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Design of a novel class of protein-based magnetic resonance imaging contrast agents for the molecular imaging of cancer biomarkers

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Abstract

Magnetic resonance imaging (MRI) of disease biomarkers, especially cancer biomarkers, could potentially improve our understanding of the disease and drug activity during preclinical and clinical drug treatment and patient stratification. MRI contrast agents with high relaxivity and targeting capability to tumor biomarkers are highly required. Extensive work has been done to develop MRI contrast agents. However, only a few limited literatures report that protein residues can function as ligands to bind Gd³⁺ with high binding affinity, selectivity, and relaxivity. In this paper, we focus on reporting our current progress on designing a novel class of protein-based Gd³⁺ MRI contrast agents (ProCAs) equipped with several desirable capabilities for *in vivo* application of MRI of tumor biomarkers. We will first discuss our strategy for improving the relaxivity by a novel protein-based design. We then discuss the effect of increased relaxivity of ProCAs on improving the detection limits for MRI contrast agent, especially for *in vivo* application. We will further report our efforts to improve *in vivo* imaging capability and our achievement in molecular imaging of cancer biomarkers with potential preclinical and clinical applications.

INTRODUCTION

Molecular imaging of disease biomarkers, especially cancer biomarkers, could potentially improve our understanding of the disease and drug activity during preclinical and clinical drug treatment and patient stratification.^{1–4} For the preclinical setting, applications of molecular imaging are useful to conduct novel therapeutic analysis. A noninvasive way to monitor disease progression and effect of drug treatment could be very helpful for developing therapeutics for prevention and treatment of disease and understanding the molecular level biological basis of pathological processes. The success in translating *in vitro* discovery, to cellular response, to preclinical small animal research, and finally to the human body, requires a sensitive and reliable imaging technique.^{5–10} Additionally, the application of molecular imaging for human diagnosis will allow clinicians to tailor disease treatment specifically for individuals that express certain biomarkers. A noninvasive imaging

technique with high sensitivity and specificity is important to perform both preclinical and clinical molecular imaging analysis.

Among clinical imaging modalities capable of imaging all parts of the human body: computed tomography (CT), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET), MRI is potentially the ideal technique for molecular imaging with preferred capability and superb soft tissue contrast. MRI is able to obtain three-dimensional images and their dynamic changes with outstanding depth penetration (from 1 mm to 1 m) and high resolution. The resolution of the preclinical MRI scanner can reach 20 μm or even less.¹¹ Without the use of ionizing radiation, MRI enables the noninvasive and repetitive assessment of biological or pathological processes of the same living subject at different time points for monitoring treatment response and disease progression with preferred safety and convenience.^{5–9} Today, MRI has been applied to acquire anatomic structures, compare volume changes, tumor metabolism, and probe the vascular properties of tumors by dynamic contrast enhanced-MRI (DCE-MRI) technique.¹² MRI has also gained the potential to be the most powerful technique for allowing direct translation of preclinical findings to clinical applications by significantly reducing the number of animals required to evaluate a new compound and associated experimental errors. With improvement of the technology, MRI has become one of the leading imaging techniques for diagnostics, monitoring treatment, and progression of many types of diseases, such as central nervous system (CNS) disorders, cardiovascular disorders, and cancer.^{4–9,13} Applications of noninvasive MRI techniques with high resolution becomes even more important for imaging-guided targeted therapy and drug delivery against biomarkers, molecular targets, and personalized medicine.

The current application of MRI, however, is largely limited because it lacks the proper sensitivity. Most MRI images are generated on the basis of the different relaxation properties of protons in different organs and tissues based upon the water found in those tissues. Such differences are very small with a large background water concentration of 18 M, which results in significantly lower sensitivity than other imaging modalities, such as PET/SPECT. In order to increase the sensitivity of MRI scans, more than 30% of scans utilize the injection of MRI contrast agents intravenously.¹⁴ Based on paramagnetic, ferromagnetic, or super paramagnetic properties of metal ions, these MRI contrast agents change the longitudinal (T_1) and transverse (T_2) relaxation times of water *in vivo* and thus produce images with altered signal intensities. The lanthanide gadolinium (Gd^{3+}) is the most frequently used metal ion for MRI contrast agent due to its very high magnetic moment and a symmetric electronic ground state. Its capability to create bright MR images by decreasing T_1 without causing substantial line broadening makes it more desirable than other contrast agents such as superparamagnetic iron oxides with T_2/T_2^* shortening which produce dark images. To date, while remarkable progress for developing contrast agents has been made in the last 20 years,^{15–20} MRI contrast agents capable of molecular imaging with high sensitivity and specificity remain elusive to the market.

To extend the application of MRI in clinical diagnostics and preclinical drug development, especially for molecular imaging of biomarker changes, contrast agents must be developed with several desired features. First, MRI contrast agents should have significantly improved

sensitivity which is controlled by the degree an MRI contrast agent alters the relaxation of water. This parameter is called relaxivity. Current clinically approved MRI contrast agents have per Gd relaxivity (r_1) values around $4\text{--}6\text{ mM}^{-1}\text{ s}^{-1}$ which is significantly lower than theoretical maximum value around $100\text{ mM}^{-1}\text{ s}^{-1}$ for one water molecule coordinated in the Gd^{3+} inner shell (Figure 1). On the basis of a recent simulation of the relationship between relaxivity and detection limits by Sherry et al.,²¹ the *in vitro* detection limits for contrast agents are about 10, 4, and $0.69\text{ }\mu\text{M}$ if the per Gd^{3+} r_1 relaxivity of contrast agents are 5, 20, or $100\text{ mM}^{-1}\text{ s}^{-1}$, respectively. With relaxivity values of approximately $4\text{ mM}^{-1}\text{ s}^{-1}$, an injection dose of $0.1\text{--}0.2\text{ mmol/kg}$ of clinical MRI contrast agents is required to generate a detectable contrast with a local Gd^{3+} concentration of about $100\text{ }\mu\text{M}$. Second, an ideal contrast agent should have the proper size for efficient tissue penetration and distribution to enable accurate detection of changes of the biomarkers during biological and pathological processes. Third, an ideal contrast agent should target to biomarkers with high specificity and high binding affinity for molecular imaging. Fourth, an ideal contrast agent should have favorable pharmacokinetics for targeting biomarkers and excretion.

