# 8 Magnetic Resonance Signal Amplification Probes

A. A. Bogdanov Jr., J. W. Chen, H. W. Kang, R. Weissleder



The ability to image specific molecular biomarkers in vivo would have important implications for the earliest detection of cancer, in assessing specific targeted therapies, and for monitoring dynamic changes in expression patterns during disease progression. However, many molecular biomarkers are expressed in low numbers, necessitating novel imaging signal amplification strategies.

Among various signal amplification strategies described so far (reviewed in Aime et al. 2002; Bogdanov and Weissleder 1998) we chose to focus on: (1) internalization of superparamagnetic particles via receptor-mediated endocytosis (Kang et al. 2002) and (2) magnetic resonance (MR) signal amplification mediated by enzymes (Bogdanov et al. 2002).

## 8.1 Receptor-Mediated Internalization

### 8.1.1 Transferrin Receptor Imaging

Initially, the receptor-mediated amplification approach has been investigated by using transferrin receptor (TfR) as a molecular shuttling system (Fig. 1). The expression of this receptor has been a subject of extensive research in molecular oncology because of its role in mediating macromolecular transport across the microvascular endothelium of the blood-brain barrier (Huwyler et al. 1996) and is overexpressed in many tumors. The latter observation suggested potential targetability of TfR for cancer therapy or tumor detection (Kresse et al. 1998; Weissleder et al. 2000). The potential of the receptor for gene expression imaging by MRI was also identified (Koretsky et al. 1996). MRI research suggested that receptor overex-

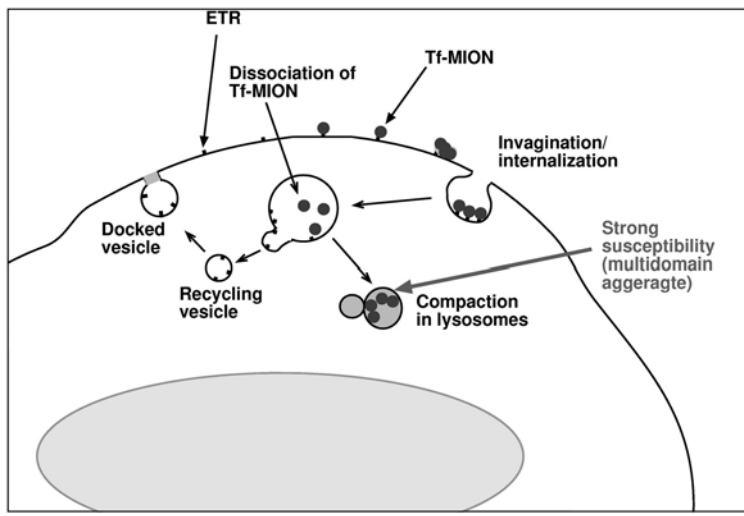


**Fig. 1.** Amplification through cellular internalization of iron oxide nanoparticles. Amplification is achieved as a result of iron oxide (magnetite) superparamagnetic susceptibility effect plus through nonregulated uptake and storage of iron oxide in intracellular compartment (formation of interacting multiple-domain systems)

pression could lead to iron accumulation in cells detectable by imaging (Kresse et al. 1998). The molecular basis of the observed effects is in the ability of TfR to internalize into the cells and to recycle back to the cell surface very rapidly. However, intracellular iron transport is tightly controlled and is a subject of feedback downregulation with resultant low efficiency of transferrin receptor as a vehicle for paramagnetic iron delivery into cells. We hypothesized that it would be possible to (1) circumvent the iron-mediated downregulation of transferrin receptor by "masking" iron in dextran-coated iron oxide nanoparticles (MION) conjugated with transferrin (Tf-MION), and (2) that Tf-MION would amplify the MR signal as a result of internalization and compartmentalization inside the cell. The amplification effect was expected because of the susceptibility effects generated by iron oxide microscopic magnetic field gradients from iron oxide particles that become stronger as the superparamagnetic crystalline domains (and magnetic moments) of several particles interact as they are confined in an intercellular vesicle (Moore et al. 1998). Diffusion of water protons in the vicinity of such gradients results in efficient transverse relaxation and susceptibility (the increase of R2 and R2*). Therefore, efficient internalization of iron oxide would result in increased transverse relaxivity of cells and, as a consequence, better detectability of receptor-positive cells. The results obtained in mouse xenografts suggested that transgene expression in tumors could be visualized noninvasively by MR imaging (Weissleder et al. 2000). The studies demonstrated that specific recognition of cell-surface receptor by targeted MR imaging probe with subsequent internalization could lead to dramatic relaxation time changes and susceptibility effects.

