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Polymer-Bound Dirhodium Tetracarboxylate Films for Fluorescent Detection of Nitric Oxide

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A polymer-bound dirhodium complex, [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C–P)], was prepared via ligand exchange of [$Rh_2(O_2CCH_3)_4$] with the side chains of a methyl methacrylate/methacrylic acid copolymer (O_2C–P) followed by axial coordination of the fluorophore, *N*-dansylpiperazine (Ds-pip). Emission from Ds-pip is quenched when coordinated to the dirhodium complex but can be restored upon displacement by analytes. Exposure of [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C–P)] films to aqueous nitric oxide (NO) evokes a 2.2-fold increase in integrated emission. The polymer matrix excludes potentially interfering analytes including reactive oxygen or nitrogen species, which cannot readily permeate the film.

Introduction

Interest in nitric oxide (NO) biology has accelerated at a rapid pace. In the past four years alone, NO biology has received more attention than photosynthesis.[1] Despite this burgeoning fascination, sensors for the direct detection of NO under physiological conditions are noticeably scarce.[2,3] We have devoted considerable effort to developing and applying transition metal complex-based fluorescent turn-on sensors of NO.[4−11] Recently, we reported fluorescent sensors that directly detect NO in aqueous solution near[9] and at[11] physiological pH with nanomolar sensitivity, as well as in living cells,[11] albeit irreversibly. Sensors that can reversibly detect NO, and thus allow spatiotemporal resolution, have also been realized.[6] Dirhodium tetracarboxylates reversibly exchange axially coordinated fluorophores with NO with attendant emission enhancement (Scheme 1). Unfortunately, water can also displace these axial ligands with consequent fluorescence restoration, precluding direct use of this system in aqueous environments. Isolation of the sensor from an aqueous solution using a Silastic polymer permitted detection of aqueous NO.[6,12] The current report describes the preparation of a polymer-bound dirhodium tetracarboxylate designed to exclude water and other potentially interfering analytes. Films of this material detect NO under physiologically relevant conditions with unprecedented selectivity versus other reactive nitrogen species.

Scheme 1. Mechanism of Fluorescence Enhancement

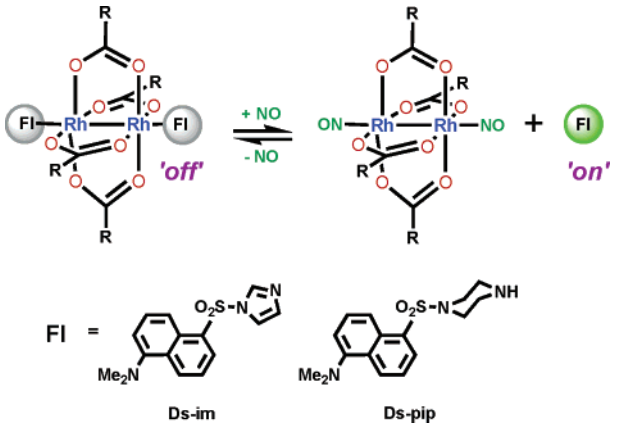

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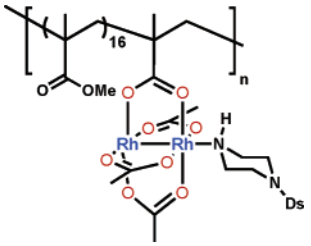
Chart 1. Structure of $[\{Rh_2(O_2CCH_3)_3(Ds\text{-}pip)\}_n(O_2C-P)]$



