Communication

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An Olefin Metathesis-Based Fluorescent Probe for the Selective Detection of Ethylene in Live Cells

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Supporting Information Placeholder

ABSTRACT: Ethylene is an important plant hormone that is involved in a variety of developmental processes including agriculturally important ripening of certain fruits. Owing to its significant roles, a number of approaches have previously been developed to detect ethylene via molecular interactions. However, there are no current approaches for detection that are selective via a discrete homogeneous molecular interaction. Here we report two profluorescent chemodosimeters for the selective detection of the plant hormone ethylene. The approach consists of a BODIPY fluorophore with a pendant ruthenium recognition element based on a Hoveyda-Grubbs 2nd generation catalysts. A marked increase in fluorescence is observed upon exposure to ethylene and selectivity is observed for ethylene over other alkenes, providing a unique approach towards ethylene detection. Imaging in live cells demonstrated that ethylene could be detected from multiple relevant sources.

Ethylene is one of the five major plant hormones and is intricately involved in complex signaling pathways controlling a disparate array of plant phenotypes. The expression of ethylene in the cell triggers many responses ranging from the regulation of cellular growth processes such as inhibition of cell division and leaf abscission to environmental stresses such as pathogen response and drought.[1] However, it is most commonly known as a ripening agent of climacteric fruit such as kiwi fruits, apples, tomatoes, mangos, and bananas. As a consequence of its essential role in multiple facets of plant growth, development, and post-harvest characteristics, a number of methods for ethylene detection have been developed. The primary means of ethylene detection rely on gas chromatography methods or laser based systems.[2] GC is the most prevalent method and can sensitively detect ethylene in the low parts per billion range. The ability to detect ethylene along with advancements in genetic approaches has led to an increased understanding of associated signaling pathways in fruit ripening over the past decades.[3] However, practical limitations imposed by many current methods have spurred interests in developing molecular approaches towards ethylene detection. An appropriately designed method for detecting ethylene in solution has the potential to allow for ethylene detection at the cellular level.

Plants detect ethylene with copper containing ethylene receptors (ETRs) that bind to the π bond of ethylene via interactions with metal *d* orbitals. Therefore it is not surprising that the few recently reported approaches towards detection of this small and unique phytochemical have utilized an interaction with transition metals as the basis for detection. The affinity of many late transition metals for π systems is well-described by the Dewar-Chatt-Duncanson model of bonding,[4] which coupled with the quenching effects of these metals on fluorescence is an attractive strategy for ethylene detection. Burstyn and coworkers developed a silver impregnated poly(vinyl phenyl ketone) (PVPK) film for sensing ethylene.[5] It is suggested that the Ag^+ ion is coordinated to both the carbonyl and aromatic ring, but upon exposure to ethylene the Ag^+-arene binding is disrupted resulting in a decrease in fluorescence. Esser and Swager used a copper scorpionate complex bound to a conjugated fluorescent polymer to detect ethylene.[6] Competitive coordination to ethylene disrupts the binding of the metal to the polymer, which results in an increase in fluorescence. The Swager group has also adapted this system to a chemoresistive sensor.[7] Kodera and coworkers used a Ag^+ ion bound by an N,S,S-macrocyclic ligand to interact with a pendant anthracene.[8] Upon binding of ethylene measureable changes were observed in the absorbance and fluorescence spectra of the anthracene moiety. While the coordination based approaches are attractive in terms of developing a reversible sensor for ethylene (Figure 1), these are not

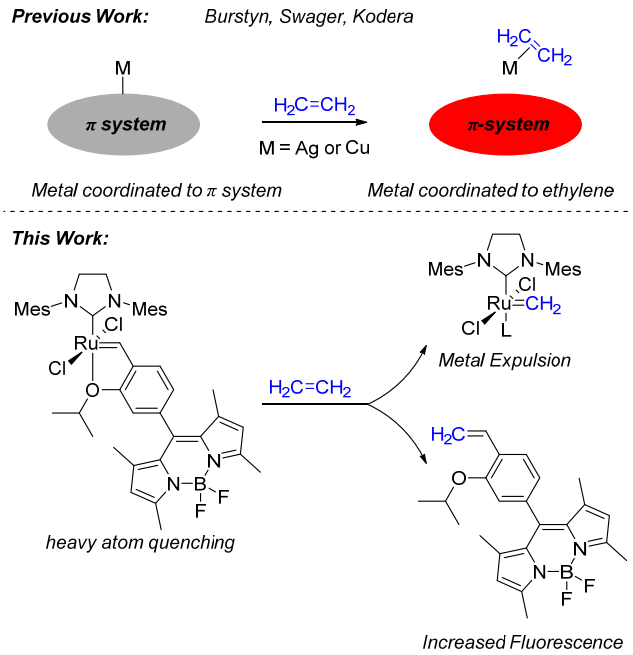


Figure 1. Molecular strategies for detection of ethylene.