There have been many efforts in improving relaxivity of MRI contrast agents by covalently linking Gd-chelates to nano-carriers, such as dendrimers,²² liposomes,²³ nanoparticle emulsions²⁴ viral capsids,²⁵ and nanotubes.²⁶ Non-covalent binding between Gdchelators and protein, such as MS-325, have shown dramatic increase of relaxivity.²⁷ For recent progress please see these excellent reviews.^{14,19,28–31} However, only limited literature reports using protein residues function as ligands to bind Gd^{3+} . In this review, we will focus on reporting our current progress on designing a novel class of protein-based Gd^{3+} MRI contrast agents (ProCAs) (Figure 1) equipped with several desirable capabilities for *in vivo* application of MRI and to meet a pressing need to develop MRI contrast agents with sufficient sensitivity and specificity to image disease biomarkers. ProCAs exhibits more than 10 times higher relaxivity and dose efficiency than that of the clinical MRI contrast agents. We will first discuss our strategy for improving the relaxivity by a novel protein-based design. We will then discuss the stability of ProCAs. We next discuss the effect of increased relaxivity of ProCAs on improving the detection limits for MRI contrast agent, especially for *in vivo* application. We will further report our efforts to improve *in vivo* imaging capability and our achievement in molecular imaging of cancer biomarkers with potential preclinical and clinical applications.

RELAXATION THEORY AND CHALLENGES FOR INCREASING RELAXIVITY

Proton relaxivity r_1 (in units of $\text{mM}^{-1}\text{ s}^{-1}$) represents the efficiency of a paramagnetic contrast agent to enhance the relaxation rate of water protons. Water molecules directly interact with metal ion such as Gd^{3+} to change the relaxation properties. The r_1 is composed of the contributions from inner sphere r_1^{IS} , second coordination sphere r_1^{2nd} , and outer sphere r_1^{OS} . As shown in Figure 1(a), the relaxivity (r_1) of the contrast agents are influenced by many factors, such as rotational correlation time (τ_R), water number (q), and second and outer sphere water. The inner sphere and second sphere relaxivity can be characterized by Solomon-Bloembergen-Morgan (SBM) equation.^{33–37} The outer sphere effects of a Gd^{3+} -based agent is usually characterized by a hard sphere model, where the relaxivity is mainly

determined by the diffusion constant of the water and the closest distance of proton nuclear spin and the gadolinium electron spin. For details, please read this excellent review.³⁸

τ_R is one of the major factors influencing high relaxivity. Contrast agents based on small chelators such as DTPA have a τ_R of approximately 54 ps at 37°C.³⁹ This value is much smaller than the electron relaxation time (T_{1e}) (T_{1e} is about 2.8 ns at 60 MHz based on simulation) and the residence time of the bound water (τ_m) (143 ns).^{39,40} Therefore, τ_R determines the value of overall correlation time (τ_c), which subsequently determines T_{1m} and thus restricts the relaxivity to values less than 10 mM⁻¹s⁻¹, regardless of the adjustment of the other parameters for typical Gd³⁺-based agents. As shown in Figure 1(b), when τ_R increases from 100 ps to 10 ns, r_1 increases from 3.7 to 46.1 mM⁻¹s⁻¹ at 60 MHz (τ_m = 10 ns). The theoretical calculation by the SBM equation^{33–37} shows that the maximum per Gd³⁺ relaxivity, r_1 , that can be achieved is up to 100 mM⁻¹s⁻¹ for Gd³⁺ contrast agents with $q = 1$ and optimized τ_R (around 10 ns). Therefore, τ_R is the limiting factor for r_1 at clinical magnetic field strengths. Optimizing τ_R can effectively increase per Gd³⁺ relaxivity.

Because optimizing τ_R can increase relaxivity and τ_R increases with molecular weight, one of the common strategies for increasing relaxivity is by covalent conjugation of Gd-chelators to macromolecules such as polymer,⁴¹ dendrimers,²² proteins²⁵ or non-covalent conjugation to macromolecules such as proteins (e.g., serum albumin).²⁸ Gd³⁺-chelators have been covalently or non-covalently bonded to proteins to alter their *in vitro* and *in vivo* properties of MRI contrast agents. Covalent conjugation of small chelating molecules to proteins such as albumin, immunoglobulin G (IgG), or polylysine to some degree increases relaxivity.³⁸ For example, covalently linking of Gd-chelates to albumin increases the relaxivity and changes the distribution of Gd³⁺ with applications in DCE-MRI to probe tumor vasculature changes.^{42,43} Non-covalent and reversibly binding small chelator MS-325 to albumin increases the blood circulation time and improves the per Gd³⁺ relaxivity up to 42 mM⁻¹s⁻¹ at 20 MHz 37°C.⁴⁴

However, the improvement in proton relaxivity is much smaller than the theoretical prediction based on the molecular-weight increase. Bryant et al. have shown that r_1 and r_2 of a polyamidoamine (PAMAM) dendrimer conjugated with DOTA derivative and grown to high generation numbers ($G = 5, 7, 9$, and 10) do not continue to increase with macromolecular size but reach a plateau.⁴⁵ Uncontrolled local motion of Gd-vector and slow water exchange rate τ_m are suggested to be the challenging factors to prevent a further increase of relaxivity.^{19,28,46} Recently, a substantial increase of molecular weight up to several million Daltons using polymerized liposomes and nanoparticle emulsions were shown to result in per Gd³⁺ T_1 relaxivities of about 11–50 mM⁻¹s⁻¹. Naturally, several million Daltons of contrast agent are far too big in size for proper *in vivo* distribution, disease tissue penetration, and excretion. In developing new contrast agents, increasing relaxivity without compromising desired *in vivo* diffusion rate and good tissue/organ penetration associated with unfavorable size is challenging and of the utmost importance.

