Imaging Techniques

DOI: 10.1002/anie.200906712

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. Non-invasive 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 the 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. Herein 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). It is widely used as a stable and heterologously expressible reporter enzyme in conjunction with optically absorbent, fluorescent, or luminescent substrates.[3] For the optimal detection of SEAP activity by MRI, we modified an existing sensor for adenosine (Ado), which is produced by 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 direct action on an MRI contrast agent. The process is reversed upon the removal or degradation of Ado, is nondestructive to the Ado sensor, and is relatively fast, because SEAP substrates can be used at concentrations well above their K_m values without affecting the background MRI signal (Figure 1a).

The Ado sensor that we used is actuated by an Ado-binding DNA aptamer.[4] In the absence of Ado at saturating concentrations, this aptamer cross-links superparamagnetic iron oxide nanoparticles (SPIOs) 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. Experimental[7] and theoretical[8] studies showed that if nanoparticles with a diameter greater than about 50 nm are used, disaggregation accompanies an increase in the T_2 relaxation rate ($R_2 = 1/T_2$); the use of smaller SPIOs leads to the opposite change in R_2.[8–10] A prototypical Ado sensor formed from SPIOs with a mean diameter of (106 ± 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 ± 0.2) mM (Figure 1b). To improve on this apparent affinity, we used hybridization rules to predict thermodynamically favorable

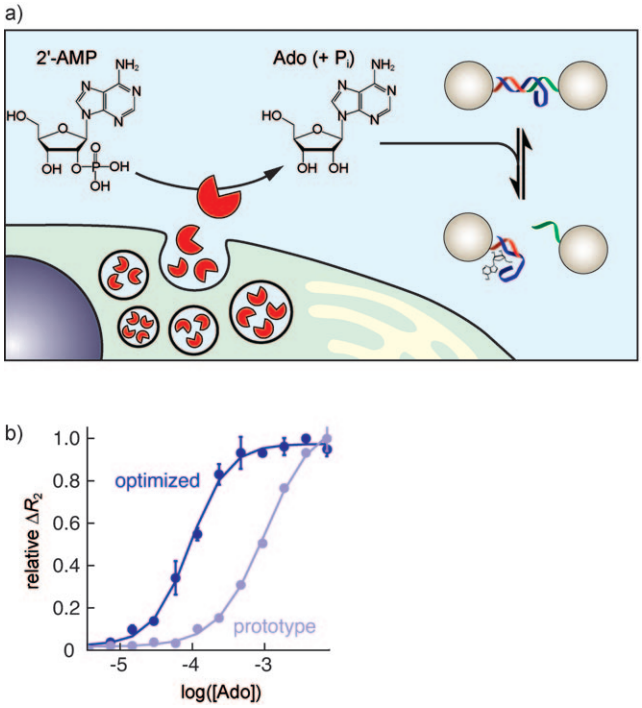

Figure 1. SEAP-based reporter-gene system for MRI. a) Secreted alkaline phosphatase (SEAP, red) is expressed and secreted from genetically modified cells (left). Extracellular SEAP cleaves 2′-AMP or a related substrate to generate adenosine (Ado, plus inorganic phosphate, P_i). Ado is then detected by a SPIO-based MRI sensor (right) actuated by an adenosine-binding aptamer (dark blue). Sensing can be reversed by the destruction or removal of Ado. b) Relative changes in the T_2 relaxation rate reported as a function of Ado concentration from prototype (light blue) and optimized (dark blue) SPIO-based Ado sensors. Relative $\Delta R_2 = [(R_2)_{obs} - (R_2)_{min}]/[(R_2)_{max} - (R_2)_{min}]$, in which $(R_2)_{obs}$ is the R_2 value observed at each Ado concentration, and $(R_2)_{max}$ and $(R_2)_{min}$ denote the maximal and minimal recorded R_2 values. Titration curves were fitted to a Hill equation to yield EC_{50} values of (1.0 ± 0.2) mM and (91 ± 14) μM for the Ado responses of the prototype and optimized sensors, respectively. Error bars (standard error of the mean, SEM) for some data points are obscured by symbols in the graph.