### 8.1.2 Inducible Endothelial Receptor Imaging

One of the most promising molecular imaging applications that can be accomplished using MRI is "precision" imaging of cell-surface molecules associated with the expression of mitotic or otherwise activated phenotypes. This important task can be potentially accomplished by using cell-surface targeted agents. We hypothesized that the prominent marker of endothelial activation and proinflammatory

molecule, E-selectin (ELAM-1) expression in tumor endothelium can be of prime importance for imaging tumor angiogenesis. The expression of E-selectin is transcriptionally upregulated as a result of endothelial cell exposure to several cytokines (e.g., IL-1$\beta$ and TNF$\alpha$) and lipopolysaccharide (Luscinskas et al. 1989; Montgomery et al. 1991). As demonstrated previously, several monoclonal antibodies to human E-selectin detect inducible E-selectin expression in human endothelial cells with high specificity (Bevilacqua et al. 1987; Peters 1998). E-selectin-targeted immunoliposomes and anti-E-selectin-hirudin conjugates prepared using H18/7 antibody were previously shown to bind to activated human endothelial cells in vitro at levels exceeding 200-fold those of control cells. These findings suggest that H18/7 antibody or its fragments can potentially serve as excellent targeting ligands for designing imaging probes.

One of the properties of E-selectin that made iron oxide-mediated targeting approach especially attractive is the ability of E-selectin to internalize soon after expression on the cell surface (Chuang et al. 1997; von Asmuth et al. 1992). Internalization of E-selectin is a tubulin-dependent process governed by the signal expressed in the cytoplasmic domain of E-selectin. It has already been established that E-selectin-bound mAb can be recovered in intracellular compartments with a tubular morphology (apparently, tubular lysosomes). Therefore, we set forth to design a highly superparamagnetic conjugate of CLIO (cross-linked iron oxide) and $F(ab')_2$ fragment isolated from H18/7 antibody.

In experiments involving the binding of CLIO-$F(ab')_2$ to human endothelial umbilical cord cells (HUVEC) that are either IL-1$\beta$ activated or control cells, we observed a very highly specific binding to activated endothelial cells in vitro (Table 1). By using a $^{125}$I-labeled CLIO-$F(ab')_2$ preparation we determined that on the average $0.14 \pm 0.05\%$ of initial iron oxide added was bound to IL-1$\beta$-treated HUVEC if CLIO-$F(ab')_2$ was added to cells at the concentration of 1.2–2.4 μg iron/ml. In the absence of IL-1$\beta$ pretreatment, only 0.02%–0.06% of iron was taken up by the endothelial cells at the same concentration of iron added. Thus, our results indicate that CLIO-bound antibody preserved its specificity (approximately 50-fold higher binding to activated than to control cells) and that the HUVECs bind CLIO-$F(ab')_2$ with high efficiency (approximately

**Table 1.** Different binding of CLIO-F(ab′)$_2$ to HUVEC cells in the presence or absence of IL-1$\beta$ pretreatment

| Preparation | CLIO-F(ab′)$_2$ [a] | |
|---|---|---|
| HUVEC, IL-1$\beta$ treatment | + | – |
| Iron binding, ng/mln cells [b] | 104 ± 12 | 2.1 |
| F(ab′)$_2$ binding, ng/mln cells [b] | 5.5 ± 0.8 | 0.1 |
| T2, ms | 29 ± 2 | > 1,500 |

[a] CLIO-F(ab′)$_2$: Fe 0.6 mg/ml, F(ab′)$_2$ 40.8 mg/ml, Fe/F(ab′)$_2$ = 15.
[b] Data presented as mean ± SD ($n$ = 2).

