Published in final edited form as:

Chem Commun (Camb). 2019 July 23; 55(60): 8860–8863. doi:10.1039/c9cc00375d.

A Dual-Responsive Probe for Detecting Cellular Hypoxia using ^{19}F Magnetic Resonance and Fluorescence

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Abstract

We report the first dual-responsive ^{19}F MRI and fluorescence imaging probe for cellular hypoxia. The Cu^{2+} -based probe exhibits no ^{19}F MR signal and reduced fluorescence signal due to paramagnetic quenching; however, the probe turns-on in both modes following reduction to Cu^+ . This bimodal agent can differentiate hypoxic and normoxic cells in both modalities.

Cellular hypoxia, or oxygen deficiency, is a detrimental physiological condition that stems from inadequate vasculature preventing proper oxygen flow and nutrient delivery to cells. Hypoxic cells express increased levels of hypoxia inducible factor (HIF-1), which regulates a cell's ability to either adapt to the hypoxic environment or die via apoptosis.^{1, 2} Hypoxia is associated with genetic instability, resistance to chemotherapy, and malignant proliferation of cells and thus is an important clinical target for imaging.^{3–6} To date, a number of agents have been developed to image hypoxia using a range of modalities.^{7–13} For example, ^{64}Cu ATSM is used as an imaging agent for positron emission tomography (PET).¹⁴ This probe functions via selective intracellular accumulation in hypoxic cells following reduction of Cu^{2+} ATSM to $[\text{Cu}^+\text{ATSM}]$ and subsequent ligand dissociation. In normoxic cells, the complex can be reduced to $[\text{Cu}^+\text{ATSM}]$, but is re-oxidized to Cu^{2+} ATSM more efficiently than ligand dissociation.^{15–18} However, radioactive probes such as this have associated safety concerns and PET imaging has limited spatial resolution.¹⁹

There has been growing interest in bimodal imaging agents as they can be used to provide complementary diagnostic information from multiple imaging modalities.^{20–22} Magnetic Resonance Imaging (MRI) is an excellent *in vivo* imaging modality since the technique provides images with high contrast and spatial resolution without the use of ionizing radiation.²³ While MRI typically is used to detect ^1H signals in the body, ^{19}F MRI is a promising alternative as ^{19}F is biostable and has excellent receptivity (83% of ^1H).^{24–26} Further, there is no detectable fluorine in the body, resulting in zero biological background signal, thus enabling quantitative hotspot imaging. Although ^{19}F MRI is effective for whole animal imaging with high contrast, it does not provide data at the cellular level.

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[†]Electronic Supplementary Information (ESI) available: Experimental details, NMR, electrochemistry, cell culture studies, electronic spectra, and flow cytometry. See DOI: 10.1039/x0xx00000x

Conflicts of interest

The authors declare no conflict of interest.

Fluorescence, on the other hand, is excellent for cellular imaging as the technique provides high specificity, excellent contrast, and subcellular spatial resolution.^{23, 27} Fluorescence can be used to track intracellular probe localization within individual cells. Indeed, a number of fluorescent probes have been reported that detect hypoxic environments, with the best probes displaying greater than 600x fluorescence turn-on after reduction.^{28, 29} Since the penetration depth of fluorescence imaging is limited, probes with bimodal responsive functionality could provide diagnostic information that spans depth penetration and resolution scales. Specifically, MRI can identify hypoxic cancerous growth using whole body imaging and fluorescence can be used to confirm the presence of hypoxic cells during tumor excision and biopsy.

Paramagnetic metals can be used to modulate both ^{19}F and fluorescence signal to make biosensors.^{30–39} To develop bimodal hypoxia-responsive probes for ^{19}F MRI and fluorescence, we chose the CuATSM scaffold as the paramagnetic Cu^{2+} center will quench both the ^{19}F MR signal via paramagnetic relaxation enhancement (PRE)^{7, 8} and the fluorescence signal due to electron transfer from the excited fluorophore to the paramagnetic metal center (Scheme 1).³⁶ Following reduction to Cu^+ and ligand dissociation, both of these effects will be relieved, resulting in signal turn-on in both modalities. Herein, we describe **CuATSMF₃-FI**, the first dual-responsive ^{19}F MRI fluorescence imaging probe that displays increases in ^{19}F and fluorescence signals in hypoxic cells.

