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An Aza-Cope Reactivity-Based Fluorescent Probe for Imaging Formaldehyde in Living Cells

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Supporting Information

ABSTRACT: Formaldehyde (FA) is a reactive carbonyl species (RCS) produced in living systems that has been implicated in epigenetics as well as in the pathologies of various cancers, diabetes, and heart, liver, and neurodegenerative diseases. Traditional methods for biological FA detection rely on sample destruction and/or extensive processing, resulting in a loss of spatiotemporal information. To help address this technological gap, we present the design, synthesis, and biological evaluation of a fluorescent probe for live-cell FA imaging that relies on a FA-induced aza-Cope rearrangement. Formaldehyde probe-1 (FAP-1) is capable of detecting physiologically relevant concentrations of FA in aqueous buffer and in live cells with high selectivity over potentially competing biological analytes. Moreover, FAP-1 can visualize endogenous FA produced by lysine-specific demethylase 1 in a breast cancer cell model, presaging the potential utility of this chemical approach to probe RCS biology.

Formaldehyde (FA), the simplest aldehyde, is a reactive carbonyl species (RCS) that has long been known as a human toxin and carcinogen that is released into the environment from natural (e.g., biomass combustion, solar degradation of humic substances, vegetation and microbe emissions) as well as anthropogenic (e.g., FA production and fumigation, vehicle exhaust, etc.) sources.[1] At the same time, FA is also produced endogenously in the body by demethylase and oxidase enzymes that regulate epigenetics[2,3] and metabolism[4,5] such as lysine-specific demethylase 1 (LSD1),[6] JmjC domain-containing proteins,[7−10] and semicarbazide-sensitive amine oxidase.[11] Active degradation by formaldehyde dehydrogenase/*S*-nitrosoglutathione reductase and aldehyde dehydrogenase 2[12] enzymes gives physiological FA levels ranging from 100 μM in blood to 400 μM intracellularly.[13−15] Elevations of FA and related RCS are implicated in a variety of disease pathologies, including various cancers,[16] neurodegenerative diseases,[15,17,18] diabetes, and chronic liver and heart disorders.[19] The rapidly growing list of modified DNA[20−22] and RNA[23] bases, particularly N-methylated bases whose demethylation pathways may involve FA production,[24−26] presage a diverse array of important contributions for FA chemistry to biology.

Despite its significance, methods for monitoring FA within intact, living biological specimens remain underdeveloped. Current methods for FA analysis rely on radiometry,[27] gas chromatography,[28,29] selected ion flow tube mass spectrometry,[30,31] and high-performance liquid chromatography,[32,33] which offer high sensitivity and selectivity but are limited by the need for sample processing and/or destruction of the intact specimens. As such, we sought to develop FA-responsive fluorescence-based probes that would give the potential to track this RCS in living samples. We now report the design, synthesis, and application of formaldehyde probe-1 (FAP-1), a first-generation reactivity-based fluorescent indicator for selective imaging of FA in solution and in living cells at physiological levels, including endogenous FA production in a cancer cell model.

We[34] and others[35−37] have exploited reactivity-based fluorescence detection for transient small-molecule analytes, including carbonyl species such as CO[38−42] and methylglyoxal,[43] and we sought to employ this general approach to FA detection. Our present design exploits an FA-induced 2-aza-Cope reaction to transform a homoallylic amine into an aldehyde coupled to a fluorogenic turn-on response.[44] Indeed, previous attempts to monitor FA have relied on formimine formation;[45,46] however, this condensation tends to have an unfavorable equilibrium constant in water, leading to difficulty in detecting low concentrations of the RCS. We reasoned that an aza-Cope rearrangement could trap the imine and lead to accumulation of a fluorescent product after hydrolysis (Scheme 1). Inspired by the work of Urano, Nagano et al. that aminomethyl silicon rhodamine dyes are weakly emissive at physiologically relevant pH due to spirocyclization,[47,48] we designed FAP-1 with a homoallylamine that would favor ring closure and low fluorescence. Upon reaction with FA, imine formation and subsequent 2-aza-Cope rearrangement and hydrolysis would yield an aldehyde product that is incapable of spirocyclization and would give a fluorescence turn-on. Spirocyclization-based strategies have been employed fruitfully to detect a wide variety of biological analytes.[49,50] FAP-1 was synthesized in seven steps, utilizing a key boronate-mediated aminoallylation to install the reactive trigger (Scheme 2).