viable for applications in more complex environments, such as a cell, due to either heterogeneity or a lack of selectivity. Small molecule fluorescent chemodosimeters provide exceptional spatial and temporal resolution, allow for better signal build up, and are well suited for potential application in biological systems.[9]

Most reaction-based fluorescent probes rely on a selective nucleophilic attack or oxidative transformations that are viable at room temperature. Such approaches have successfully resulted in small molecule fluorescent probes for reactive analytes such as H_2O_2, ClO^-, $O_2^{\cdot-}$, NO, $ONOO^-$, H_2S, formaldehyde, and biological thiols, amongst others.[9] However, ethylene is particularly challenging to detect as it is not suitably nucleophilic or otherwise reactive with other organic substrates under ambient or biological conditions. This was indeed noted by Burstyn and others leading to the use of transition metal-π interactions for ethylene detection. As such, we sought to utilize a selective reaction mediated by a transition metal to develop a fluorescent chemodosimeter for ethylene. After evaluating a number of strategies for selectively reacting with ethylene, olefin metathesis was explored as an intriguing approach.

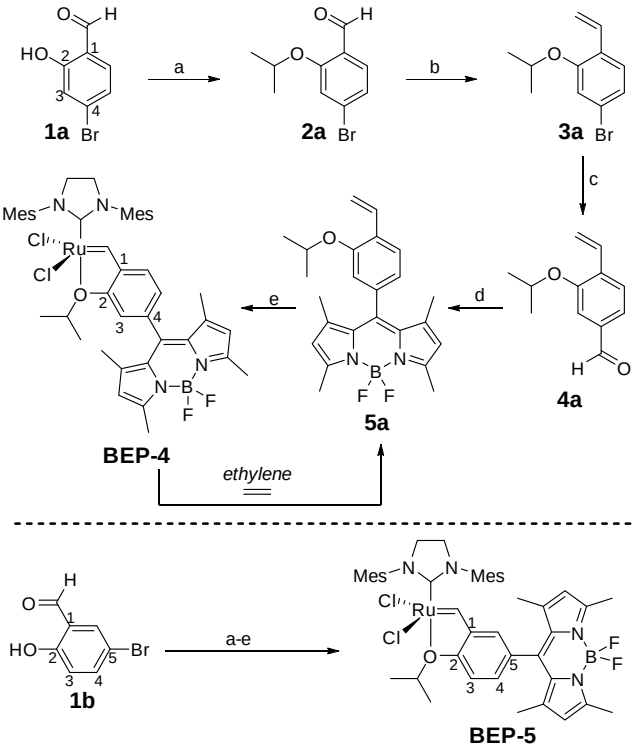
It is well-established that both steric and electronic effects influence the reactivity of olefins with metathesis catalysts.[10] Indeed it is useful to categorize olefin reactivity for predicting product outcomes of cross-metathesis reactions. These categories range from highly active type I olefins that undergo homodimerization rapidly such as ethylene and allyl alcohol to type IV olefins such as trisubstituted and electron poor olefins that do not participate in cross metathesis. This reactivity trend poises that a ruthenium catalyst should be selective for ethylene over other, more substituted alkenes.

We hypothesized that a Hoveyda-Grubbs 2nd generation type complex appended with a fluorophore would provide a sensitive and selective chemodosimeter for the detection of ethylene. The presence of a proximal heavy metal acts to quench fluorescence from the fluorophore providing the "off-state" and upon reacting with the analyte the metal is displaced resulting in an increase in fluorescence.[11] This approach utilizing the unique reactivity of a transition metal is in line with other methods used for challenging small molecule analytes including NO,[12] CO[13] and H_2S[14]. Indeed this metal-displacement approach is becoming a common strategy for chemodosimeter design.[15]

To evaluate this hypothesis, two BODIPY-based profluorescent probes, BEP-4 and BEP-5, were prepared (Scheme 1). Starting from 4-bromosalicylaldehyde **1a**, alkylation and Wittig olefination provided bromostyrene **3a**. Lithium-halogen exchange followed by a dimethylformamide quench provided aldehyde **4a**. Condensation of the aldehyde with 2,4-dimethylpyrrole followed by DDQ oxidation and chelation with BF_3 provided the penultimate BODIPY **5a**. Subsequent reaction with Grubbs 2nd generation catalyst provided the BODIPY Ethylene Probe-4 (BEP-4) (with numerical assignment arising from the position of the bromine in the initial starting material relative to the aldehyde). Additionally, to evaluate the influence of structural variation on both reactivity and selectivity the analogous BEP-5 was prepared from 5-bromosalicaldehyde **1b**.

