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Neurotransmitter-responsive nanosensors for $T_2$-weighted magnetic resonance imaging

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Supporting Information Placeholder

ABSTRACT: Neurotransmitter-sensitive contrast agents for magnetic resonance imaging (MRI) have recently been used for mapping signaling dynamics in live animal brains, but paramagnetic sensors for $T_1$-weighted MRI are usually effective only at micromolar concentrations that themselves perturb neurochemistry. Here we present an alternative molecular architecture for detecting neurotransmitters, using superparamagnetic iron oxide nanoparticles conjugated to tethered neurotransmitter analogs and engineered neurotransmitter binding proteins. Interactions between the nanoparticle conjugates result in clustering that is reversibly disrupted in the presence of neurotransmitter analytes, thus altering $T_2$-weighted MRI signals. We demonstrate this principle using tethered dopamine and serotonin analogs, together with proteins selected for their ability to competitively bind either the analogs or the neurotransmitters themselves. Corresponding sensors for dopamine and serotonin exhibit target-selective relaxivity changes of up to 20%, while also operating below endogenous neurotransmitter concentrations. Semisynthetic magnetic particle sensors thus represent a promising path for minimally perturbative studies of neurochemical analytes.

Recent years have seen substantial advances in technology for mapping neurochemical dynamics in the brain. Magnetic resonance imaging (MRI) methods in particular offer an attractive combination of minimal invasiveness, ability to monitor large fields of view, and relatively high resolution for studies in animals. A basis for efforts to directly image monoamine neurotransmitters by MRI has been a set of sensors derived from the heme domain of the bacterial cytochrome P450-BM3 (BM3h). BM3h variants engineered for selective binding to dopamine and serotonin produce ligand-dependent changes in longitudinal relaxation time ($T_1$), allowing corresponding neurotransmitters to be detected in $T_1$-weighted MRI. Although BM3h-based probes have been used to map monoamine dynamics in vivo, they produce weak effects in MRI, as reflected by the slope of $T_1$ vs. probe concentration (relaxivity, $r_1$). With relaxivity values of 1-2 mM$^{-1}$s$^{-1}$, these sensors must be applied at concentrations that substantially exceed physiological levels of neurotransmitter analytes in brain interstitium. This in turn perturbs endogenous neurotransmitter concentrations and limits sensitivity of detection.

MRI probes based on superparamagnetic iron oxide nanoparticles (SPIOs) could offer a solution to these problems. SPIOs can be detected at subnanomolar particle concentrations by transverse relaxation time ($T_2$)-weighted MRI. Reversible aggregation of SPIOs affords a versatile and sensitive mechanism for molecular detection. Recently, this mechanism was applied to detect dynamic calcium signaling events in the brain, indicating its potential for in vivo applications to other analytes.

To adapt SPIOs for sensing neurotransmitters, we initially targeted dopamine. Figure 1a depicts a candidate dopamine responsive nanoparticle (DaReNa) probe consisting of two populations of modified SPIOs, one conjugated to a dopamine-binding protein, and the other attached to a tethered analog capable of competing with dopamine for protein binding. In the absence of dopamine, the two types of SPIOs should remain dispersed, while dopamine induces clustering of SPIOs.

In the presence of dopamine, interactions between particles should be disrupted by competition,
breaking up the aggregates and resulting in $r_2$ values more like unfunctionalized SPIOs.

To synthesize DaReNas, we chose to incorporate BM3h dopamine-binding proteins as actuating domains. The most effective variant, BM3h-9D7, exhibits a reported dissociation constant ($K_d$) of $1.3 \pm 0.1 \mu M$ and 28-fold selectivity against norepinephrine. We introduced a thiol for bioconjugation of this variant to SPIOs, forming 9D7-S450C. We also sought a dopamine analog that could be tethered to thiol-reactive SPIOs while retaining affinity for 9D7. Guided by structural data, we initially synthesized 4-O-(26-mercapto-3,6,9,12,15,18,21,24-octaoxahexaclyoxy)-dopamine (DA-PEG-SH, Figure 1b and Scheme S1).

