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## A Secreted Enzyme Reporter System for MRI\*\*

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An important goal in modern biology is to understand how molecular processes commonly studied at the cellular level give rise to physiological functions in complex tissues and organisms. Noninvasive imaging of gene expression patterns in whole animals could provide information critical to this end but current methods lack sensitivity and spatiotemporal precision. Enzymatic reporter systems detectable by magnetic resonance imaging (MRI) address these limitations by combining the relatively high spatial and temporal resolution of MRI with the ability of each genetically-expressed enzyme to generate many MRI-detectable product molecules.[1,2] A challenge with imaging-based detection of some of the most popular reporter enzymes is the need to deliver MRI probes to their sites of action within cells. Here we describe a new reporter gene system for MRI that relieves this problem by harnessing an extracellular enzyme, the mammalian secreted alkaline phosphatase (SEAP).

SEAP is a truncated, secreted variant of placental alkaline phosphatase (PLAP), and is widely used as a stable and heterologously expressible reporter enzyme in conjunction with optically absorbant, fluorescent, or luminescent substrates.[3] To detect SEAP activity optimally by MRI, we modified an existing sensor for adenosine (Ado), the product of SEAP's hydrolysis of phosphorylated adenosine derivatives. In this system, the reporter enzyme is therefore detected through its generation of product molecules, as opposed to its activity on an MRI contrast agent directly. The approach is reversible by removal or degradation of Ado, nondestructive to the Ado sensor, and relatively fast, because SEAP substrates can be used at concentrations well above their  $K_m$  values without affecting background MRI signal (Fig. 1a).

The Ado sensor we used is actuated by an Ado-binding DNA aptamer,[4] which, in the absence of saturating Ado concentrations, crosslinks superparamagnetic iron oxide (SPIO) nanoparticles modified with reverse-complementary DNA segments.[5,6] Ado-dependent disaggregation of the functionalized SPIOs modulates their ability to create contrast in  $T_2$ -weighted MRI scans. If nanoparticles with diameter greater than ~50 nm are used,

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experimental[7] and theoretical[8] studies show that disaggregation accompanies an increase in  $T_2$  relaxation rate ( $R_2 = 1/T_2$ ); smaller SPIOs show the opposite change in  $R_2$ . [8-10] A prototypical Ado sensor formed from SPIOs with mean diameter of  $106 \pm 1$  nm, as measured by dynamic light scattering (DLS), showed a 50% change in relaxation rate ( $R_2 = 1/T_2$ ) at an Ado concentration ( $EC_{50}$ ) of  $1.0 \pm 0.2$  mM (Fig. 1b). To improve on this apparent affinity, we used hybridization rules to predict thermodynamically favorable modifications to the probe, and found that weakening the crosslinking segment's interaction with its 5' SPIO-conjugated binding partner resulted in a new Ado sensor with a tenfold-improved  $EC_{50}$  of  $91 \pm 14$   $\mu$ M at the same sensor concentration (Fig. 1b and Supporting Information). Similar Ado dependence was reported by changes in  $\Delta R_2$  or by changes in the ratio  $\Delta R_2/\Delta R_1$ , which was observed to be approximately independent of SPIO concentration (Suppl. Fig. 1).

The Ado sensor was insensitive to a number of SEAP substrates (Suppl. Fig. 2), including 2'-adenosine monophosphate (2'-AMP), a compound hydrolyzed to Ado by the enzyme. In the presence of 2'-AMP, the SEAP parent enzyme PLAP induced Ado sensor responses that were detected after about ten minutes by MRI (Fig. 2a).  $R_2$  changes were not observed in the absence of substrate. A second enzyme, adenosine deaminase (ADA), was used to reverse the MRI contrast changes induced by hydrolysis of 2'-AMP (Fig. 2b). After addition of ADA,  $R_2$  returned to baseline values observed before addition of substrate (Fig. 2a, shaded area). This reversal was blocked by pentostatin, an ADA inhibitor, showing that the ADA reversal was dependent on deaminase activity of the enzyme. Both PLAP- and ADA-catalyzed changes observed by MRI were closely correlated ( $r = -0.91$ ) to differences in the mean particle cluster sizes recorded by DLS from the same samples (Fig. 2c), consistent with the established relationship between  $T_2$  relaxation and cluster size for these SPIOs. [7, 8] DLS also enabled time-resolved measurements of the Ado sensor's response to alkaline phosphatase activity. Fig. 2d shows that the complete sensor response took place within 7 minutes after addition of PLAP in the presence of 2'-AMP, while no discernable changes followed addition of BSA or enzyme in the absence of 2'-AMP. These results demonstrated that SEAP/PLAP activity could be detected using the improved Ado sensor, and that reversible, nondestructive use of the system is possible in the presence of ADA.