Another way one can optimize the relaxivity for a Gd³⁺-based contrast agent is to optimize the second and outer shell effects. Some contrast agents have an inner sphere q value of zero yet still produce appreciable relaxivity.²⁸ Therefore, second shell effects significantly

contribute to the relaxivity of an agent. However, the second sphere contributions to the overall relaxivity are usually not significant for small molecules due to negligible water interaction interface, fast water diffusion, fast molecular tumbling, and extended proton Gd^{3+} -proton distance. For example, conjugation of hydrophilic chain of polyethylene glycol (PEG) to a small chelator contrast agent DTPA results in a modest increase in relaxivity.⁴⁷ In some cases, the relaxivities of small chelator-based contrast agents were decreased because of the addition of long PEG chain that limits the water exchange rate.⁴⁸

Contributions from the second and outer sphere to the relaxivity become significant when calculating a macromolecule complex with an optimized τ_c and large interface for water molecule interactions. Based on simulations at 20 and 60 MHz with $r_{\text{GdH}}^{2\text{nd}} = 5 \text{ \AA}$, $\tau_R = 10 \text{ ns}$, $\tau_m = 10 \text{ ns}$, and $q = 4$, the second sphere per Gd^{3+} relaxivity for a protein metal complex could potentially reach 3.3 and $8.8 \text{ mM}^{-1} \text{ s}^{-1}$, respectively.

Outer sphere effects of a Gd^{3+} -based agent are usually characterized by a hard sphere model, where the relaxivity is mainly determined by the diffusion constant of the water, the closest distance of proton nuclear spin, and the gadolinium electron spin. $\text{Gd}(\text{C}_{11}\text{-DOTP})^{5-}$ is known Gd^{3+} complex with $q = 0$. Owing to the contribution of outer sphere mechanism, the relaxivity of $\text{Gd}(\text{C}_{11}\text{-DOTP})^{5-}$ is about 4.6 at 20 MHz at 25°C . After binding to human serum albumin (HSA), the per Gd^{3+} relaxivity of $\text{Gd}(\text{C}_{11}\text{-DOTP})^{5-}$ -albumin complex can increase to about $40 \text{ mM}^{-1} \text{ s}^{-1}$ at 5°C . Since $\text{Gd}(\text{C}_{11}\text{-DOTP})^{5-}$ does not have any inner sphere water, such remarkable relaxivity is mainly due to second and outer sphere water. Relaxivity increases when the temperature decreases from 37 to 5°C , which indicates that the τ_m of second and outer sphere water is approaching an optimized condition when $\text{Gd}(\text{C}_{11}\text{-DOTP})^{5-}$ binds to albumin.⁴⁹ Recently, the relaxivities of the monocrystalline superparamagnetic iron oxide nanoparticle (MION) were shown to be influenced by the coating thickness of the hydrophilic PEG, due to their influence of the water retention to the core of the MION. As the thickness of PEG increases, R_2 decreases and R_1 increases.^{50,51} In this case, both physical abundance of protons and water residency time were suggested to alter the relaxation rates. The coating molecules of contrast agents could change the water abundance on the surface, hinder water diffusion, and immobilize water molecules through hydrogen bonds.

An additional tactic to increase the relaxivity for Gd^{3+} -based contrast agents is to increase q , or the number of water ions that fill the coordinate shell around Gd^{3+} . Pioneered by Meade et al. a class of smart contrast agents has been developed by modulating the number of q upon responses to chemical events such as calcium, zinc, and enzymatic actions.^{52–55} Theoretically, when q is doubled from 1 to 2 , the relaxivity of the contrast agents should also be doubled. However, this is a very tricky parameter to optimize because one must strike a balance between allowing as many water molecules to coordinate with gadolinium as possible while keeping the gadolinium ion tightly chelated within the contrast agent structure. It is important that q is increased carefully in order to ensure Gd^{3+} is not made liable to dissociation. Raymond et al.,^{19,56} developed hydroxypyridinone-based MRI contrast agents with $q = 2$ or 3 . Competition experiments between Gd^{3+} and other metal ions suggest that these contrast agents have comparable stability to clinical MRI contrast agents.

These contrast agents are also resistant to the competition from other anions, such as phosphate.

RATIONAL DESIGN OF PROTEIN-BASED MRI CONTRAST AGENTS

In the past three decades, Gd^{3+} -chelators were covalently or non-covalently bonded to proteins to alter *in vivo* properties of MRI contrast agents. For example, MS-325 can reversibly bind to albumin with increased blood circulation time and improve the relaxivity. Because of these improvements, MS-325 is applied to imaging blood vessel abnormalities.⁴⁴ Gd -chelates were also covalently linked to proteins such as albumin. Covalent linkage of Gd^{3+} to albumin increases the relaxivity and changes the distribution of Gd^{3+} . Meade et al.⁴¹ developed protein-polymer MRI contrast agents. In their design, DO3A-based Gd^{3+} chelators were linked to lysine-containing random-coil polymers. The per Gd^{3+} relaxivity is up to $14 \text{ mM}^{-1} \text{ s}^{-1}$ and per particle relaxivity is about $461 \text{ mM}^{-1} \text{ s}^{-1}$.

ProCA is different from any of these Gd^{3+} labeled proteins in which Gd^{3+} -chelates covalently or non-covalently linked to proteins. ProCA uses side chains from the scaffold protein to directly generate a Gd^{3+} binding protein and our designed protein itself serves as a chelator to tightly bind to Gd^{3+} (Figure 1(c)). Similar strategies have been applied by Caravan et al.²⁷ and Liepold et al.⁵⁷ where the Gd^{3+} binding sites were formed by the amino acid side chains of helix-loop-helix peptide or virus particles. Caravan et al. *de novo* designed a metallo-peptide with helix-loop-helix structure, which bind to DNA with a 100% increase of per Gd^{3+} relaxivity ($r_1 = 42.4 \text{ mM}^{-1} \text{ s}^{-1}$ at 60 MHz and 37°C). Liepold and his colleagues developed a Gd^{3+} -loaded Cowpea chlorotic mottle virus (CCMV), and 180 Gd^{3+} were able to load in CCMV. A T_1 relaxivity of Gd^{3+} -loaded particle is $202 \text{ mM}^{-1} \text{ s}^{-1}$ (at 61 MHz).⁵⁷