With FAP-1 in hand, we tested its fluorescence turn-on response to FA in aqueous solution buffered to pH 7.4, in which it shows good solubility (log $D_{oct/wat}$ = 0.53 ± 0.01). FAP-1 is weakly fluorescent (ε_{650} = 190 M^{-1} cm^{-1}, ϕ_{fl} = 0.36; Figures S1 and S2) and exhibits a ∼8-fold fluorescence turn-on response (λ_{max} = 645 nm, λ_{em} = 662 nm) upon treatment with 100 μM FA, a physiological concentration of this RCS, within 1 h (Figure 1).[13,14] This fluorescence enhancement is likely due to the

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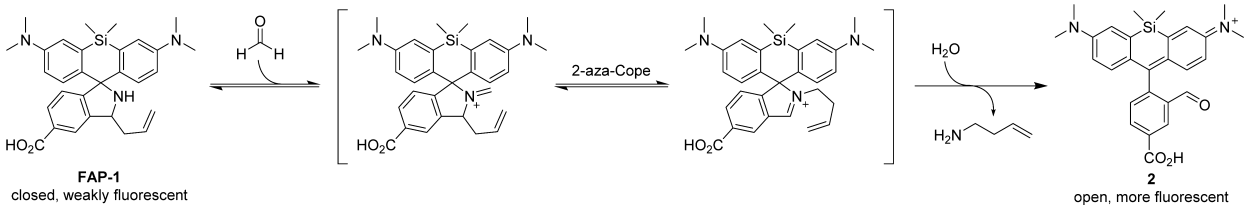
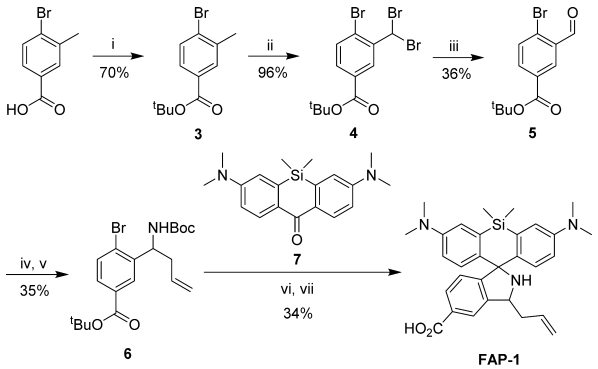
Scheme 1. Design of Formaldehyde Probe FAP-1



Scheme 2. Synthesis of Formaldehyde Probe FAP-1[a]



[a]Reagents and conditions: (i) Boc_2O, DMAP, THF, ^{t}BuOH, 70 °C, 12 h; (ii) NBS, AIBN, $PhCF_3$, 115 °C, 8 h; (iii) $AgNO_3$, H_2O, MeCN, 70 °C, 24 h; (iv) NH_3, MeOH, 0 °C, then allylboronic acid pinacol ester, rt, 10 h; (v) Boc_2O, THF, rt, 14 h; (vi) PhLi, THF, −78 °C, then ^{t}BuLi, THF, −78 °C, then **7**, −78 °C to rt, 3 h; (vii) TFA:DCM, 1:1, rt, 12 h.

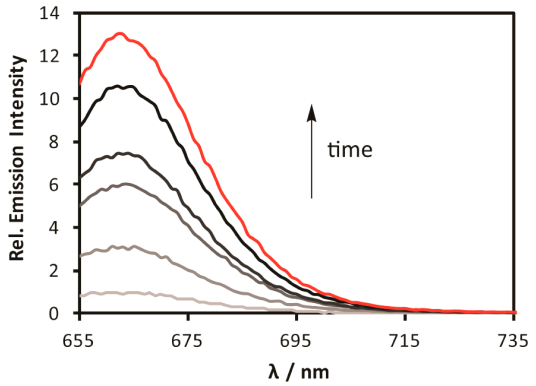


Figure 1. Fluorescence response of 10 μM FAP-1 to 100 μM FA. Data were acquired at 37 °C in 20 mM PBS (pH 7.4) with excitation at λ_{ex} = 645 nm. Emission was collected between 655 and 750 nm. Time points represent 0, 20, 45, 60, 90, and 120 (red trace) min after addition of 100 μM FA.

increase in absorptivity observed during FA treatment (Figure S1b). At extended incubation times, the turn-on response saturates at ∼45-fold (Figure S3). At a 10 μM FAP-1 concentration and a 2 h cutoff, the *in vitro* detection limit for FA was found to be 5 μM (Figure S4). To verify that the observed fluorescence turn-on response was the result of the proposed 2-aza-Cope reaction, the reaction between FAP-1 and FA was monitored by LC-MS, which shows clean conversion from FAP-1 to a product with the expected mass of aldehyde **2** (Figure S5).