Results and Discussion

A number of polymer-bound dirhodium tetracarboxylates have been investigated.[13,14] In the current study, an easily prepared, biocompatible polymer with carboxylate side chains and transparency in the visible spectrum was sought. On the basis of these criteria, a copolymer of methyl methacrylate (MMA) and methacrylic acid (MAA) was selected. Poly(MMA-*co*-MAA) copolymers with controlled comonomer ratios are readily synthesized by radical-initiated polymerization. PMMA is transparent from 285 to 800 nm and can be cast as uniform films.[15] PMMA has also been used extensively in biological and vapor-phase sensing.[16−18] Furthermore, NO-permeable PMMA membranes have been adopted as components of commercial biological NO assay devices.[19] On the basis of the precedence for carboxylate exchange with $[Rh_2(O_2CCH_3)_4]$,[20−22] it was anticipated that carboxylate side chains of poly(MMA-*co*-MAA) could be exchanged with acetate groups of $[Rh_2(O_2CCH_3)_4]$ to afford the target 3:1 mixed carboxylate complex. A copolymer (O_2C-P) of 16:1 MMA/MAA was prepared by an established protocol,[15] and carboxylate exchange with $[Rh_2(O_2CCH_3)_4]$ was accomplished by refluxing in THF. The *N*-dansylpiperazine[6] (Ds-pip, Scheme 1) axial fluorophores were readily incorporated by stirring the resultant $[\{Rh_2(O_2CCH_3)_3\}_n(O_2C-P)]$ complex with 1 equiv of Ds-pip per dirhodium center and then precipitating $[\{Rh_2(O_2CCH_3)_3(Ds\text{-}pip)\}_n(O_2C-P)]$ (Chart 1) with diethyl ether. Integration of ^1H NMR spectral signals corresponding to O_2C-P, Ds-pip, and the acetate functionalities confirmed the desired stoichiometry (Chart 1).

Films of $[\{Rh_2(O_2CCH_3)_3(Ds\text{-}pip)\}_n(O_2C-P)]$ were drop-cast from 250 μL of a dichloromethane solution (3.88 mM in dirhodium) onto 10 mm × 43 mm PMMA substrates, followed by curing in the dark for 24 h. Reproducibility of film casting was monitored by comparing the emission spectra of nine films cast in this manner (Figure S1, Supporting Information). The standard deviation (±8%) in integrated fluorescence was close to instrument error (±6%). Homogeneity within a given film was assessed by comparing the integrated emission spectra from 11 points within a representative film (Figure S2). The standard deviation of these measurements (±6%) was within error. Visually, the films are transparent and purple in color. The films display good photostability with no change in emission intensity after 10 consecutive scans followed by 1.5 h of continuous irradiation at 395 nm. The films are stable to water for 4 h, and no fluorophore or complex leaches from the film, based on optical and fluorescence spectral examination of the solution. After 24 h, the films begin to degrade and the fluorescence begins to increase, and all subsequent measurements were therefore limited to a maximum of 2 h exposure to aqueous solution. Emission spectra did not change when performed under atmospheric conditions rather than anaerobically, indicating that O_2 exposure does not have any statistical significance on the emissive properties of the sensor.

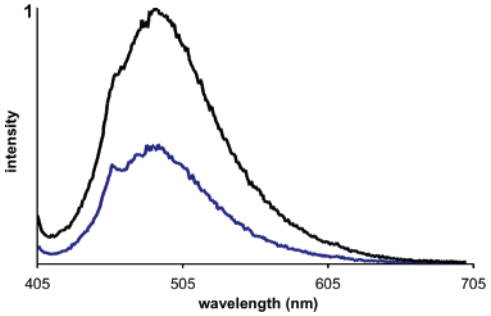


Figure 1. Emission from a film of $[\{Rh_2(O_2CCH_3)_3(Ds\text{-}pip)\}_n(O_2C-P)]$ immersed in buffer (50 mM HEPES, 100 mM KCl, pH = 7.4, blue trace) increases by 2.2-fold upon saturation of the solution with NO(g) (1.6 mM, 160 equiv, black trace).

When $[\{Rh_2(O_2CCH_3)_3(Ds\text{-}pip)\}_n(O_2C-P)]$ films immersed in 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH = 7.4) were exposed to excess NO(g) (250 μL added to yield a 1.6 mM saturated solution, corresponding to 160 equiv in solution) at 25 °C, an immediate 2.2-fold increase (average of three measurements, SD = 0.09) in integrated emission was observed (Figure 1). Purging with argon to remove NO and reverse this effect was only partially successful. Furthermore, the extent of reversibility was not the same for every film; a 50−77% return to the initial state was observed. This variability may be due to irregular morphology at the molecular level, perhaps hindering recoordination of Ds-pip. Ongoing system optimization will better define this behavior.