We hypothesize that MRI contrast agents with high relaxivity can be achieved by directly designing Gd^{3+} binding sites into stable proteins to improve the three key factors of τ_c , q , and outer coordinate shell contributions without sacrificing desired *in vivo* diffusion of water and stability of Gd^{3+} . Figure 1(c) shows the modeled structure of designed ProCA1 in domain one of CD2 using our developed computational methods. A Gd^{3+} binding pocket is formed by several oxygen atoms from carboxyl side chains such as Asp and Glu from different stretches of the host protein and one side of Gd^{3+} binding pocket is designed to open to allow Gd^{3+} to have fast water exchange. Different from previous studies using existing Gd^{3+} chelators to attach or bind to larger macromolecules, we have designed protein-based contrast agents by creating binding sites directly in proteins for Gd^{3+} with desired metal-binding affinity and relaxation properties. The development of our protein-based MRI contrast agents is based on our cumulated efforts in understanding metal coordination and key determinants for metal-binding affinity, selectivity, conformational change, and dynamic properties of metalloproteins using protein design.^{58–64} To understand metal selectivity, we have performed extensive analysis of structural parameters such as the ligand types, coordination numbers, water numbers, and bond angles and lengths of different classes of metal-binding sites in proteins.^{65,66}

In designing ProCA three main parameters were tuned in order to obtain high relaxivity. These three parameters are τ_c : a time constant that refers to local magnetic fluctuations; outer and second shell optimization: interactions between the contrast agent and water molecules outside the inner sphere; and q : the number of bound water molecules.

First, directly coordinating Gd^{3+} ions to protein ligand residues eliminates the high internal mobility of the paramagnetic moiety associated with polymers and protein conjugates. The proton T_1 relaxivity can be dramatically increased due to the increase in correlation time. We selected a rigid host structure as the scaffold protein for our contrast agent because of its high resistance to pH and salt denaturation and tolerance to mutations as well as its correlation time τ_R of 9.2 ns, which is very close to the most optimized τ_R based on the SBM equation.^{33–37} Second, contribution of the second and outer layer spheres can be explored by protein engineering and modification. Third, the different coordination shells provide us with the possibility of increasing water q without sacrificing metal-binding affinity and selectivity. We have previously shown that metal selectivity is also largely dependent on the ligand types and chemical properties such as electrostatic interactions of protein environment, as well as long range interactions.^{58,60,63,67} We successfully designed metal binding sites in a scaffold protein with strong target metal selectivity in the presence of excess physiological metal ions.^{58–64} Structure determination by solution nuclear magnetic resonance (NMR) revealed that the actual coordination geometry in a designed variant is the same as our design, verifying the computational methods and the design strategy of incorporating metal-binding sites in proteins.^{58–64} Fourth, targeted contrast agent with improved specificity to certain biomarkers can be developed by expression as a fusion protein or by grafting of the protein. Additionally, proteins are biocompatible and have been modified to protein drugs to overcome adverse effects such as immune responses due to rapid current advances in biotechnology and pharmacology.⁶⁸

The per Gd^{3+} relaxivity of ProCA1 is $117 \text{ mM}^{-1} \text{ s}^{-1}$ at 1.5 T, $48 \text{ mM}^{-1} \text{ s}^{-1}$ at 3T, and $6 \text{ mM}^{-1} \text{ s}^{-1}$ at 9.4 T, and is much higher than most of clinical MRI contrast agents. Such substantial increase of relaxivity is likely due to optimized q , τ_c , and outer sphere relaxivity. Tb^{3+} luminescent lifetime experiments indicate that ProCA1 has a $q = 2.1$. Strikingly, we overcome the instability of contrast agents when q is increased. Both r_1 and r_2 increase when temperature decrease, indicating that ProCA1 has fast water exchange. ProCA1 has much higher metal selectivity ($pGd/pMg > 10.0$, $pGd/pZn = 5.3$) than clinical MRI contrast agents ($pGd/pMg = 4.3$, $pGd/pZn = 4.2$ for Gd-DTPA). No precipitation was found when ProCA1 was supplemented with Ca^{2+} and phosphate, indicating that Ca^{2+} and phosphate cannot compete for Gd^{3+} .⁶⁴ We have also designed a novel generation of ProCA with multiple Gd^{3+} binding sites and improved stabilities (named ProCA2 and ProCA3) (Xue et al., unpublished results).

In addition to taking advantage of the larger hydration surface of the protein as opposed to a small molecule, we further increased relaxivity of ProCA1 by addition of a secondary shell with PEGylation. The PEGylation modifications dramatically increased longitudinal and transverse relaxivities of the ProCA1 at different field strengths tested (0.47, 1.4, 3.0, and 9.4 T). The r_1 and r_2 values of ProCA1-PEG0.6k show an increase of almost 66 and 110% compared with ProCA1. The r_1 and r_2 values of ProCA1-PEG2.4k increased by 100 and

125% and the r_1 and r_2 values of ProCA1-PEG12k increased by 252 and 130% from ProCA1. By comparison with Gd-DTPA, whose r_1 and r_2 values are less than $10 \text{ mM}^{-1} \text{ s}^{-1}$ at any magnetic field strength, the ProCA1 and PEGylated ProCA1 showed dramatically higher r_1 and r_2 values. ProCA1-PEG12k exhibits 19-fold higher r_1 and r_2 values compared with Gd-DTPA. PEGylated ProCA1 displayed relaxivities that are even higher than nanoparticle-based contrast agents. Additionally intriguing, at high field strength of 9.4 T, ProCA1-PEG2.4k still exhibited great increase of relaxivity values r_1 and r_2 .³²

Improving the relaxivity of the contrast agents has two advantages. First, increasing the relaxivity of the contrast agents can decrease the required injection dosage, which could potentially decrease the Gd^{3+} accumulation *in vivo*. Second, improving the relaxivity can potentially improve the detection limits for an MRI contrast agent, which greatly benefits molecular imaging using MRI. As shown in Table 1, the injection dosage of only 0.0024–0.0048 mmol Gd/kg for ProCA was used to generate excellent *in vivo* imaging (Figure 2). It is estimated that 0.02 mmol Gd/kg is needed for the molecular imaging of human epidermal growth factor receptor 2 (HER-2) biomarkers.^{64,32,69} On the other hand, clinical contrast agents usually require injection of 0.1–0.2 mmol Gd/kg to obtain magnetic resonance (MR) images with good contrast. The molecular imaging of HER-2 by three-step targeting requires as high as 0.145 mmol/kg of avidin-Gd-DTPA to clearly image tumor.⁷⁰ Nanoparticles, such as G-5 dendrimers, have an injection dosage of 0.03 mmol Gd/kg. Indeed, by improving the relaxivity, the dosage efficiency of ProCA is significantly improved compared with other contrast agents. Our HER-2 MRI results⁶⁹ indicate that by incorporating targeting moieties, ProCAs could be applied to imaging other cancer biomarker because of efficient targeting and high relaxivity.