Next BEP-4 and BEP-5 were optically characterized. As expected, the ruthenium containing complexes, BEP-4 and BEP-5 are weakly fluorescent in toluene (BEP-4: λ_{em} = 512 nm, Φ = 0.002; BEP-5: λ_{em} = 513 nm, Φ = 0.004. See SI figure S2) as compared to the styrene products of a reaction with ethylene **5a** and **5b** ((**5a**: λ_{em} = 514 nm, Φ = 0.24; **5b**: λ_{em} = 515 nm, Φ = 0.21. See SI figure S2). To evaluate the reactivity of the probes to ethylene both BEP-4 and BEP-5 were dissolved in toluene and exposed to a balloon of ethylene gas (Figure 2a and 2b). Over 60 min a 14-fold turn-on was observed for BEP-4 and 24-fold for BEP-5 at their relative λ_{em}. A maximum turn-on 113 is observed for BEP-4 and 78 for BEP-5. Interestingly, while BEP-4 shows a larger overall increase in fluorescence, it appears that BEP-5 reacts faster. For example after 1 h BEP-4 reaches 12% of its maximum, while BEP-5 is at 30% of maximum turn-on. While a number of other

Scheme 1. Synthesis and Reactivity of BEP-4 and BEP-5.



Conditions: (a) 2-iodopropane, K_2CO_3, Cs_2CO_3, DMF, RT, 8 h. (b) Ph_3PMeBr, *n*-BuLi, THF, -78 °C to RT to 30 °C 1 h, -78 °C add **2a** to RT 8 h (c) *n*-BuLi, THF, -78 °C to RT, -78 °C quench w/ DMF. (d) 2,4-dimethylpyrrole, 1 drop TFA, CH_2Cl_2, RT, 90 min; DDQ, 10 min; then Et_3N, $BF_3{\cdot}Et_2O$, RT 2 h. (e) Grubbs 2nd Gen., CuCl, DCM, 40 °C, 1 h.

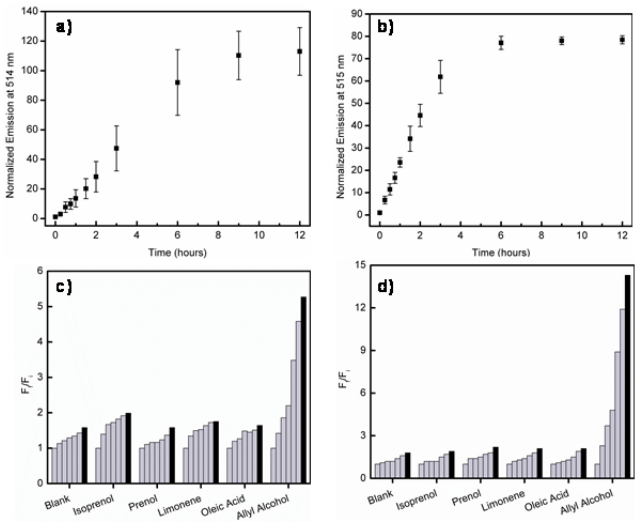


Figure 2. BEP-4 and BEP-5 optical characterization in toluene. Turn-on fluorescence response of 2 μM (a) BEP-4 or (b) BEP-5 under an atmosphere of ethylene. λ_{ex} =475 nm. Normalized emission at max λ_{em} vs time. Error bars represent SD at each time point from three experiments. . Fluorescence responses of 2 μM (c) BEP-4 or (d) BEP-5 to allyl alcohol and biologically relevant alkene species. Bars represent normalized integrated fluorescence intensity responses between 490

and 630 nm with λ_{ex} = 475 nm for the respective analytes (100 μM) at t = 0, 5, 10, 15, 30, 45, and 60 min (black).