FAP-1 shows good selectivity for FA over potentially competing biological RCS, including 4-hydroxynonenal, dehydroascorbate, glucosone, oxaloacetate, and methylglyoxal as well as over simple carbonyl-containing molecules including acetaldehyde, pyruvate, and glucose (Figure 2). FAP-1 is not

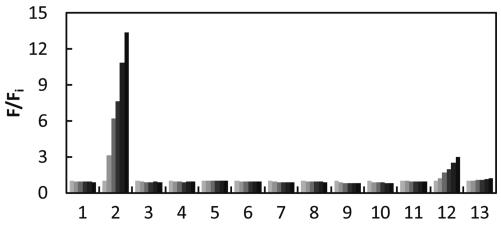


Figure 2. Fluorescence response of 10 μM FAP-1 to biologically relevant RCS and related molecules. Bars represent relative emission from 655 to 700 nm at 0, 20, 45, 60, 90, and 120 (black) min after addition. Data shown are for 100 μM of all species unless otherwise denoted. Data were acquired in 20 mM PBS (pH 7.4) at 37 °C with excitation at λ_{ex} = 645 nm. Legend: (1) PBS; (2) FA; (3) acetaldehyde; (4) 4-hydroxynonenal; (5) dehydroascorbate; (6) glucose, 1 mM; (7) glucosone; (8) oxaloacetate; (9) pyruvate; (10) H_2O_2; (11) glutathione, 5 mM; (12) methylglyoxal; (13) methylglyoxal, 10 μM.

responsive to 10 μM methylglyoxal, which is above its single-digit micromolar physiological range[51] but does show a small response to superphysiological levels (100 μM) of this RCS. In addition, we exposed FAP-1 to oxidizing and reducing conditions that could be encountered in the cell—specifically 100 μM H_2O_2 and 5 mM glutathione—and observed no change in fluorescence (Figure 2).

Having established that the 2-aza-Cope-based trigger of FAP-1 can selectively detect FA in solution at physiological levels, we next evaluated its ability to visualize changes in FA in living cells using confocal microscopy (Figure 3). Treatment of HEK293T cells with 10 μM FAP-1 for 30 min followed by washing to remove excess probe and addition of various concentrations of FA (200 μM to 1 mM) showed a significant and dose-dependent fluorescence turn-on in FA-treated cells over control cells (Figure 3a−d,f), demonstrating the ability of FAP-1 to detect FA in a cellular context. Notably, these FA concentrations fall well within a physiological concentration range, which is estimated at ∼100 μM in blood,[13] 400 μM intracellularly,[14] and up to 700−800 μM in several cancer tissues.[52] To rule out the possibility of photoactivation and/or photobleaching interfering with fluorescence intensity measurements, we conducted photostability studies in HEK293T cells. FAP-1 exhibits consistent fluorescence intensity during 100 scans with 6% laser power (used for all imaging experiments) but exhibits slight photobleaching at 50% laser power (Figure S6). Moreover, cell viability was verified using Hoechst 33342 staining, which clearly showed intact and viable nuclei (Figure 3e) as well as a propidium iodide assay, which indicated no difference between FA-treated and untreated

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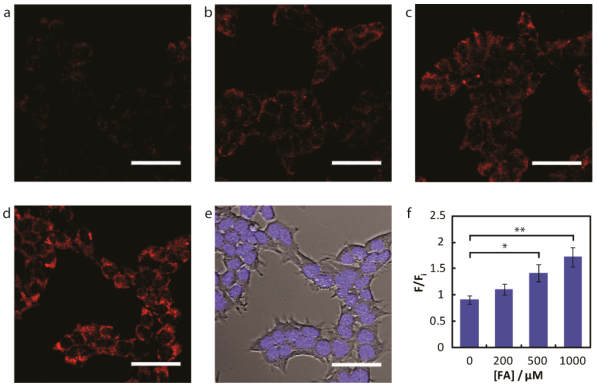

Figure 3. Confocal microscopy of FA detection in live HEK293T cells using FAP-1. Cells were treated with 10 μM FAP-1 in BSS for 30 min at 37 °C, followed by an exchange into fresh BSS and addition of varying FA concentrations. Images are taken 30 min after addition of (a) vehicle, (b) 200 μM FA, (c) 500 μM FA, and (d) 1 mM FA. (e) Bright-field image of cells in (d) overlaid with image of 1 μM Hoechst 33342. Scale bar represents 50 μm in all images. (f) Mean fluorescence intensities of HEK293T cells treated with varying concentrations of FA for 30 min relative to mean fluorescence intensity before FA addition; error bars denote SEM, $n = 3$. * $P < 0.005$, ** $P < 0.0005$.