STABILITY OF PROTEIN-BASED MRI CONTRAST AGENTS

Free Gd^{3+} is toxic with LD_{50} about 0.2 mmol/kg in mice; thus, it is essential that designed MRI contrast agents have strong metal-binding affinities and selectivity. We applied several spectroscopic methods to evaluate the metal-binding affinity and selectivity of ProCAs. The first generation of ProCA, ProCA1, has a very good metal selectivity for Gd^{3+} over physiological metal ions, such as Zn^{2+} , Mg^{2+} , and Ca^{2+} ($\log(K_{\text{Gd}}/K_{\text{Zn}}) = 5.34 \log(K_{\text{Gd}}/K_{\text{Mg}}) > 10.06$ and $\log(K_{\text{Gd}}/K_{\text{Ca}}) > 9.84$), although the binding affinity is weaker than clinical MRI contrast agents. The high Gd^{3+} stability and selectivity of ProCA is further supported by the observation that the r_1 is not changed in the presence of 10 mM Ca^{2+} .⁶⁴ The new generation of ProCA, ProCA3, has a thermodynamic stability constant (pGd) of 21.6, (Xue, unpublished data) which is comparable to most of the clinical MRI contrast agents and much high than Gd-DTPA-BMA (pGd = 16.6).

The stability of protein in the circulation system is essential for the *in vivo* application of ProCA. To test the serum stability, ProCAs were incubated with 75% human serum for 3 and 6 h. The stability of the ProCAs was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, and the ProCAs remains intact after 6 h of incubation. Taken together, ProCAs are stable for *in vivo* application.

MOLECULAR IMAGING OF BIOMARKERS BY MRI

As discussed above, to achieve molecular imaging of biomarkers, targeted contrast agents with high specificity are required in addition to its sensitivity. To date, many studies have been devoted to developing targeted MR contrast agents to achieve molecular imaging by MRI (Table 1). Commonly, antibody, peptide or small ligand, and small protein domains such as affibodies have been used to achieve targeting.^{4,22,24,70,76–82,84,83,85} To enhance the sensitivity of the contrast agents, these targeting moieties are usually linked to high payload MRI contrast agents, such as nanoparticles. Conjugation of fibrin antibody to emulsion nanoparticles has been successfully applied to image vulnerable plaques.²⁴ Also, conjugation of arginine-glycine-aspartic acid (RGD) peptide to emulsion nanoparticles is successfully applied to image integrin, which is up-regulated in many diseases such as cancer and atherosclerosis.^{81–84} However, owing to the large size and low tissue penetration, nanoparticles may be limited to imaging biomarkers on the blood vessel. Many strategies have been applied to overcome the limitation of extravasation and diffusion barriers for the macromolecule. Bhujwalla and coworkers applied a multistep targeting and prelabeling strategy using antibody-tagged magnetic particles.^{70,86} EP-3533, a small collagen targeting peptide conjugated with Gd³⁺-chelates, is able to target to collagen in the collagen-rich scar in liver.^{76,77} In addition, synthesized small molecules such as peptoid-(Gd)₈-dendron were also applied for molecular imaging to vascular endothelial growth factor receptor 2 (VEGFR2), the angiogenesis biomarker highly expressed in tumors.⁸⁰ However, although great achievement has been made over the last decade, the development of MRI contrast agents for molecular imaging by MRI is largely hampered by relatively low sensitivity compared with PET/SPECT, inadequate perfusion to diseased tissue, instability of the peptide targeting moieties, and targeting specificity and selectivity.

Figures 3(a) and 4(b) show our developed targeted contrast agents against two biomarkers of HER2 and GRPR that are over-expressed in several different types of cancer cell surfaces. We will show our effects on the molecular imaging of cancer biomarkers by targeted ProCAs in the next few sections.

Molecular Imaging of HER-2 Expression Level by Targeted Contrast Agent ProCA1.affi for Breast Cancer Diagnostics

Biomarkers such as the epidermal growth factor receptors EGFR and HER2/Neu are highly expressed in various diseases including breast and ovarian cancers and play important roles in disease progression and survival. HER-2 is a major prognosis biomarker expressed in 30% breast cancer and 60–70% of ductal carcinoma *in situ* (DCIS) tissue. Monitoring the spatial and temporal changes of several molecular biomarkers such as HER-2/EGFR sharing the same signaling pathway during cancer progression and treatment is the key for understanding the molecular basis of cancers, early and accurate diagnosis, and for developing effective drugs with synergistic effects to treat this deadly disease. They are also major drug targets for targeted therapy. The clinical application of targeted therapy is largely limited because current methods for assessment of these cancer biomarkers involve invasive methods, such as biopsy, and because the effectiveness of the target therapy largely depends on the preselection of patients over-expressing these biomarkers. To date, one of five

HER2/Neu clinical tests, including biopsy and immunostaining (IHC, immunohistochemistry) provides incorrect results, leading to improper selection of appropriate patients for personalized treatment using biomarker targeted therapies.^{87,88} To achieve MRI of HER-2, Bhujwalla and coworkers has applied a three-step strategy for the targeting: biotinylated trastuzumab binds to HER-2, avidin binds to biotin, and bG4D-Gd-SA binds to avidin.^{70,86} There is an urgent need to develop noninvasive and accurate methods to facilitate diagnosis and selection of patients and to monitor biomarker levels/distribution and their changes upon treatment by targeted drugs.