Synthesis of **CuATSMF₃-FI** (Scheme S1) was carried out from previously reported **1** with 2,2,2-trifluoroethanamine hydrochloride via transamination to afford **2** (70%).⁷ This was subsequently reacted with *tert*-butyl(2-aminoethyl)carbamate to yield **3** (62%). Compound **3** was complexed with $\text{Cu}(\text{OAc})_2$ to obtain **4** (75%), which was then deprotected and reacted with the succinimidyl ester of 5-(and 6-) carboxyfluorescein to yield **CuATSMF₃-FI** (61%). Acetylated **CuATSMF₃-FIac** was achieved via Fischer esterification of **CuATSMF₃-FI** with acetic anhydride in pyridine to give a 98% yield. All intermediates were purified via C18 reverse-phase chromatography using acetonitrile and Milli-Q grade water as eluents. The purity of all compounds was validated using HRMS and NMR (See Supporting Information). **CuATSMF₃-FI** is stable in acidic environments as determined by LC/MS (Figure S1A and S23) and ^{19}F NMR (Figure S1B).

We first investigated the redox characteristics of **CuATSMF₃-FI**. Recent work has shown that the introduction of a $-\text{CF}_3$ group to CuATSM ($E_{1/2} = -0.63$ V vs. SCE) causes a cathodic shift in potential due to the electron-withdrawing effects of fluorine (CuATSMF₃ $E_{1/2} = -0.56$ V).⁷ Indeed, cyclic voltammetry (CV) of **CuATSMF₃-FI** also shows a similar shift in potential, with a quasi-reversible $\text{Cu}^{2+}/\text{Cu}^+$ peak at $E_{1/2} = -0.55$ V in DMF (Figure S2). This indicates that the primary contributor of the redox shift is the fluorine moiety and the fluorescein does not significantly impact the $E_{1/2}$ of the complex. Moreover, this suggests **CuATSMF₃-FI** should retain hypoxia selectivity as previous studies demonstrated that CuATSM scaffolds with $E_{1/2} < -0.50$ V will accumulate more in hypoxic cells compared to normoxic cells.^{17, 40}

To characterize the complex and validate the probe's functionality as a bimodal agent, we next investigated how the ^{19}F NMR and fluorescence properties of **CuATSMF₃-FI** changed

during reduction. ^{19}F NMR spectra for **CuATSMF₃-FI** were obtained in the absence and presence of excess sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). Complex **CuATSMF₃-FI** displayed no peaks in its ^{19}F NMR spectrum (Figure 1A top); however, in the presence of reductant, a sharp triplet peak was observed at -70.2 ppm (Figure 1A bottom), corresponding to the free ligand, which forms due to the instability of the Cu(I) reduction product in aqueous solution.^{7, 8} Longitudinal (T_1) and transverse (T_2) relaxation times of reduced **CuATSMF₃-FI** were 730 ms and 150 ms, respectively (Table S1).⁷ In aqueous solution, **CuATSMF₃-FI** displays some baseline fluorescence ($\Phi = 0.196$, Figure S3), which increases 3.5-fold following reduction with 2 mM dithiothreitol (DTT) or glutathione (GSH) (Figure 1B). 2 mM GSH does not change the fluorescence of 5(6)-carboxyfluorescein itself (Figure S6), indicating that the observed increase in fluorescence is due to reduction at the Cu center and ligand dissociation. Thus, our probe displays an increase in both ^{19}F NMR and fluorescence signal intensities following reduction.

CuATSMF₃-FI was subsequently examined by Electron Paramagnetic Resonance (EPR) spectroscopy to confirm that reduction occurs at the Cu^{2+} center. The room temperature EPR spectrum of **CuATSMF₃-FI** in DMF gave the four characteristic Cu^{2+} peaks including hyperfine splitting with a $g_{av} = 2.06$ (Figure S4), consistent with previous reports for Cu^{2+} complexes with square planar geometry.^{6-8, 41, 42} A similar spectrum was observed in 10% DMSO in HEPES buffer at room temperature (Figure 1C). Upon addition of one equivalent of $\text{Na}_2\text{S}_2\text{O}_4$ or excess DTT to **CuATSMF₃-FI**, the peaks corresponding to the Cu^{2+} species disappear, consistent with a one electron reduction of Cu^{2+} .

