Tumor-targeted enzyme mediated MR signal amplification using paramagnetic substrates.

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SYNOPSIS. A novel combination of a pre-targeting system of cancer-specific antibody-enzyme conjugates and a paramagnetic substrate has been designed for tumor-specific receptor MR imaging. Monoclonal antibody conjugates linked to horseradish peroxidase (HRP) or glucose oxidase (GO) were synthesized by using bisaromatic hydrazone bonds. Purified conjugates were tested in vitro and in vivo using A431 a human squamous carcinoma model in the presence of bis-(5-hydroxytrypatmide)-DTPA(Gd) paramagnetic HRP substrate (5HT-DTPAGd). L6 (anti-L6 protein family, a pan-carcinoma specific monoclonal antibody, mAb) was tested for tumor cell pre-targeting and showed strong and specific regional tumor T1 signal enhancement on MRI after the injection of 0.2-0.25 mmol dose of 5HT-DTPAGd/kg.

INTRODUCTION. Antibody-mediated targeting of cancer has a goal of either specific therapeutic intervention or detection of cancer-specific molecular marker expression for tumor detection, typing and staging. We previously reported MR signal amplification effect (MRAMP) caused by paramagnetic substrates that undergo rapid oxidation and oligomerization in the presence of oxidized forms of catalytically-active peroxidase and myeloperoxidase [1,2]. In this research we hypothesized that a concept of pre-targeting of mAb-enzyme conjugates could be used in vivo in a combination with bifunctional paramagnetic electron donor compounds (i.e. carrying two polymerizing residues per one paramagnetic molecule). The use of two conjugates was dictated by the need for site-specific delivery of the source of enzymatic hydrogen peroxide production (GO) and the oxidizing enzyme (HRP). We anticipated that enzyme-induced oxidation and polymerization of the substrate at the tumor site would result in tumor-specific signal enhancement.

METHODS. Substrate 5HT-DTPAGd was synthesized using DTPA cyclic anhydride in the presence of 5-hydoxytryptamine and triethylamine as shown in Fig. 1A [3], chelated with Gd and purified by HPLC. The relaxivities of paramagnetic products were measured using gadolinium complex concentrations ranging from 2.5 mM to 0.1 mM in the presence of peroxidase (HRP) and GO. Oligomerization of was achieved by incubating the desired solution of a monomer with an excess of hydrogen peroxide or GO (1 μg) and HRP (1μg ≈ 4Units) for 1 hour at 40°C. T_1 and T_2 were measured at two different field strengths: 0.47T and 1.5T. Enzymes were covalently conjugated to mAbs using HydraLinK (EMD) and purified on a Superdex 200 FPLC column (GE); Fig 1B. Conjugates were tested by SDS-electrophoresis and in A431 cell culture by cross-titration cell ELISA using HRP substrate. Mice (*nu/nu*) were implanted with $2 \cdot 10^6$ A431 cells s.c. over the femoral muscle and imaged under 1.5% isofluorane anesthesia at two weeks after implantation. MR images were acquired using a Bruker Biospin 2.0T/45 cm imaging spectrometer equipped with ±20G/cm self-shielded gradients. T1-weighted, spin-echo MRI was performed with the following acquisition parameters: TR/TE = 400/8.0 ms, FOV = 3 cm x 3 cm, matrix = 256x128, NEX = 8; 12 images acquired consecutively over a 1-h period.

Table 1. Properties of 5HT-DTPAGd as paramagnetic HRP substrate

Reaction Components	$r_{1p}/mM^{-1}s^{-1}$(0.47T, 40°)	r_{1p} Increase	Apparent kinetic K(k1+k-1)/s^{-1}
5HT-DTPA-Gd(PBS)	4.3	-	NA
5HT-DTPA-Gd/HRP(0.5U)/H_2O_2	10.8	251%	1.6e-3
5HT-DTPA-Gd(PBS)/HRP(0.5U)/GOX/Glucose	9.1	212%	5.6e-3

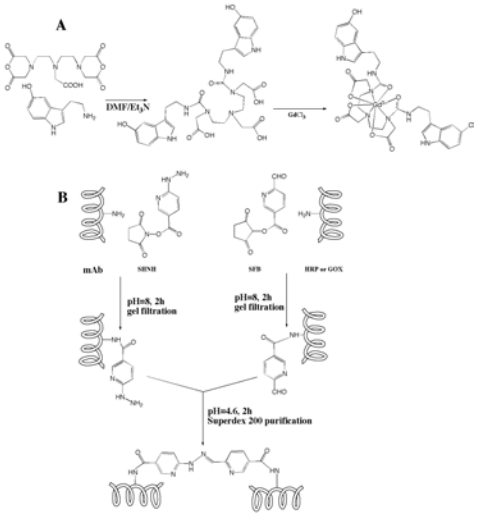

Fig. 1. **A** - Synthesis of 5HT-DTPA (DMF/triethylamine, RT 48 h) and subsequent chelation of Gd. **B**- A schema of mAb conjugate synthesis using HydraLinK.