Although many studies have sought to probe the effects of nitric oxide in biology, the fundamental mechanism of NO transport remains to be elucidated.[23−25] Given the dangers of using NO(g) and the difficulties in removing higher nitrogen oxides from this gas,[26] many researchers opt instead

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to use nitrosothiols (RSNOs). However, the chemical details of "NO" delivery by these species have not been established.[27−32] Some researchers claim that RSNOs will effectively heterolyze along the S−N bond, directly transferring a nitrosonium (NO^+) fragment to another thiolate (termed nitrosation), whereas others contend that homolysis occurs to liberate 0.5 equiv of disulfide (RSSR) and NO, which reacts with another thiolate in an electron-releasing process (termed nitrosylation). The latter pathway requires a transition metal or reducing agent to facilitate homolytic decomposition of the nitrosothiol.[33,34] A metal center embedded in a polymer matrix should be inaccessible for direct nitrosation and not promote RSNO homolysis. Consequently, one would expect that when an NO sensor film is exposed to a solution of RSNO, the sensor will be unresponsive. Indeed, we find that exposing a film of [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] to 1.5 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP, in rigorously metal-free buffer) produces a very small turn-on after 1 h. Only a 30% increase in fluorescence was observed even after 2 h, indicating production of little NO, presumably due to slow thermal decomposition of SNAP. This result indicates that SNAP is unable to access the metal centers. Our film-based sensor therefore has the capacity to distinguish NO from nitrosothiols, a decided advantage for deconvoluting their respective biological functions.

Nitric oxide is just one RNS in a family of many. Peroxynitrite is one of the most highly oxidized forms of nitrogen and is often produced in vivo by rapid reaction of NO with superoxide. This RNS can sometimes produce false positives and is generally considered an interfering analyte for NO sensing. An ideal NO sensor should be able to discriminate higher nitrogen oxides from the desired analyte, nitric oxide. Exposure of a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$-(O_2C−P)] film to 2.4 mM 5-amino-3-(4-morpholino)-1,2,3-oxadiazolium chloride (SIN-1-Cl), a commercially available peroxynitrite donor,[35] afforded no change in integrated emission. Exposure to peroxynitrite itself (94 μM) leads to a slight (∼13%) decrease in integrated fluorescence emission, demonstrating that the film-encapsulated sensor will not produce a false turn-on response when peroxynitrite, rather than NO, is being generated.

Nitroxyl (HNO) may play an important role in biology, with a signaling pathway possibly orthogonal to that for nitric oxide.[36,37] Because many sensors require nitric oxide to function as a one-electron reducing agent to produce a fluorescence turn-on and HNO can serve as a one- or two-electron reducing agent, HNO could possibly produce spurious fluorescence turn-on in many of the NO sensors that have been detailed. Surprisingly, few of the other NO sensors to date have been screened for selectivity. To probe more thoroughly the possible orthogonality of the NO/HNO signaling pathways, sensors that are selective for one over the other are required. The [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$-(O_2C−P)] film initially displays no change in fluorescence when exposed to 1.4 mM Angeli's salt ($Na_2N_2O_3$), which decomposes rapidly at pH 7.4 to generate HNO. Even after 1 h, only a 12% decrease was noted. This result demonstrates that the sensor film responds to HNO much more slowly and in the opposite way than with NO. These RNS selectivity experiments strongly suggest that the [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] sensor film will selectively sense gaseous NO over other oxides of nitrogen and its functionally orthogonal cousin HNO, permitting highly detailed studies into the mechanisms of generation, transport, delivery, and action of NO(g) alone.

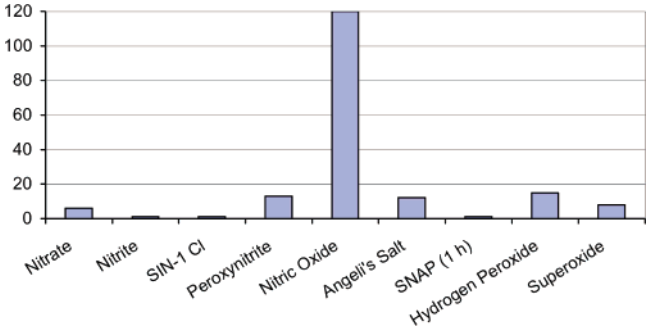

Figure 2. Absolute percent turn-on of film fluorescence in response to various reactive nitrogen species and reactive oxygen species after 1 h exposure time, compared to the immediate response to NO(g). Detailed procedures are provided in the Experimental Section, and individual fluorescence spectra associated with these data are provided in the Supporting Information.