To demonstrate the extent of turn-on in reductive environments, phantom ^{19}F MR images of **CuATSMF₃-FI** in the absence and presence of sodium dithionite were acquired. Images were taken on a 7 T preclinical MRI using a rapid acquisition with relaxation enhancement (RARE) sequence. As shown in Figure 2A, a 4.5 mM solution of **CuATSMF₃-FI** displayed zero detectable fluorine signal (signal to noise ratio (SNR) of 1.8), whereas a 4.5 mM solution of **CuATSMF₃-FI** with excess $\text{Na}_2\text{S}_2\text{O}_4$ displayed a SNR of 67.4. As expected, SNR increased linearly with increasing **CuATSMF₃-FI** concentration (Figure 2B). MR data collected demonstrates that a turn-on can be visualized in reductive environments.

To evaluate the bimodal functionality of **CuATSMF₃-FI** in cells, experiments were performed with HeLa cervical cancer cells to confirm a biological turn-on response in hypoxic environments. For cellular studies, we synthesized the acetyl ester **CuATSMF₃-FIAc**, a derivative of **CuATSMF₃-FI** with greater cell permeability. HeLa cells were incubated with **CuATSMF₃-FI** and **CuATSMF₃-FIAc** for 4 hours and their cytotoxicity was determined using an MTT assay (Figures S7 and S8). The complexes showed negligible cytotoxicity up to 200 μM . Cell uptake studies were performed with 30 μM **CuATSMF₃-FIAc** for one hour in normoxia (20% O_2) and hypoxia (0.1% O_2 , representative of severe tumor hypoxia).² Intracellular copper concentration was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). Normoxic cells with **CuATSMF₃-FIAc** contained 0.13 ± 0.01 fmol Cu^{2+} per cell, while hypoxic cells contained 0.18 ± 0.02 fmol Cu^{2+} per cell, demonstrating a 40% increase in uptake ($p < 0.05$, t-test). The corresponding fluorescence signal for cells treated under the same conditions was monitored by flow cytometry and fluorescence microscopy (Figures 3A, 3B, S9, and S10). Flow cytometry

revealed a 60% increase in fluorescence ($p < 0.05$, t-test) from our probe in hypoxic cells vs. normoxic cells (Figure 3A, Table S2). This result was further supported by fluorescence imaging where much brighter signal was observed in hypoxic cells (Figure 3B right) vs. normoxic cells (Figure 3B left). Interestingly, fluorescence was observed throughout the cells and was not specifically localized to one organelle or intracellular region. This is consistent with previous reports of localization of CuATSM labeled with a fluorescent pyrene moiety.^{43, 44} We note that the fluorescence of the CuATSMF₃-FI scaffold is pH dependent, with decreased fluorescence observed at acidic pH (Figure S1A). Previous studies have shown that hypoxic cells induce a slight acidic environment,⁴⁵ which may dampen the overall hypoxia-induced fluorescence response of **CuATSMF₃-FI**. Future work will incorporate pH-insensitive fluorophores into this scaffold.

To further demonstrate the complex's selectivity for hypoxia, 60 μ M of **CuATSMF₃-FIAc** was incubated in HeLa cells for ¹⁹F NMR studies. Figure 3C shows three representative ¹⁹F NMR spectra from control DMSO-treated cells (bottom), normoxic cells treated with **CuATSMF₃-FIAc** (middle), and hypoxic cells treated with **CuATSMF₃-FIAc** (top). There are no visible peaks for the control sample and a small peak for the normoxic sample (SNR 5.4 ± 0.7). The hypoxic sample shows a sharp ¹⁹F peak (SNR 39.7 ± 2.5). This data indicates that there is minimal reduction of the Cu²⁺ complex in normoxic cells; however, there is a quantifiable peak in hypoxic cells (greater than seven-fold increase in SNR), demonstrating that the bimodal probe is reduced under severe hypoxic cellular conditions. We did not observe signal turn-on in HeLa cells under 2% oxygen, representative of more mild hypoxic conditions. Thus, we have demonstrated the ability to use **CuATSMF₃-FI** to provide enhanced signal in severe hypoxic cells using both magnetic resonance and fluorescence outputs.