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[**] We thank the Raymond and Beverley Sackler Foundation for their generous support. Additional funding was provided by NIH grant DP2-OD002114 to A.J. MRI = magnetic resonance imaging.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200906712.

modifications to the probe, and found that weakening of the interaction between the cross-linking segment and its 5′ SPIO-conjugated binding partner resulted in a new Ado sensor with a tenfold-improved EC_{50} value of (91 ± 14) μM at the same sensor concentration (Figure 1b; see also the Supporting Information). Similar Ado dependence was reported by changes in ΔR_2 or by changes in the ratio $\Delta R_2/\Delta R_1$, which was observed to be approximately independent of the SPIO concentration (see Figure 1 in the Supporting Information).

The Ado sensor was insensitive to a number of SEAP substrates (see Figure 2 in the Supporting Information), 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 10 minutes by MRI (Figure 2a). Changes in R_2 were not observed in the absence of the substrate. A second enzyme, adenosine deaminase (ADA), was used to reverse the MRI contrast changes induced by the hydrolysis of 2′-AMP (Figure 2b). After the addition of ADA, R_2 returned to baseline values observed before the addition of the substrate (Figure 2a, shaded area). This reversal was blocked by pentostatin, an ADA inhibitor, showing that the reversal with ADA 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 for the same samples (Figure 2c). This correlation is 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 response of the Ado sensor to alkaline phosphatase activity. The complete sensor response took place within 7 minutes after the addition of PLAP in the presence of 2′-AMP, whereas no discernable changes followed the addition of BSA or the enzyme in the absence of 2′-AMP (Figure 2d). These results demonstrated that SEAP/PLAP activity could be detected by 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, in which SEAP could be applied as genetically encoded reporter enzyme. SEAP was expressed transiently 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. The time course of R_2 changes over 4 days following SEAP transfection corresponded to independent measurements of SEAP activity with fluorescence-based assays ($r = 0.88$; Figure 3a). Changes were reversible and consistent with the dynamic range of the sensor (see Figure 3 in the Supporting Information). As a further demonstration of the SEAP reporter system in cultured cells, the SEAP gene was placed under the control of a tetracycline-inducible promoter and coexpressed with the tetracycline repressor. When 2′-AMP and the Ado sensor were used to detect SEAP activity, the R_2 values for tetracycline-induced cells were significantly increased with respect to those for uninduced cells and similar to those for cells constitutively expressing SEAP in the absence of the repressor (Figure 3b). Again, an independent measurement of SEAP activity on the basis of an optical readout demonstrated that levels of the enzyme corresponded

