, complete preclinical characterization of an imaging agent is necessary before proceeding to clinical trials. Results obtained from these studies are also necessary to optimize the in vivo efficiency of an agent. Investigators should characterize an agent as much as possible although some laboratories do not have the resources, equipment, or expertise to perform all the work. Parameters required for characterization of the imaging agents developed for other imaging modalities are discussed separately elsewhere (Chopra et al. 2011; Leung et al. 2012).

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