HER-2 targeted contrast agent ProCA1.affi was created by fusing the C-terminal of ProCA1 with a HER-2 affibody. We use HER-2 affibody instead of an antibody for several important reasons. It has a comparable binding affinity to an antibody and strongly and selectively binds to HER-2 with a K_d of 22 pM.⁸⁹ On the other hand, it has a size of 5.8 kDa which is significantly smaller than antibodies (~150 kDa). The fusion protein, named ProCA1.affi, has a molecular weight of 17 kDa which means ProCA1.affi is more ideal for tumor penetration. Furthermore, the binding site by affibody is different from Herceptin, a therapeutic antibody, allowing a clinician to monitor the change of HER-2 expression during drug treatment. Because ProCA1.affi has high relaxivity, high tumor penetration, and high binding affinity to HER-2, ProCA1.affi can potentially be applied to image HER-2 expression level in tumor.⁶⁹

Figure 3 shows that ProCA1.affi is able to differentially enhance several cancer cells with different expression levels. We implant tumors with different expression levels in each mouse. SKOV-3 has high HER-2 expression level (10^6 receptors/cell), whereas MDA-MB-231 tumor has low HER-2 expression level (10^4 receptors/cell). As shown in Figure 3, after tail vein injection of contrast agents with 10-fold lower injection dose than Gd-DTPA, ProCA1.affi can specifically enhance the SKOV-3 tumor which has high expression level of HER-2. To evaluate the specificity of HER-2 enhancement in tumor, HER-2 in xenograft mice were first blocked with HER-2 affibody without contrast agents, then ProCA1.affi was injected for the MRI, no enhancement were found in tumor after blocking (Figure 3(d)). These results support that tumor enhancement is due to ProCA1.affi and HER-2 interaction, and tumor enhancement is not due to blood perfusion, blood vessel permeability, or necrosis. These results indicate that ProCA1.affi can be used to evaluate the expression level of HER-2 biomarkers by the molecular imaging of MRI. ProCA1.affi could be further applied to quantitatively evaluate tumor biomarker expression and receptor occupancy using MRI.⁶⁹

Optimizing Peptide Targeting Capability to Tumor Biomarkers by Grafting Approach

Fusing or conjugating short peptide fragments with affinity to biomarkers (peptide targeting) has been commonly used in molecular imaging because of its small size and advances in peptide synthesis.²⁸ However, the application of this approach faces two limitations: (1) peptides are more easily degraded by enzymatic cleavage with a short half-life *in vivo* and (2) the undefined structure from a peptide could decrease binding specificity and binding affinity to the target biomarker. In the effort to improve peptide targeting, we applied a grafting approach to overcome these two limitations (Figure 4(b)).

Gastrin-releasing peptide receptor (GRPR) is a biomarker for many cancers such as prostate, breast, and small cell lung cancer.^{90,91–93} Its natural peptide ligand, gastrin-releasing peptide (GRP), binds to GRPR with high affinity.⁹⁴ We use grafting approach to link GRP peptide in the middle of ProCA1 to achieve molecular imaging of GRPR in a prostate tumor model (Figure 4(a) and (b)). As we put peptide in the middle of the ProCA by grafting approach, the targeting peptide is effectively protected from protease degradation. We added flexible linkers to the end of the peptide to give some flexibility to the peptide for targeting. Interestingly, when the peptide is grafted in the middle of ProCA1, much higher targeting capability is observed than when GRP is linked to the C-terminal (Figure 4(c)). Using these strategies, we successfully imaged GRPR in prostate tumor cells through intratumoral injection.⁹⁰ Moreover, our unpublished data shows that GRP is stable for at least 6 h when it is grafted in ProCA1, while literature shows the proGRP (a precursor of GRP containing GRP peptide) decreased by 6–28% after 2 h at room temperature.⁹⁵ Thus, grafting approach protect peptide from degradation. The grafting approach could be applied to other peptide-based targeting for the molecular imaging of tumor biomarkers.

Size is Essential for Contrast Agents Distribution and Excretion

The locations of cancer biomarkers are diversified. For example, VEGFR is a biomarker expressed on blood vessel surface of a tumor, while HER-2 is a biomarker deeply expressed in a tumor outside of the blood vessel. Thus, the ideal half-life for targeting varies in different biomarkers. In principle, longer circulation gives more time for Gd^{3+} dissociation and toxicity. Ideally, the contrast agents should have a short blood circulation time if the biomarker is expressed on the blood vessel surface. On the other hand, for molecular imaging of a tumor biomarker deeply expressed outside of the blood vessel, the contrast agents should have sufficient blood half-life to allow the contrast agents to penetrate deeply into the tumor and then quickly be excreted out of the body to reduce the toxicity. ProCA1 in mice has a half time varying from 30 min to hours depending on the surface modification³² and exhibits enhancement in various organs (Figure 2). At time of less than 1 h, an enhancement of the bladder was observed suggesting its renal excretion. Such *in vivo* behavior and half-life are very different from the minutes timescale observed in small chelators or days from nanoparticles that were uptaken by macrophages and accumulated in the liver and spleen. De Jong et al.⁹⁶ studied the distribution of gold nanoparticles with a diameter of 10, 50, 100, and 250 nm. After 24 h, 10-nm gold nanoparticles distributed in various organs such as the blood, liver, kidney, spleen, heart, lung, and brain, whereas 50-nm gold nanoparticles mainly distributed in the blood, lung, liver, and spleen. The larger particles with a diameter larger than 50 nm mainly located in the blood, liver, and spleen⁹⁶. Besides influencing tumor penetration and organ distribution, the size of the contrast agents could also influence renal excretion. A molecule with a molecular size greater than 7 nm in diameter (about 60 kDa) is not readily able to pass through the glomeruli, and the blood half-lives of these molecules in mice are usually much longer than 80 min.⁹⁷ For example, lysozyme has a molecular weight of 15 kDa and hydrodynamic diameter of 3.4 nm, and the blood half-life is 12 min. When the molecular weight is increased to 152 kDa as an example of IgG, the blood half-life is 330 min. There is an increasing concern about the toxicity of nanoparticles with a size greater than 15 nm since the renal excretion is prevented for these large macromolecules because of the risk of accumulation in the human body.⁹⁷

Size and Tissue Penetration is Essential for Molecular Imaging of Cancer Biomarkers

Besides high relaxivity and high dose efficiency, the size of the contrast agents is also essential for the molecular imaging of tumor biomarkers. To quantitatively evaluate tumor biomarker expression levels, contrast agents should be first homogeneously distributed in the tumor. Then, the nontargeted contrast agents should be easily washed out of the tumor to avoid unnecessary false positive enhancement under MRI.