solvents were evaluated, characterization was conducted in toluene due to lower vapor pressure and common use in olefin metathesis reactions. However, it should be noted that both probes respond to ethylene in an 80/20 mixture of water and acetone (Figure S3). Owing to the difficulty in accurately dosing small amounts of ethylene to a solution of probe, allyl alcohol was used as a fast-reacting monosubstituted type I alkene benchmark. In a typical experiment a 2 μM probe solution of BEP-4 or BEP-5 would be treated with 100 μM of isoprenol, prenol, limonene, oleic acid or allyl alcohol. This series of alkenes serves to represent a range of alkenes species to evaluate probe selectivity and extent of turn-on. We were delighted to observe the probe solutions exhibit a significant increase in florescence only when exposed to the type I alkene, allyl alcohol. Isoprenol, prenol, limonene, and oleic acid showed little to no turn-on as compared to the control sample. These results suggest that both BEP-4 and BEP-5 demonstrate promising selectivity for ethylene over other potential terpene and terpenoid compounds that would be present in plant samples.

Turn-on in florescence is presumably due to metathesis with ethylene or allyl alcohol and cleavage of the covalent connection between the BODIPY and the ruthenium producing the corresponding products **5a** and **5b**. To support this BEP-5 was reacted with an excess of ethylene to return the styrene **5b**. It should be noted that the control samples for BEP-4/5 demonstrate a slow background turn-on. Exposure of a BEP-4 solution to open atmosphere does result in isolable aldehyde product, which is also observed in the mass spectrum of the crude mixture. However, exclusion of moisture and oxygen does not appear to completely ablate this effect and while exposure to a long-wave handheld UV lamp does increase this background turn-on, carefully limiting exposure of the probes to light does not provide a completely stable background.

With these findings in mind we went on to evaluate the ability of the two probes to detect an environment of ethylene. This was done by injecting ethylene into an airtight cuvette via a gastight syringe and observing fluorescence intensity after 60 minutes (Figure 3). Using a series of different ethylene injections ranging from 5 μL to 500 μL resulted in a dose dependent increase of fluorescents as seen in figure 3. Limits of detection were calculated to be 29 μL for BEP-4 and 20 μL for BEP-5. This corresponds to 13 ppm and 9 ppm ethylene, respectively, in the headspace of the cuvette.

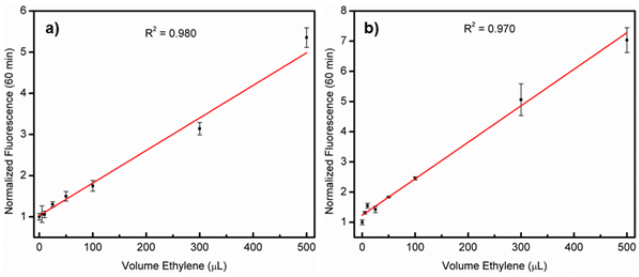


Figure 3. BEP-4 and BEP-5 in toluene show turn-on response to ethylene gas injected via gas tight syringe. Turn-on fluorescence response of 2 μM (a) BEP-4 or (b) BEP-5. λ_{ex} =475 nm, λ_{em} = 513 nm (BEP-4) and 514 nm (BEP-5). Endpoints represent normalized fluorescence 60 min after the addition of ethylene. Error bars denote SD.

Finally, we evaluated the ability of BEP-4 and BEP-5 to detect exogenous ethylene in live cells via confocal microscopy. Excitingly, both BEP-4 and BEP-5 were successful in detecting ethylene in a live cell environment with a variety of ethylene sources. HEK239T cells were exposed directly to ethylene gas or vapor from a ripe banana and mango. The cells were then treated with 2 μM BEP-5 and imaged. Ethylene gas resulted in a 74% increase in fluorescence and gas from a sealed chamber holding a ripe banana and mango resulted in a 61% increase in mean fluorescence compared to a control that was protected from ethylene exposure (see Figure S5 and S6). Additionally, BEP-4 and BEP-5 were able to detect ethylene from the ethylene releasing molecule ethephon, which undergoes hydrolysis to ethylene and is used as an agricultural ethylene source, in PC12 cells. Depending on concentration of probe used, BEP-4 displayed a 14-22% increase in mean fluorescence and BEP-5 showed a 25-31% increase in mean fluorescence relative to the appropriate control (See Figure S7). Two significant differences between mammalian cells and plants is the presence of a cell wall and chlorophyll, which can interfere in some imaging experiments. *Chlamydomonas reinhardtii,* represents a valuable model system as it has both a carbohydrate rich cell wall and chlorophyll. In the green algae *C. reinhardtii* BEP-4 was able to detect exogenous ethylene gas and ethylene from ripe fruit (Figure 4). BEP-5 was also able to detect exogenous ethylene gas, however no statistically significant turn-on was observed for ethylene from fruit (Figure S9). These results are especially exciting considering that other examples of olefin metathesis in biological systems often required significant efforts to design compatibility.[16] We hypothesize that the relatively hydrophobic nature of BEP-4/5 drives membrane diffusion and cellular accumulation. Additionally, the fact that these probes do not necessitate turnover of a catalytic cycle via a prone methylidene intermediate is a potential benefit, which may pose a challenge in other attempts to perform catalytic olefin metathesis in aqueous environments.[17]