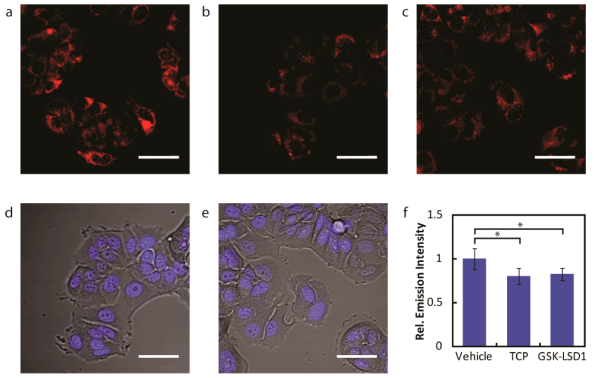

Figure 4. Confocal microscopy of FAP-1 in TCP- and GSK-LSD1-treated MCF7 cells. Cells were pretreated with inhibitor (TCP or GSK-LSD1) for 20 h, followed by exchange into fresh BSS with 10 μM FAP-1 and incubation for 60 min at 37 °C. Images are of cells treated with (a) vehicle, (b) 20 μM TCP, (c) 1 μM GSK-LSD1. (d and e) Bright-field images of cells in (b) and (c), respectively, overlaid with images of 1 μM Hoechst 33342. Scale bar represents 50 μm in all images. (f) Mean fluorescence intensities of MCF7 cells treated with (1) vehicle (2) 20 μM TCP, and (3) 1 μM GSK-LSD1. Error bars denote SEM, $n = 6$. * $P < 0.005$.

cells (Figure S7a). To further probe the cellular distribution of FAP-1, we performed colocalization studies using commercial organelle-targeted dyes. FAP-1 was found to be excluded from the nucleus (Figure S8m–r, Table S1) but showed overlap with endoplasmic reticulum-, Golgi apparatus-, lysosome-, and mitochondria-targeted dyes (Figure S8a–l, Table S1). In addition, the distribution of FAP-1 was not appreciably affected by the addition of 1 mM FA (Figure S8p–r).

We next moved on to show that FAP-1 could be applied to image endogenous FA levels in a disease model. Specifically, elevated FA levels in certain cancers have been attributed to overexpression of LSD1, where pharmacological inhibition of LSD1 can lead to an observable decrease in FA.[53] To determine whether FAP-1 was able to visualize changes in endogenously produced FA, we employed the MCF7 human breast cancer cell line that is known to overexpress LSD1.[54] Upon treatment of MCF7 cells with 20 μM tranylcypromine (TCP), an LSD1 inhibitor with an IC_{50} of 2 μM,[55] a ~20% decrease in FAP-1 fluorescence signal compared to control cells was observed. Additionally, treatment with 1 μM GSK-LSD1, a more potent LSD-1 inhibitor (IC_{50} of 42 nM),[56] also attenuated FAP-1 fluorescence (Figure 4). Taken together, the data show that FAP-1 is capable of detecting endogenously produced FA in a disease model.

To close, we have presented the design, synthesis, and properties of FAP-1, a new type of fluorescent indicator for selective and sensitive detection of FA via a 2-aza-Cope reaction. FAP-1 features a robust fluorescence turn-on response to biologically relevant concentrations of FA as well as selectivity over potentially competing analytes. Furthermore, FAP-1 is capable of detecting exogenous and endogenous FA in live cells. Current efforts are underway to utilize FAP-1 and develop next-generation versions to probe the biology of reactive carbonyl species, specifically in the context of epigenetics, aging, and disease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05340.

Experimental details, including synthesis and characterization, selectivity assays, spectroscopic methods, cellular imaging methods, and cell viability assays (PDF)

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Notes

The authors declare no competing financial interest.

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