Tumors have abnormal blood vasculature compared to normal tissue. The growth of a tumor is much faster than blood vessels; therefore the rapid proliferation of tumor cells forces the blood vessel apart. Tumor and stroma cells also secrete enzymes and growth factors to facilitate the formation of new blood vessels and extracellular matrix. The extracellular matrix could slow down the penetration of the molecule to the inside of the tumor. Furthermore, high interstitial fluid pressure in the tumor forms a barrier for drug and imaging reagent penetration to the tumor from the blood vessel.⁹⁸ Enhanced permeability and retention (EPR) effects show that a molecule with a molecular size from 40 kDa to more than 778 kDa can accumulate in the tumor with high concentration.⁹⁹ However, the optimized size for imaging tumor biomarkers could be much smaller. To evaluate tumor biomarkers outside of the blood vessel, the ideal contrast agents should be able to first efficiently penetrate to the tumor and then the unbound form should be quickly washed away. Proteins with molecular weight less than 40 kDa could be quickly washed out from tumor. What is the possible good size for imaging reagents have efficient tumor penetration? Dreher et al.¹⁰⁰ used fluorescent labeled dextrans with a molecular weight difference between 3.3 kDa to 2 MDa to evaluate the role of the drug size on the tumor accumulation and penetration. Consistent with EPR effects, 40–70 kDa dextrans has the highest accumulation in tumors within 30 min. However, the dextrans with a molecular weight larger than 40 kDa are mainly accumulated along the blood vessel of the tumor. Dextrans with a molecular weight of 4.7 and 10 kDa can become deeply (larger than 35 μm) distributed in the tumor within 30 min, whereas 2-MDa dextrans can only penetrate 5 μm .¹⁰⁰ Thus, imaging reagents with a large size are not ideal for MRI imaging of tumors. Currently, MRI molecular imaging using Gd^{3+} -based nanoparticles mainly target to cardiovascular and tumor vasculature biomarkers such as fibrin and integrin^{22,24,70,76–82,84,83,85} with limited penetration to tumor tissue. The molecular imaging of MRI to evaluate tumor biomarkers is limited by the lack of MRI contrast agents with high dose efficiency, high tissue penetration, proper blood retention time, and good renal excretion profile.

To test the penetration of contrast agents in tumors, we linked the HER-2 targeting moiety, affibody, to ProCA (named ProCA1.affi) and then compared the tumor penetration of antibody (MW about 150 kDa) and ProCA1.affi (MW about 17 kDa) at different time points. We iv-injected either HER-2 antibody or ProCA1.affi into the SKOV-3 tumor-bearing mice. ProCA1.affi can be stained in the tumor after 4-h postinjection. On the contrary, no antibody in tumors can be stained by immunofluorescence at the same time point. ProCA.affi are evenly distributed in tumors with high concentration 24-h postinjection, whereas the antibody is only localized in the region near the blood vessel with much lower accumulation (Figure 5). These results indicate that ProCA1.affi exhibits unprecedented tumor penetration, and such unique features have great potential for

quantitative/semiquantitative evaluation of biomarker changes during disease progression and drug treatment.

CONCLUSION AND PERSPECTIVES

The application of MRI for molecular imaging is limited by the lack of MRI contrast agents with high relaxivity for sensitivity, biomarker selectivity, good tissue penetration, and *in vivo* properties. We have created a novel class of contrast agents by designing a Gd^{3+} binding pocket into a protein scaffold. High relaxivities of ProCA are achieved by tuning τ_R , q , and second and outer sphere relaxivity.

To avoid large molecular size, we graft affibody or peptide instead of an antibody to our contrast agents to target cancer biomarkers. We also carefully designed the proper protein size to optimize blood retention time and tumor penetration. The molecular imaging of several tumor biomarkers is achieved by our careful design of targeted ProCA. Currently, ProCAs have been developed to target HER-2 and GRPR which are over-expressed in many types of cancers including breast and prostate cancers. However, ProCA certainly is not limited to these two biomarkers. Theoretically, ProCAs could be targeted to various over-expressed biomarkers due to the ease of grafting targeting moieties onto the protein structure.

With the advantage of spatial resolution and depth penetration, MRI has been applied in both preclinical drug development and clinical diagnostics. The high relaxivity and organ distribution feature of nontargeted ProCA make it a promising tool for imaging tumor metastasis with high sensitivity, specificity, and dose efficiently. Owing to the longer blood retention time and slower tumor penetration than clinical contrast agents, nontargeted ProCA can also be applied to DCE-MRI to evaluate tumor vasculature with increased accuracy. Targeted imaging by MRI has great potential to evaluate the drug effects on cancer biomarkers changes with high accuracy. Our results show that targeted ProCA is able to semiquantitatively differentiate biomarker expression levels, which is a great advantage for semiquantitative evaluation of biomarker expression level changes in preclinical disease models after drug treatment. The repetitive administration of targeted ProCA in the same animal in preclinical studies could potentially save the cost of animals and enable tracking of biomarker changes over time in small animal studies. Besides, targeted ProCA may have a potential application to clinical diagnostics for patient selection and monitoring drug treatment.

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FIGURE 1.