In conclusion, we synthesized a fluorinated and fluorescent CuATSM derivative that provides bimodal imaging information for detection of hypoxic cell environments. Attaching a fluorophore to a fluorinated CuATSM did not perturb the coordination environment and **CuATSMF₃-FI** retained hypoxic cellular redox selectivity. The initial fluorescence increased following reduction of the Cu²⁺ and subsequent demetallation. In the presence of a chemical reductant, the quenched Cu²⁺ NMR and MRI signals are restored due to relief of the PRE effect. Live cell fluorescence analysis demonstrated that HeLa cells incubated with **CuATSMF₃-FIAc** emitted quantifiable increase in fluorescence and no specific probe localization was observed. An increase in signal was also observed in the ¹⁹F MR modality as a robust ¹⁹F NMR signal was observed in hypoxic HeLa cells and not normoxic cells. Ongoing work with dual-responsive ¹⁹F MRI probes includes increasing fluorine density for improving MR sensitivity, enhancing fluorescence signal differentiation, and exploring other bimodal combinations for imaging and theranostic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by start-up funds from UT-Austin (EQ) and a grant from the Welch Foundation (F-1883) (EQ). We acknowledge the Biomedical Imaging Center and the Microscopy and Imaging Facility of the Center for Biomedical Research Support at UT Austin for access to instruments. We thank Kanchan Aggarwal for guidance with flow cytometry and Prof. Jonathan Sessler for use of his plate reader. Some NMR spectra were acquired on a Bruker AVIII HD 500 instrument acquired through a National Institutes of Health equipment grant (J. Sessler, 1 S10 OD021508–01).

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Figure 1.

(A) ^{19}F NMR spectra of 5 mM $\text{CuATSMF}_3\text{-FI}$ in the absence (top) and presence (bottom) of excess $\text{Na}_2\text{S}_2\text{O}_4$ performed in 3:2 $\text{d}_6\text{-DMSO}$:HEPES (pH 7.2, 25 mM). (B) Normalized fluorescence spectra of 2 mM DTT and 2 mM GSH reduction of 1.5 μM $\text{CuATSMF}_3\text{-FI}$ in HEPES (pH 7.2, 50 mM). (C) Electron Paramagnetic Resonance (EPR) spectra of 1 mM $\text{CuATSMF}_3\text{-FI}$ in 10% DMSO in HEPES (pH 7.2, 50 mM) with one equivalent $\text{Na}_2\text{S}_2\text{O}_4$ (light green) and excess DTT (dark green) at 298 K.

Figure 2.

(A) Phantom ^{19}F MR images of 4.5 mM **CuATSMF₃-FI** (7 T MRI RARE pulse sequence: 1500 ms repetition time (TR), 3.75 ms echo time (TE), RARE factor 32) performed in 3:2 DMSO:HEPES (pH 7.2, 50 mM) in the absence (left, circled) and presence (right) of excess $\text{Na}_2\text{S}_2\text{O}_4$ as a reductant. (B) Phantom ^{19}F MR images with increasing concentration (top to bottom: 3.6 mM, 2.7 mM, 1.8 mM, 0.9 mM) in 3:2 DMSO:HEPES (pH 7.2, 50 mM) with excess $\text{Na}_2\text{S}_2\text{O}_4$.

Figure 3.

(A) Flow cytometry single cell fluorescence distribution data from HeLa cells incubated for 1 hour with 30 μM **CuATSMF₃-FIAc** in normoxic (20% O_2 , black) and hypoxic (0.1% O_2 , green) environments. (B) Fluorescence images of normoxic (left; brightfield top, fluorescence bottom) and hypoxic (right) HeLa cervical cancer cells after one hour 30 μM incubation of **CuATSMF₃-FIAc**. Scale bar 200 μm . (C) ^{19}F NMR spectrum of lysed HeLa cells in anhydrous d_6 -DMSO with no **CuATSMF₃-FIAc** (bottom), one hour 60 μM **CuATSMF₃-FIAc** in normoxia (middle), and one hour 60 μM **CuATSMF₃-FIAc** in hypoxia (top).

Scheme 1.

Design strategy for hypoxia selective bimodal ¹⁹F MR and fluorescent probe. The initial modulated ¹⁹F signal and quenched fluorescence "turns-on" upon hypoxic reduction of Cu²⁺ to Cu⁺ and subsequent demetallation of the ligand scaffold.