We next sought to test the system in a cellular context where SEAP could be applied as genetically-encoded reporter enzyme. SEAP was transiently expressed in nonadherent HEK-293 cells and its activity was measured in conditioned supernatants by performing MRI in the presence of 2'-AMP and the Ado sensor. Fig. 3a shows that the four day time course of  $R_2$  changes following SEAP transfection corresponded to independent measurements of SEAP activity using fluorescence-based assays performed ( $r = 0.88$ ). Changes were reversible and consistent with the dynamic range of the sensor (Suppl. Fig. 3). As a further demonstration of the SEAP reporter system in cultured cells, the SEAP gene was placed under control of a tetracycline-inducible promoter and coexpressed with the tetracycline repressor. When 2'-AMP and the Ado sensor were applied to detect SEAP activity,  $R_2$  values from tetracycline-induced cells were significantly increased with respect to uninduced cells, and similar to cells constitutively expressing SEAP in the absence of the repressor (Fig. 3b). Again, an independent measurement of SEAP activity using an optical readout demonstrated that levels of the enzyme corresponded to the changes measured by MRI (Suppl. Fig. 4). These experiments demonstrated that genetic control of SEAP expression in cell culture could be effectively detected by MRI.

We have shown that reversible detection of an established secreted reporter enzyme, SEAP, is possible using an MRI contrast agent that selectively monitors products of SEAP-mediated hydrolysis of phosphorylated purines. The MRI sensor mechanism allowed tracking of SEAP expression induced by transient transfection and tetracycline-inducible

gene regulation in cultured cells. The system generates strong  $T_2$ -based contrast changes, does not involve cell delivery or catalytic destruction of contrast agents, and is both reversible and moderately fast, because of its product-dependent sensing mechanism. This form of detection, and the resulting reversibility, differ from earlier efforts to measure enzymatic activity using contrast agents and conjugates that are themselves modified by reporter[1] or endogenous marker[11-14] enzymes. MRI detection of SEAP reporter activity could be useful in opaque cell or tissue culture environments where optical assays are unreliable. MRI-based assays may be particularly beneficial for screening applications where data from three dimensional sample arrays may be acquired in parallel.[15] MRI measurements using the new system might also be effective for monitoring alkaline phosphatase activity in widely-used SEAP or PLAP-expressing tissue and animal models. [16,17] In these complex contexts, implants[18] containing the Ado sensor could be used to avoid interference by endogenous factors. Systemic mapping experiments may also be feasible, supported by the possibility of performing ratiometric  $\Delta R_2/\Delta R_1$  measurements[19] with the SPIO-based Ado sensor (Suppl. Fig. 1).

## Experimental Section

Ado sensors were assembled by conjugating biotinylated DNA strands to streptavidin-coated magnetic nanoparticles. MRI was performed on an Avance 4.7 T scanner. Detailed protocols are available as Supporting Information.