(a) Schematic representation of a Gd^{3+} chelate surrounded by bulk water with one inner sphere and three second sphere water molecules and several outer sphere water molecules. Modification of contrast agents by PEG could change the water properties which could further influence relaxivity. (b) Simulated magnetic field-dependent relaxivity r_1 of clinical MRI contrast agents and ProCA based on the given τ_R , τ_m , τ_v , and γ^2 and Solomon-Bloembergen-Morgan (SBM) theory using combinations of τ_R (100 ps for clinical MRI contrast agents and 10 ns for ProCA), τ_m (0.1, 1, 10, and 100 ns), τ_v (10 ps), and γ^2 ($5 \times 10^{19} \text{ s}^{-2}$) under a magnetic field strength from 0.01 MHz to 10,000 MHz. (c) Model structure of ProCA1 with PEG modification.

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FIGURE 2.

Multiple organ enhancement of magnetic resonance imaging (MRI) of mice after injection of ProCA1 for 20 min (b), 3 h (c), 24 h (d) compared with pre-scan (a). (e) MRI intensity changes of kidney (black) and liver (gray) before and after injection of ProCA1. (Reprinted with permission from Ref 32. Copyright 2012 Elsevier)

FIGURE 3.

Molecular imaging of HER-2 by ProCA1.affi. (a) Model structure of ProCA1.affi. (b) Magnetic resonance imaging (MRI) of HER-2 xenograft tumor (SKOV-3 and MDA-MB-231) before and after injection of ProCA1.affi. (c) Tumor intensity changes over time postinjection of ProCA1.affi. SKOV-3 has much higher HER-2 expression and SKOV-3 has more MRI signal enhancement than that of MDA-MB-231. The MRI signal intensities of SKOV-3 or MDA-MB-231 tumor regions from six adjacent slides were quantified by Image J software. The average signal intensities and the standard deviation were then calculated from these six adjacent slides. (d) MRI of the mouse SKOV3 tumor pre-blocked by affibody ZHER_{2:342} (bottom) and without blocking (top). (Reprinted with permission from Ref 69. Copyright 2011 PloS ONE)

FIGURE 4.

Design (a, b) and binding test (c) of ProCA1.GRP for the molecular imaging of gastrin-releasing peptide receptor (GRPR). GRP peptide was linked either at C-terminal (a, named ProCA1.GRPC) and in the middle of ProCA1 with grafting approach (b, named ProCA1.GRP(52)). (c) Radioactive binding test of ProCA1, ProCA1.GRPC, and ProCA1.GRP(52) to GRPR high-expression cells (PC3 and DU154) and GRPR low-expression cells (H441). ProCA1.GRP(52) shows the best binding among three contrast agents. (Reprinted with permission from Ref 90. Copyright 2010 Springer)

FIGURE 5.

Immunofluorescence imaging of ProCA1.affi (left) or HER-2 antibody (right) in SKOV-3 xenograft tumors in mice after IV injection. ProCA1.affi and HER-2 antibody are stained with red color. Blood vessel is stained with green color. ProCA1.affi is evenly distributed in tumor 24-h postinjection, whereas HER-2 antibody only accumulated in near the blood vessel. (Reprinted with permission from Ref 69. Copyright 2011 PloS ONE)

TABLE 1
The Size, Organ/Tissue Enhancement, Biomarker Recognition, Relaxivity, and Dosages of Typical MRI Contrast Agents

Targeting or Not	Contrast Agents	Size	Organ/Tissue Enhancement	Biomarker Recognition	Per Gd ³⁺ , r ₁ , mM ⁻¹ s ⁻¹	Field Strength, T	Temperature, °C	Dosage, μmol Gd/kg	References
Nontargeted contrast agents	Gd-DTPA	547 Da	Whole body, brain, CNS, kidney	None	3.5	1.5	20	100–200	71
	MS-325		Blood vessel	None	42	0.47	37	30	44, 72
	Gadomer-17	35 kDa	Blood vessel	None	17.3	0.47	40	25–100	73
	PAMAM-G4	59 kDa	Liver, kidney, blood vessel	None	29	1.5	20	30	15, 45
	NanoglobularMRI CAs (G3)	3.2 nm 27 kDa	Blood vessel, tumor, vasculature	None	10	3	RT	10–30	74
ProCA	Gold nanoparticles	1.9 nm	Kidney, bladder	None	4.1	7	RT	[Gd ³⁺] 5 mM, 300 μl	75
	ProCA1, ProCA1.affi, ProCA1.GRP	12 kDa	Kidney, liver, and tumor biomarkers	Nontarget, HER-2, GRPR	117	1.4	RT	2.4	64
		2–3 nm							
Targeted contrast agents	EP-3533		Collagen-rich scar, collagen-rich liver	Collagen	18.7	0.47	37	25 μmol particle/kg, 20 μmol particle /kg	76, 77
	EP-3600		Myocardium	Collagen	21.3	1.4	37	12.3 μmol particle/kg	78
	Gd-DOTA-R832		Inflammation testions in liver	VCAM-1	8.5	0.47	37	100	79
	Peptoid-(Gd)8-dendron	~8.6 kDa	Tumor	VEGFR2	13.8	0.54	37	8 μmol particle /kg, 32 μmol Gd/kg	80
	α _v β ₃ -Integrin-targeted nanoparticles	270 nm	Tumor	α _v β ₃ -integrin	20 (1,800,000)	0.47	40		81
		167 and 236 nm	Tumor	α _v β ₃ -integrin				2 mL/kg, 0.2 nM	82
		273 nm	Tumor	integrin	19.1	0.47	37	3	83
					0.47 T				
		273 nm	Angiogenesis in atherosclerosis	α _v β ₃ -integrin	17.7	1.5		0.5 mL/kg	84
	bGdD-Gd-SA	4182 Da	Her-2 positive tumor	HER2 by multiple step				145	70
	Gd-perfluorocarbon nanoparticles	<250 nm	Vulnerable plaques	Fibrin	18			0.25–0.5 mL/kg	24
	Dendrimer nanoclusters	75–150 nm	Subcutaneous tumor xenografts	Folate receptor	12.3	1.4	40	300	22
	P947		Atherosclerotic plaques	MMPs, ACE, and APN					85

CNS, central nervous system; MRI CA, magnetic resonance imaging contrast agents; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; MMP, matrix metalloproteinases; ACE, angiotensin-converting enzyme; APN, aminopeptidases N