Reactive oxygen species (ROS) are another subset of biological agents that could potentially interfere with fluorescent sensors relying upon fluorophore displacement from a transition metal. However, exposure of the films to hydrogen peroxide or superoxide in aqueous buffer lead to slight decreases of 15% and 8%, respectively, in integrated emission. To maximize the lifetime of superoxide (added in the form of KO_2) in solution, a concentrated DMSO stock solution stabilized with 18-crown-6[38] was prepared immediately prior to use. The total concentration of superoxide was limited to 10−100 μM in the stock solution and 100 nM in rigorously metal-free buffer. A comparison of the fluorescence response of films to all RNS and ROS investigated is shown in Figure 2. Individual fluorescence spectra for all of these measurements are given in the Supporting Information.

Conclusions

To the best of our knowledge, [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$-(O_2C−P)] is the first example of a solid-bound metal

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complex capable of turn-on fluorescent detection of NO under physiologically relevant conditions. Although the turn-on and reversibility of this system require improvement for some target applications, this study validates the potential of the film-based strategy utilizing this sensor composition. Encapsulation of the dirhodium complex shields the sensor from interference by restricting access of potentially interfering analytes (such as RNS, ROS, or water) and can distinguish NO from other common sources. Functional films such as those described herein are promising candidates for incorporation as components of fiber optical sensory devices for in vivo monitoring. Detailed characterization/optimization and practical application of these films would be of interest.

Experimental Section

SNAP, SIN-1-Cl, peroxynitrite (as a solution in 0.3 M NaOH), and $Na_2N_2O_3$ (Angeli's salt, AS) were purchased from Cayman Chemical Company. Peroxynitrite was stored at −80 °C and quantified by UV−vis spectroscopy immediately prior to use.[39] Potassium chloride (Puratronic, 99.997%) was purchased from Alfa Aesar, and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, free acid, Ultrapure Bioreagent) was purchased from J. T. Baker. Water was collected from a Milli-Q Biocel (Millipore, $\rho \geq 18.2$ MΩ). Buffer was prepared in freshly collected Millipore water (≥18.2 MΩ) with ultrapure bioreagent or electronic grade components and rendered transition metal-free by passage through a column of Chelex 100 resin. All reagents and chemicals were used as received. Ds-pip[6] and PMMA-*co*-MAA[40] were prepared by reported methods.

Synthesis of [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)]. A solution of polymer HO_2C−P (50 mg, 30 μmol MAA units) was heated to reflux for 16 h in 2 mL of THF under N_2 with [$Rh_2(OAc)_4$] (14 mg, 32 μmol). After the mixture cooled to room temperature, Ds-pip (13 mg, 41 μmol) was added and the solvent was removed under reduced pressure. The crude solid was then rinsed twice with diethyl ether to remove excess Ds-pip. An excess of the Ds-pip was necessary to prevent underloading observed under stoichiometric conditions. Drying the resultant pale violet solid in vacuo gave the desired rhodium-loaded polymer in 85% yield. ^{1}H NMR ($CDCl_3$): δ 8.75 (d, 1H, J = 9 Hz); 8.48 (d, 1H, J = 10 Hz); 8.3−8.2 (m, 2H); 7.7−7.5 (m, 2H); 3.61 (br s, 52H), 3.25 (br s, 6H); 2.1−1.7 (m, 28 H); 1.59 (br s, 9H); 1.5−1.1 (br m, 9H); 1.02 (br s, 16H); 0.85 (br s, 32H).

Selectivity Studies. SNAP (NO/NO^+ Donor). To a gastight cuvette containing a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film on one face was added solid SNAP (1.0 mg, 4.5 μmol) in an inert-atmosphere drybox. The cuvette was equipped with a septum cap and sealed, removed from the drybox, and a basal fluorescence scan was acquired. Under a positive nitrogen purge was added 3 mL of degassed buffer (50 mM HEPES, 100 mM KCl, pH 7.4) to afford a homogeneous solution ([SNAP]$_f$ = 1.5 mM), and a fluorescence scan was immediately acquired (blue trace, Figure S3). Further spectra were obtained after 1 (red trace, Figure S3) and 2 h (black trace, Figure S3). Only a 30% increase in fluorescence intensity was induced after 2 h.