0.1 pg iron/cell). The shortening of T2 relaxation times of water was proportional to the amount of iron associated with the cells (Table 1). Cells treated with CLIO-F(ab′)$_2$ conjugate were pelleted and studied using MR imaging to determine whether differential binding of CLIO-F(ab′)$_2$ to nontreated versus IL-1$\beta$-treated cells would be reflected in differential effects on MR signal intensity. T2-weighted MR spin-echo images reveal remarkable differences in signal intensities of IL-1$\beta$-treated and control cells. Endothelial cells stimulated with IL-1$\beta$ showed a substantially stronger contrast enhancement. There was no detectable signal in pellets of cells incubated with CLIO-F(ab′)$_2$ without pretreatment with IL-1$\beta$. This result is thus in agreement with the quantitative analysis of CLIO-F(ab′)$_2$ binding to HUVECs (Table 1). Since an increase of CLIO-F(ab′)$_2$ uptake after IL-1$\beta$ treatment could be a result of additional cytokine-mediated activation of adsorptive endocytosis or pinocytosis, we tested this using several control preparations. We compared binding of H18/7 F(ab′)$_2$ conjugated to cross-linked iron oxides (CLIO). Results of MR imaging clearly demonstrate that the profound signal intensity changes detected in IL-1$\beta$-activated HUVECs incubated with CLIO-F(ab′)$_2$ do not stem from the augmentation of nonspecific uptake mechanisms since cells incubated in the presence of control nanoparticles did not show any measurable uptake before or after IL-1$\beta$ treatment. Interestingly, the excess of free H18/7 F(ab′)$_2$ inhibited binding and uptake by IL-1$\beta$-treated cells only by 20% on the average, suggesting a multipoint interaction of CLIO-F(ab′)$_2$ with the cell surface, i.e., a higher avidity of the resultant conjugate.

The obtained data demonstrate that: (1) H18/7 or control $F(ab')_2$ fragments can be covalently modified with *N*-Succinimidyl-acetyl-thioacetate (SATA) and attached to superparamagnetic nanoparticles using thiol-disulfide exchange reaction, (2) anti-E selectin nanoparticles bound with high specificity to IL-1$\beta$-stimulated HUVECs, and (3) specific interaction between CLIO-$F(ab')_2$ and HUVEC can be directly imaged using MRI. Our experiments provided evidence of the feasibility of E-selectin imaging and justified further development of MR-targeted agents for monitoring proinflammatory markers associated with tumor vascular endothelial proliferation, angiogenesis, and atherosclerosis.

## 8.2 Enzyme-Mediated MR Signal Amplification

Several MR amplification strategies can be used to increase the atomic relaxivity of paramagnetic lanthanides, which is critical for detecting low numbers of target molecules. This is commonly achieved by modulating the rotational correlation time ($\tau_r$). So far, three different ways of decreasing rotational mobility of metal complexes had been considered: (1) dispersing paramagnetic chelated metal ions into a high viscosity compartments, (2) covalent attachment of paramagnetic complexes to macromolecules, and (3) noncovalent binding of chelated complexes to macromolecules. We hypothesized that there exists a fourth strategy leading to a similar $\tau_r$ increase. This strategy would include condensation (or polymerization) of low relaxivity complexes into higher relaxivity products. Therefore, we originally developed and subsequently explored a generic method of signal amplification that relies on enzyme-mediated substrate conversion into magnetically active oligomers, exemplified by DOTA(Gd) tyramide polymerization (MRamp). The major advantage of MRamp is in that a single substrate can potentially be used as a reporter for thousands of targets, using an antibody-enzyme conjugate as the primary reporter, similar to that of a traditional enzyme-linked immunoassay (Fig. 2). Specifically, we assumed that the oxidoreductases (e.g., peroxidases, designated as $E^{\bullet}$ below, of which horseradish peroxidase and myeloperoxidase have been already tested) would catalyze the reduction of peroxide using a low-