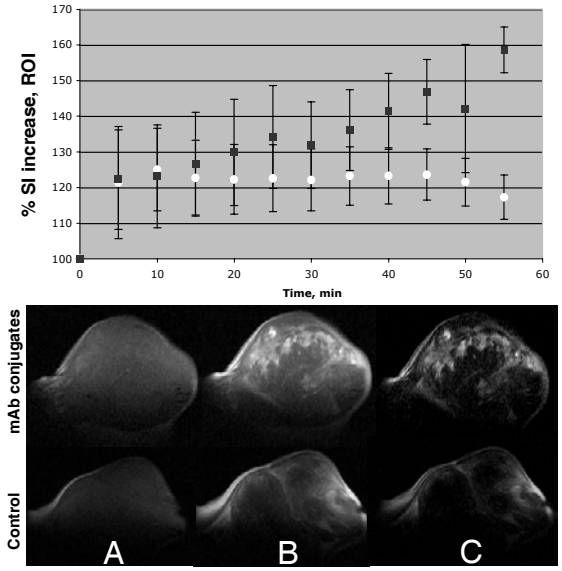

Fig. 2 Top: Plot of signal intensity (SI) vs. time within tumor ROI. Squares – treated; circles – control. Data shown as mean±SD (n=4). Bottom: Representative 2.0T MRIs of mouse A431 tumor xenograft pre- and post-injection of 5HT-DTPAGd substrate. MRIs in upper row from animal pre-injected with L6 mAb conjugates. Lower row shows control tumor MRIs. Pre- (A, scan 1), post- (B, scan 12), and subtraction (C, scan 12 – scan 1).

RESULTS AND DISCUSSION. Relaxivities of enzymatic reaction products (Table 1) were 2.1-2.5 times higher than initial 5HT-DTPAGd substrate. R1 increase proceeded 3.5-times faster in the presence of GO/glucose than hydrogen peroxide. Similar data were obtained when R2 values were measured. The above results suggest the formation of macromolecules with longer molecular rotational correlation times (τ_r) and, consequently, higher relaxivity. Preliminary in vitro imaging suggested that by using HRP/GO enzymatic system HRP could be detected at 1.5T at the level of about 0.4-0.04 units (100-10 ng) in the presence of 5HT-DTPAGd substrate. Both enzymes were independently conjugated to mAbs using bisaromatic hydrazone bonds. The testing of the purified conjugates was accomplished by using human squamous carcinoma cell cultures (A431) pan-carcinoma L6 antigen (L6 mAb) ($1.5\text{-}2 \cdot 10^6$ receptors/cell). By using cross-titration of conjugates on live cells by ELISA we optimized ratios between mAb-HRP and mAb-GO conjugates to achieve maximum signal and saturation of receptor binding sites. Animals with implanted human xenografts were pre-injected IV with a total of 5-10 μg of conjugates 2 hours before imaging or received no injection of antibodies (control). After injection of 5HT-DTPAGd, control tumors exhibited rapid signal enhancement followed by a gradual wash-out of paramagnetic substrate (Fig. 2, lower graph, circles) with a slow elimination of residual substrate. In animals pre-injected with mAb-conjugates, a strong regional enhancement of tumor signal intensity was observed over time [a 160% increase at approx. at 55 min post contrast (Scan 12) compared to the pre-injection image] consistent with a relaxivity increase in the presence of HRP/GO system. Subtraction images (Fig. 2C) showed areas of paramagnetic product deposition which corresponded to the areas of antibody extravasation and binding to the tumor. The observed changes in signal enhancement over time (Fig. 2; Top) suggest the utility of MR contrast amplification approach to high-resolution tumor-specific receptor imaging with potential applications in "MR staining" of tissues and molecular typing of cancers.

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