SIN-1-Cl ($ONOO^-$ Donor). To a gastight cuvette containing a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film on one face was added solid SIN-1-Cl (1.5 mg, 7.3 μmol) in an inert-atmosphere drybox. The cuvette was equipped with a septum cap and sealed, cycled out of the drybox, and a basal fluorescence scan was acquired. Under a positive nitrogen purge was added 3 mL of degassed buffer (50 mM HEPES, 100 mM KCl, pH 7.4) to afford a homogeneous solution ([SIN-1-Cl]$_f$ = 2.4 mM), and a fluorescence scan was immediately acquired (red trace, Figure S4). Another spectrum was measured 1 h later (blue trace, Figure S4). No change in integrated emission was induced after 1 h.

Angeli's Salt (HNO Donor). To a gastight cuvette containing a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film in PMMA on one face was added solid Angeli's salt (0.5 mg, 4.1 μmol) in an inert-atmosphere drybox. The cuvette was equipped with a septum cap and sealed, the cuvette was cycled out of the drybox, and a basal fluorescence scan was acquired. Under a positive nitrogen purge was added 3 mL of degassed buffer (50 mM HEPES, 100 mM KCl, pH 7.4) to afford a homogeneous solution ([AS]$_f$ = 1.4 mM), and a fluorescence scan was immediately acquired (black trace, Figure S5). Another spectrum was acquired 1 h later (blue trace, Figure S5). A 12% decrease in integrated emission was induced after 1 h.

Peroxynitrite. To a quartz cuvette containing a [{$Rh_2(O_2CCH_3)_3$-(Ds-pip)}$_n$(O_2C−P)] film was added 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4), and a basal fluorescence spectrum was recorded (black trace, Figure S6). A 75 μL aliquot of freshly thawed peroxynitrite solution (3.76 mM in 0.3 M NaOH, previously stored at −80 °C) was added ([$ONOO^-$]$_f$ = 94 μM), and a fluorescence scan was immediately acquired. Another spectrum was acquired 1 h later (blue trace, Figure S6). A 13% decrease in integrated emission was induced after 1 h.

Nitrite. To a quartz cuvette containing a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film was added 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4), and a basal fluorescence scan was acquired (black trace, Figure S7). The buffer was removed via syringe and replaced with 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4) containing 10 mM $NaNO_2$, and a fluorescence scan was run. Another spectrum was recorded 1 h later (blue trace, Figure S7). No change in integrated emission was induced after 1 h.

Nitrate. To a quartz cuvette containing a [{$Rh_2(O_2CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film (diluted in PMMA) was added 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4), and a basal fluorescence scan was recorded (black trace, Figure S8). The buffer was removed via syringe and replaced with 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4) containing 10 mM $NaNO_3$, and a fluorescence scan was acquired. Another spectrum was run 1 h later (blue trace, Figure S8). No change in integrated emission was observed after 1 h.

Hydrogen Peroxide. To a quartz cuvette containing a [{$Rh_2(O_2$-$CCH_3)_3$(Ds-pip)}$_n$(O_2C−P)] film (diluted in PMMA) was added 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4), and a basal fluorescence scan was recorded (black trace, Figure S9). A 3.5 μL aliquot of 30% H_2O_2(aq) ([H_2O_2]$_f$ = 9.2 mM) was added, and a fluorescence scan was run (blue trace, Figure S9). A 15% decrease in integrated emission was observed after 1 h.

Superoxide. To a gastight cuvette containing a [{$Rh_2(O_2CCH_3)_3$-(Ds-pip)}$_n$(O_2C−P)] film (diluted 10-fold in PMMA versus other films) was added 3 mL of buffer (50 mM HEPES, 100 mM KCl, pH 7.4), and a basal fluorescence scan was recorded (black trace, Figure S10). Following the basal scan, 30 μL of a KO_2 solution in DMSO (100 μM, stabilized with 18-crown-6)[38] was added and a fluorescence scan was acquired (blue trace, Figure S10). An 8% decrease in integrated emission was induced after 1 h.

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Supporting Information Available: Results of film casting reproducibility/homogeneity and fluorescence spectra for selectivity studies with RNS and ROS. This material is available free of charge via the Internet at http://pubs.acs.org.

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