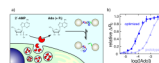
## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

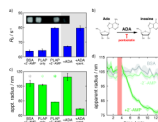
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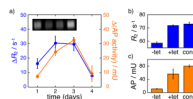
**Figure 1.**

SEAP-based reporter gene system for MRI. a) Secreted alkaline phosphatase (SEAP, red) is expressed and secreted from genetically modified cells (left). Extracellular SEAP cleaves 2'-AMP or a related substrate to generate adenosine (Ado, plus inorganic phosphate, P<sub>i</sub>). Ado is then detected by a superparamagnetic iron oxide (SPIO)-based MRI sensor (right), actuated by an adenosine-binding aptamer (dark blue). Sensing can be reversed by destruction or removal of Ado. b) Relative  $T_2$  relaxation rate changes reported as a function of Ado concentration from prototype (light blue) and optimized (dark blue) SPIO-based Ado sensors. Relative  $\Delta R_2 = [(R_2)_{obs} - (R_2)_{min}] / [(R_2)_{max} - (R_2)_{min}]$ , where  $(R_2)_{obs}$  is the  $R_2$  observed at each Ado concentration and  $(R_2)_{max}$  and  $(R_2)_{min}$  denote maximal and minimal recorded  $R_2$  values. Titration curves were fitted to a Hill equation to yield EC<sub>50</sub> values of  $1.0 \pm 0.2$  mM and  $91 \pm 14$   $\mu$ M for Ado responses of the prototype and optimized sensors, respectively. Error bars (s.e.m.) for some data points are obscured by symbols in the graph.



**Figure 2.**

Specificity and reversibility of reporter enzyme sensing. a) MRI data were obtained from 24 (mg Fe)/L SPIO-based Ado sensor incubated with mixtures of 0.5 U PLAP ( $\sim 10 \mu\text{M}$ ) and 2 mM 2'-AMP, or control conditions lacking substrate (PLAP only) or substituting 15  $\mu\text{M}$  BSA instead of enzyme. To test reversibility of the system (gray shaded area), 0.5 U of ADA ( $\sim 2 \mu\text{M}$ ) alone (+ADA) or in the presence of 500  $\mu\text{M}$  of the ADA inhibitor pentostatin (+ADA +pent.) were added after 60 min. preincubation of Ado sensor with substrate and PLAP (error bars represent s.e.m,  $n = 3$ ). The inset shows the image of the corresponding microtiter wells ( $TE = 30 \text{ ms}$ ). b) Schematic showing the action of ADA on Ado. The inosine product is not detected by the Ado sensor, and the reaction is inhibited by pentostatin. c) DLS results showing apparent radii displayed by Ado sensors corresponding to conditions in panel a. Color coded asterisks denote conditions studied kinetically in panel d. The radius of Ado sensors without enzyme and substrate was  $60.7 \pm 0.4 \text{ nm}$  ( $n = 3$ ). d) Time courses of Ado sensor particle clustering measured by DLS before and following addition (red vertical bar) of SEAP with 2'-AMP (dark green,  $n = 4$ ). SEAP without substrate (light green,  $n = 3$ ), or BSA with 2'-AMP (gray,  $n = 2$ ). Shaded areas represent s.e.m. over multiple measurements.



**Figure 3.**

MRI measurement of SEAP reporter expression. a) SEAP was transiently expressed from 293-F cells and monitored by MRI for four days. Conditioned supernatants from transfected and mock-transfected control cells were added to the SPIO-based Ado sensor [14.4 (mg Fe)/L] in the presence of 2'-AMP and scanned to obtain  $R_2$  values (blue). As an independent control, alkaline phosphatase (AP) activity was measured in parallel using a standard fluorometric assay (orange).  $\Delta R_2$  and  $\Delta(\text{AP activity})$  reflect differences from baseline  $R_2$  values measured from mock-transfected cells ( $n = 3$ ). Data from three measurements are reported. b) In a second test of reporter gene detection by MRI, the Ado sensor [24 (mg Fe)/L] was mixed with supernatants from 293-F cells expressing SEAP under a tetracycline-inducible promoter.  $R_2$  values were recorded in the absence (-tet) and presence (+tet) of tetracycline, and also from cells that constitutively expressed the reporter gene (const;  $n = 3$  in each case). c) Control measurements of AP activity were performed using a colorimetric assay. DLS and image data are available as Supporting Information.