Ligand binding to 9D7-S450C was examined by optical titration, exploiting absorbance changes arising from BM3h heme-analyte interactions. Measured $K_d$ values for binding of dopamine and DA-PEG-SH to 9D7-S450C are $1.3 \pm 0.1 \mu M$ and $108 \pm 1 \mu M$, respectively, indicating that addition of the PEG-SH tether to dopamine substantially interferes with binding. To form a ligand-protein pair with improved affinity, we introduced mutations L17A and Q189T to create 9D7*, which we predicted from the 9D7-dopamine crystal structure would exhibit improved tolerance for PEGylated ligands (Figure 1c and Table S1).

We also examined the effect of eliminating the 3-hydroxy group from DA-PEG-SH, resulting in the compound O-(26-mercapto-3,6,9,12,15,18,21,24-octaoxahexaclyoxy)-tyramine (Tyr-PEG-SH, Figure 1b and Scheme S2). Tyramine has previously been shown to display minimal binding to dopamine receptors, suggesting lesser potential for physiological side-effects. Optical titration of dopamine and Tyr-PEG-SH onto 9D7* yields $K_d$ values of $2.1 \pm 0.6 \mu M$ and $7.8 \pm 2.6 \mu M$, respectively (Figure 1d), indicating that the desired improvements were achieved while also preserving dopamine specificity (Figure S1).

Tyr-PEG-SH and 9D7* were subsequently conjugated to maleimide-terminated photocrosslinked lipid-coated SPIOs. Following thiol-maleimide conjugation, the concentration of conjugated Tyr-PEG ligands per mM SPIO Fe was $\sim 5 \mu M$. Meanwhile, a standard protein assay indicated that the concentration of 9D7* domains per mM Fe was $\sim 3 \mu M$.

A functional DaReNa was formed by 1:1 mixing of 9D7*- and Tyr-PEG-functionalized SPIOs. The resulting nanosensor was characterized by MRI and dynamic light scattering (DLS). The average DaReNa relaxivity is $208 \pm 2$ (mM Fe)$^{-1}$s$^{-1}$, whereas $r_2$ values of 9D7*-SPIO and Tyr-PEG-SPIO conjugates alone range from 130-140 (mM Fe)$^{-1}$s$^{-1}$ (Figure 2a). These values correspond to greater darkening of MRI signal by DaReNa compared with individual SPIO species, and are also consistent with DLS results demonstrating that DaReNa components cluster when mixed (Figure 2b). The mean hydrodynamic diameter ($D_h$) of DaReNa is $138 \pm 4$ nm, while $D_h$ values of $52 \pm 2$ nm and $32 \pm 1$ nm are observed for 9D7*-SPIOs and Tyr-PEG-SPIOs alone. The observed relationship between particle size and $r_2$ is expected in a motional averaging relaxation regime.

Dopamine causes unclustering of DaReNa particles and lowers $r_2$ accordingly. In DLS, addition of 200 µM dopamine to the DaReNa solution (100 µM Fe = $\sim 1$ nM nanoparticles) reduces the mean $D_h$ by about 25%, to $104 \pm 4$ nm (Figure 2b). Corresponding changes in nanoparticle aggregation are directly observed using transmission electron microscopy of similar samples (Figure 2c). Dopamine concentrations above 40 µM also cause significant decreases in relaxivity ($t$-test $p \leq 0.02, n = 3$), with 500 µM dopamine producing an $r_2$ of $152 \pm 2$ (mM Fe)$^{-1}$s$^{-1}$, 20% lower than in the absence of dopamine (Figure 2d). Addition of equivalent amounts of serotonin or norepinephrine produce substantially smaller changes in $r_2$, indicating selectivity of the DaReNa response to dopamine (Figure 2e). Importantly, DaReNa responses to dopamine are achieved at a nanoparticle concentration of only 1 nM and a total 9D7* concentration of 0.2 µM, about 100 times lower than concentrations used for T$_1$-based dopamine sensing and low enough to have minimal effect on dopamine signaling in realistic applications.
To evaluate the kinetics of the DaReNa responses, we began by examining the time course of light scattering changes after mixing 9D7* and Tyr-PEG-functionalized particles or challenging DaReNa with dopamine (Figure S2). Results obtained with a dead time of ~20 s indicate that substantial size alterations occur soon after changes in conditions, but that slow evolution of the scattering signal continues. Because light scattering tends to be dominated by large species, we also obtained more precise kinetic measurements using biolayer interferometry (BLI). 9D7* was immobilized on the surface of a BLI probe and binding and unbinding of Tyr-PEG-functionalized SPIOs were measured on a time scale of seconds. Measurements reveal an association rate of 0.016 ± 0.001 s⁻¹ in the absence of dopamine and a dissociation rate of 0.027 ± 0.004 s⁻¹ following challenge with 100 µM dopamine (Figure 2f). These results, which correspond to time constants of 38-63 s, are comparable to kinetic measurements from the 9D7* parent protein itself, and are thus suitable for functional imaging of responses to standard block stimuli or pharmacological challenges in vivo.⁷¹⁷