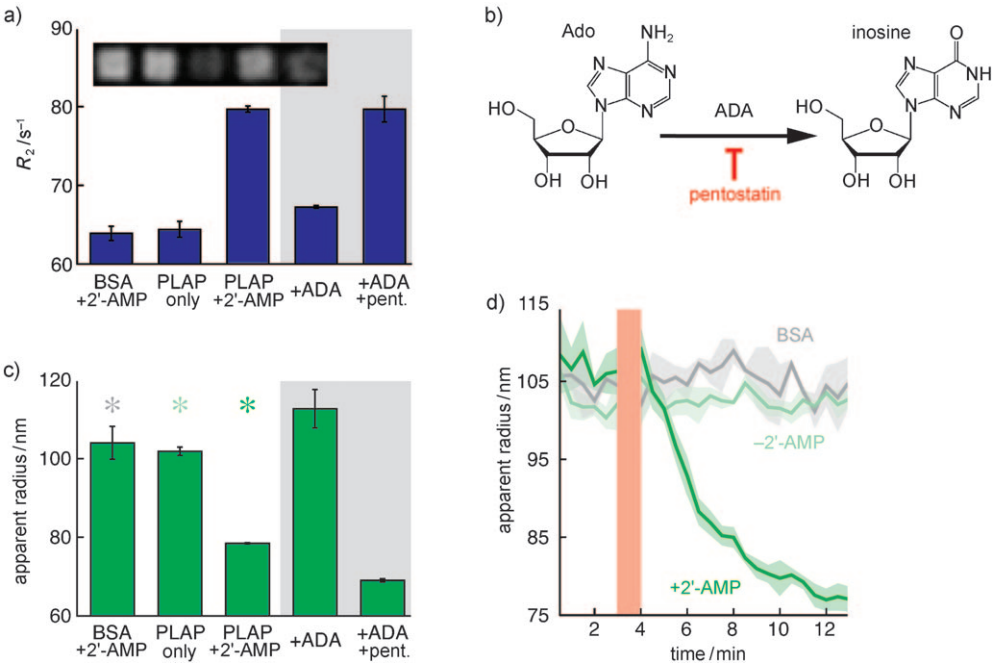

Figure 2. Specificity and reversibility of reporter-enzyme sensing. a) MRI data were obtained from mixtures of the SPIO-based Ado sensor (24 mg of Fe/L) incubated with PLAP (0.5 U, ca. 10 μM) and 2′-AMP (2 mM), or under control conditions in the absence of the substrate (PLAP only) or with BSA (15 μM) instead of the enzyme. To test the reversibility of the system (gray shaded area), ADA (0.5 U, ca. 2 μM) alone (+ADA) or in the presence of the ADA inhibitor pentostatin (500 μM; +ADA +pent.) were added after preincubation of the Ado sensor with the substrate and PLAP for 60 min (error bars represent the SEM, $n = 3$). The inset shows the image of the corresponding microtiter wells (time to echo, TE = 30 ms). b) Scheme 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 the apparent radii displayed by Ado sensors under the conditions in (a). Color-coded asterisks denote the conditions studied kinetically [results shown in d)]. The radius of Ado sensors without the enzyme and the substrate was (60.7 ± 0.4) nm ($n = 3$). d) Time course of Ado-sensor-particle clustering measured by DLS before and following the addition (red vertical bar) of SEAP with 2′-AMP (dark green, $n = 4$), SEAP without a substrate (light green, $n = 3$), or BSA with 2′-AMP (gray, $n = 2$). Shaded areas represent the SEM over multiple measurements.

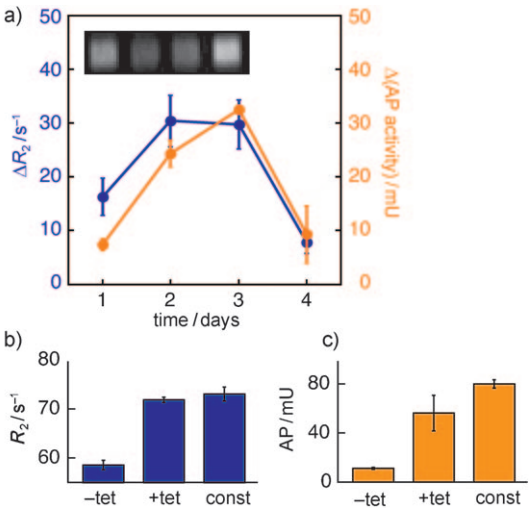

Figure 3. MRI measurement of SEAP-reporter expression. a) SEAP was expressed transiently from 293-F cells and monitored by MRI for 4 days. Conditioned supernatants from transfected and mock-transfected control cells were added to the SPIO-based Ado sensor (14.4 (mg Fe)/L) in the presence of 2′-AMP and scanned to obtain R_2 values (blue). As an independent control, alkaline phosphatase (AP) activity was measured in parallel by using a standard fluorometric assay (orange). ΔR_2 and Δ(AP activity) reflect differences from baseline R_2 values measured for mock-transfected cells ($n=3$). 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 the control of a tetracycline-inducible promoter. R_2 values were recorded in the absence (−tet) and presence (+tet) of tetracycline, and also for cells that constitutively expressed the reporter gene (const; $n=3$ in each case). c) Control measurements of AP activity were performed by using a colorimetric assay. DLS and image data are available as Supporting Information.

to the changes measured by MRI (see Figure 4 in the Supporting Information). These experiments demonstrated that the genetic control of SEAP expression in cell culture could be detected effectively by MRI.

We have shown that the reversible detection of an established secreted reporter enzyme, SEAP, is possible by using an MRI contrast agent that selectively monitors products of the SEAP-mediated hydrolysis of phosphorylated purines. The MRI sensor mechanism enabled 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 the 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) differs from earlier efforts to measure enzymatic activity through the use of 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 in which data from three-dimensional sample arrays may be acquired in parallel.[15]

MRI measurements with 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, especially given the possibility to perform ratiometric $\Delta R_2/\Delta R_1$ measurements[19] with the SPIO-based Ado sensor (see Figure 1 in the Supporting Information).

Experimental Section

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

Received: November 28, 2009
Revised: January 26, 2010
Published online: April 22, 2010

Keywords: alkaline phosphatase · aptamers · enzymatic reporters · gene reporters · magnetic resonance imaging

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