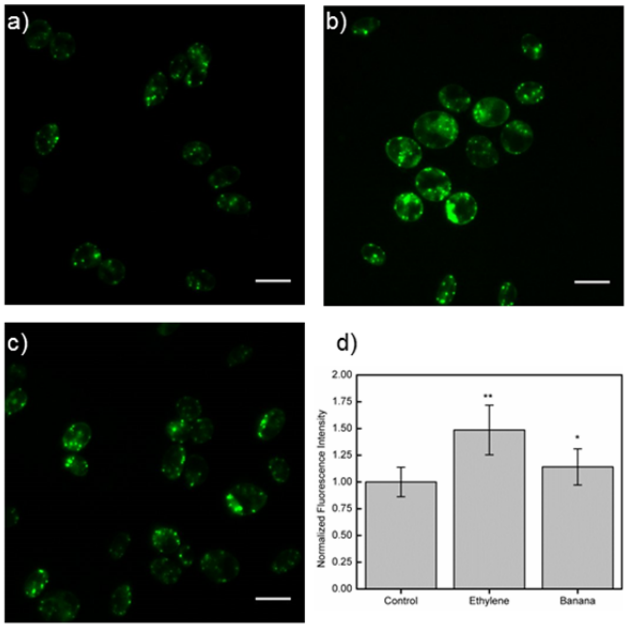


Figure 4. Confocal microscopy of BEP-4 in live *Chlamydomonas reinhardtii* cells. Pelleted cells were treated with 20 μM BEP-4 in PBS for 15 min at 37 °C, excess unreacted probe was removed, cells were resuspended, plated and imaged. (a) Control (b) cells diffused with ethylene gas for 15 min, media removed and cells were treated with probe as in the control (c) cells diffused with gas from banana for 1 h, media removed and cells were treated with probe as in control.. Scale bar represents 10 μm in all images. (d) Normalized mean fluorescence intensities of Chlamydomonas cells with each treatment. Error bars denote SD (n = 2). **p < 0.01. *p < 0.05.

In summary two new chemodosimeters based on Hoveyda-Grubbs 2nd generation catalysts were developed for the selective detection of

ethylene. The probes do not appear to react significantly with olefins that are not rapidly reacting type I olefins, however a steady increase in background fluorescence is observed. A dose dependent increase in fluorescence upon exposure to ethylene gas is observed. Slight structural variation provides some variance in fold turn-on and rate of reaction with ethylene. While these initial findings are promising, structure-activity studies to improve sensitivity by varying the ligand identities on Ru are being investigated. Both BEP-4 and BEP-5 were capable of detecting ethylene in a cellular environment. Efforts to apply these probes in plant cells, such as *Arabidopsis thaliana* are currently underway.

ASSOCIATED CONTENT

The supporting information is available free of charge on the ACS Publications website at DOI:
Experimental details, including synthesis and characterization, selectivity assays, spectroscopic methods, cellular imaging methods.

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Notes

The authors declare no competing financial interest.

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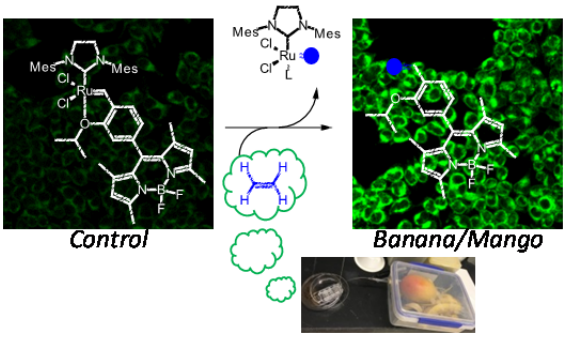
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