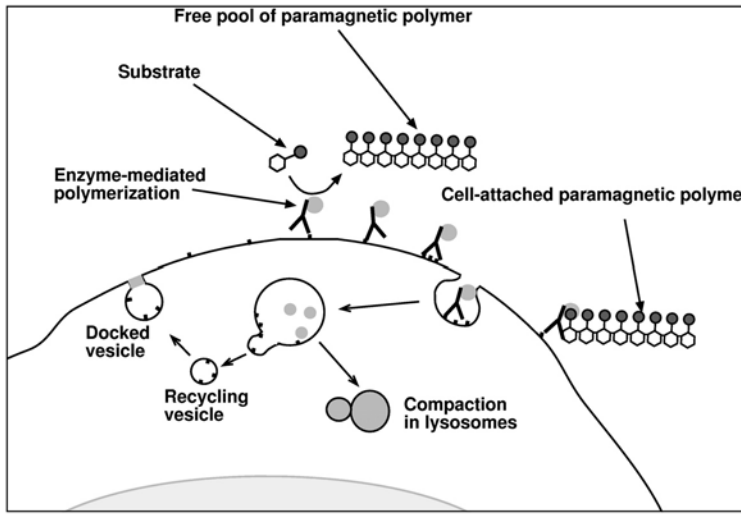


**Fig. 2.** Magnetic resonance amplification as a result of enzyme-mediated polymerization of paramagnetic substrates: hypothetical mechanisms of amplification after binding of enzyme-tagged, target-specific molecules (e.g., antibodies) to the cell surface

relaxivity, paramagnetic substrate (AH) as a donor of electrons (reaction 1).

$$2\,\mathrm{AH} + [\mathrm{E}^{\bullet}\mathrm{H_2O_2}] \rightarrow 2\,[*\mathrm{A}] + 2\,\mathrm{H_2O} + \mathrm{E} \quad (1)$$

$$\mathrm{n}[*\mathrm{A}] \rightarrow [\mathrm{A}]\mathrm{n} \quad (2)$$

Oxidized substrate molecules (*A) would then self-polymerize (oligomerize) into the larger, high relaxivity paramagnetic oligomers ([A]n, reaction 2). Catalytic generation of hydrogen peroxide (serving as oxidizer in this reaction) can be used by including glucose oxidase as the second enzymatic component in this reaction in vitro and in vivo. Relaxometric measurements and theoretical calculations have shown a 2.5-fold increase in relaxivity at imaging field strengths that is primarily modulated by an increase in the rotational correlation time as a result of the oligomerization.

Another class of enzymes that catalyze polymerization of monophenols, tyrosinases (monophenol, oxygen oxidoreductases), could be potentially utilized as an alternative to the glucose oxidase/peroxidase system. Unlike peroxidase, tyrosinase catalyzes two consecutive reactions using monophenols for hydroxylation and subsequent oxidation to quinones. Self-polymerizing quinones bearing a paramagnetic label can form large-molecular weight products and thus contribute to an increase of atomic relaxivity of paramagnetic lantanides.

Therefore, the potential advantages of MRamp are: (1) it utilizes low molecular weight lanthanide complexes which are converted into large molecules "on site," (2) the development of these low molecular weight precursors is clinically viable, (3) the observed relaxivity changes are higher or similar to that of other amplification strategies, (4) the oligomerized products can be designed to reside locally, and (5) the method can be used in a variety of generic ways, potentially allowing the read-out of many different targets. So far, we have shown that the method holds promise in the (1) MR detection of a model ligand using an enzyme-linked immunoadsorbent assay format, (2) imaging of a proinflammatory marker, E-selectin, on the surface of endothelial cells, and (3) detection of endogenous enzymes – tyrosinase and myeloperoxidase. Two primary targets are currently under investigation: a mutant, constitutively active ΔEGFR and angiogenesis-modulated E-selectin. ΔEGFR expression strongly upregulates VEGF expression which, in turn, upregulates E-selectin and tube formation by endothelial cells. These targets were chosen because of their importance in tumor proliferation, modulation by chemotherapy (e.g., EGFR tyrosine kinase inhibitors) and the current lack of imaging probes directed against them. To further increase the sensitivity of MRamp, we are investigating novel paramagnetic substrates to exploit changes in T1 (gadolinium) and T2/T2* effect (dysprosium).

### 8.2.1 Myeloperoxidase-Specific Substrates

Myeloperoxidase (MPO) is an endogenous enzyme of clinical interest because it is secreted by activated macrophages and neutrophils

in response to injury. MR imaging of MPO activity in vivo has the potential to reveal detailed knowledge of inflammatory diseases such as rheumatoid arthritis and atherosclerosis. To investigate the application of the MRamp strategy to imaging MPO, we developed and tested various substrates against MPO. We found that DOTA(Gd) 5-hydroxytryptamine, in the presence of MPO and peroxide, such as that produced by glucose oxidase, rapidly oligomerizes. The oligomer demonstrates an increase in T1 relaxivity similar to that of DOTA(Gd) tyramide with horseradish peroxidase. Furthermore, the degree of enhancement appears to be proportional to the amount of MPO present. In vitro imaging experiments performed at 1.5 T in phantoms containing matrigel demonstrates the ability of the 5-hydroxytryptamine substrate to polymerize at the surface of the Matrigel$^{TM}$ in the presence of MPO and glucose oxidase, resulting in the selective enhancement on T1 weighted images of the interface/border of the matrigel.