As an additional precursor to in vivo applications, we determined that intracranial infusion of DaReNa to relevant areas of the rat brain is feasible. Targeted injection of 10 µL sensor at 1 mM SPIO iron concentration produces broad staining in the striatum, where dopamine concentrations within detection range of the sensor have previously been recorded (Figure 3).¹⁸ T₂-weighted contrast is visible over a diameter of ~2 mm and corresponds to T₂ relaxation rate (R₂) increases by about a factor of three near the center of the injected region, revealing the ability of DaReNa to spread effectively in tissue. Noninvasive brain delivery strategies could be used in the future to further improve the distribution of sensor particles.¹⁹
transmitters to SPIOs. DaReNas and SeReNas operate at

For sensitivity at ligand binding capacities about 100:

while 50 µM dopamine and norepinephrine produce changes

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nin [55x246]∫g to SPIOs. [79x92]→ weighted MRI can be formed by conjugating neuro-

an serotonin analog, O-(26-mercapto-3,6,9,12,15,18,21,24-octaoxahexacosyl)-serotonin (SHT-PEG-SH, Figure 4a and Scheme S3). This molecule is similar to O-alkylserotonin derivatives with sharply reduced affinity for serotonin receptors, but it exhibits high affinity for a previously identified serotonin-binding BM3h variant, BM3h-3DB10, which is selective against binding to dopamine and norepinephrine. 3DB10 was modified for bioconjugation via the S450C mutation to produce 3DB10*. Optical titration of 3DB10* with serotonin and SHT-PEG-SH gives $K_d$ values of 0.95 ± 0.88 µM and 3.2 ± 5.0 µM, respectively, indicating that the protein is able to tolerate PEGylation of serotonin without substantial loss of affinity (Figure 4b).

SeReNas were generated by conjugating 3DB10* and SHT-PEG-SH to SPIOs. Absorbance measurements indicated approximately 9 µM SHT-PEG ligands and 3 µM 3DB10* domains per mM SPIO Fe. SeReNas display $r_2$ and $D_t$ values substantially greater than those of 3DB10*- or SHT-PEG-conjugated SPIOs (Figure S3). In contrast to DaReNas, SeReNas display less cooperative behavior when titrated with their ligand. Values of $r_2$ decrease almost linearly with serotonin, ranging from 183 ± 3 (mM Fe)$^{-1}$s$^{-1}$ without ligand to 163 ± 8 (mM Fe)$^{-1}$s$^{-1}$ in 500 µM serotonin (Figure 4c). Serotonin concentrations as low as 5 µM produce significant $r_2$ changes ($t$-test $p = 0.02$, $n = 3$), and the maximum $r_2$ change over the full range is 11% of the ligand-free value. These responses are selective: 50 µM serotonin produces a 9.2% decrease in $r_2$, while 50 µM dopamine and norepinephrine produce changes of <1.9% (Figure 4d). As with the DaReNas, SeReNas display sensitivity at ligand binding capacities about 100-fold lower than previously used in $T_1$-weighted sensing approaches.

These results demonstrate that neurotransmitter sensors for $T_2$-weighted MRI can be formed by conjugating neurotransmitter binding proteins and cognate tethered neurotransmitters to SPIOs. DaReNas and SeReNas operate at nanoparticle concentrations of 1 nM and binding capacities of 0.2 µM, suitable for minimal perturbations to endogenous neurotransmitter concentrations ranging from micromolar in interstitium to millimolar near synapses. The probes could be further refined to enhance their sensitivity, dynamic range, and kinetics. Manipulations of nanoparticle size and functionalization density, for instance, could be used to improve response times to the single-second or subsecond scale. The combination of protein and tethered ligand engineering techniques applied here could also be used to construct MRI sensors for additional targets.

ASSOCIATED CONTENT

Supporting Information. Additional data, detailed methods, and characterization of synthetic compounds. This material is available free of charge at http://pubs.acs.org.

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VH and SO contributed equally, and IGA and AB also contributed equally.

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