### 8.2.2 Dysprosium-Based Substrates

Dysprosium has a large magnetic susceptibility, making it a potential T2/T2* imaging agent. We have investigated the potential of the MRamp strategy to cause T2/T2* shortening (reverse amplification) utilizing the substrate DOTA(Dy) tyramide. In the presence of a peroxidase such as horseradish peroxidase, there is a 30% shortening of T2/T2* at both 1.5 T and 7 T, and this shortening is further amplified when the substrates are compartmentalized in liposomes. Because T2/T2* relaxivity increases with field strength, a dramatic signal difference is obtained at 7 T on T2 weighted images. Combining with the ability to target enzyme activity, dysprosium-based MRamp substrates are potential high field enzyme reporter agents.

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## References

Aime S, Cabella C, Colombatto S, Geninatti Crich S, Gianolio E, Maggioni F (2002) Insights into the use of paramagnetic Gd(III) complexes in MR-molecular imaging investigations. J Magn Reson Imaging 16:394–406

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA, Jr (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci 84:9238–9242

Bogdanov A, Jr, Weissleder R (1998) The development of in vivo imaging systems to study gene expression. Trends Biotechnol 16:5–10

Bogdanov A, Jr, Matuszewski L, Bremer C, Petrovsky A, Weissleder R (2002) Oligomerization of paramagnetic substrates results in signal amplification and can be used for MR imaging of molecular targets. Mol Imaging 1:16–23

Chuang PI, Young BA, Thiagarajan RR, Cornejo C, Winn RK, Harlan JM (1997) Cytoplasmic domain of E-selectin contains a non-tyrosine endocytosis signal. J Biol Chem 272:24813–24818

Huwyler J, Wu D, Pardridge W (1996) Brain drug delivery of small molecules using immunoliposomes. Proc Natl Acad Sci 93:14164–14169

Kang HW, Josephson L, Petrovsky A, Weissleder R, Bogdanov A, Jr (2002) Magnetic resonance imaging of inducible E-selectin expression in human endothelial cell culture. Bioconj Chem 13:122–127

Koretsky A, Lin Y, Schorle H, Jaenisch R (1996) Genetic control of MRI contrast by expression of the transferrin receptor. In: Proceedings of the Fourth Meeting of the International Society for Magnetic Resonance in Medicine, Berkeley, CA, 1996. International Society for Magnetic Resonance in Medicine, New York, pp 69

Kresse M, Wagner S, Pfefferer D, Lawaczeck R, Elste V, Semmler W (1998) Targeting of ultrasmall superparamagnetic iron oxide (USPIO) particles to tumor cells in vivo by using transferrin receptor pathways. Magn Reson Med 40:236–242

Luscinskas FW, Brock AF, Arnaout MA, Gimbrone MA (1989) Endothelial-leukocyte adhesion molecule-1-dependent and leukocyte (CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. J Immunol 142:2257–2263

Montgomery KF, Osborn L, Hession C, Tizard R, Goff D, Vassallo C, Tarr PI, Bomsztyk K, Lobb R, Harlan JM (1991) Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Proc Natl Acad Sci 88:6523–6527

Moore A, Basilion J, Chiocca A, Weissleder R (1998) Measuring transferrin receptor gene expression by NMR imaging. Biochim Biophys Acta 1402: 239–249

Peters AM (1998) The use of nuclear medicine in infections. Brit J Radiol 71:252–261

von Asmuth EJ, Smeets EF, Ginsel LA, Onderwater JJ, Leeuwenberg JF, Buurman WA (1992) Evidence for endocytosis of E-selectin in human endothelial cells. Eur J Immunol 22:2519–2526

Weissleder R, Moore A, Mahmood U, Bhorade R, Benveniste H, Chiocca EA, Basilion JP (2000) In vivo magnetic resonance imaging of transgene expression. Nature Med 6:351–355