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TAML Activator-Based Amperometric Analytical Devices as Alternatives to Peroxidase Biosensors

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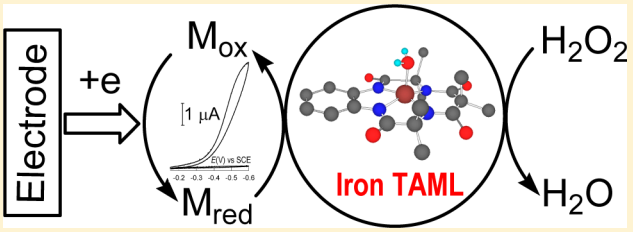
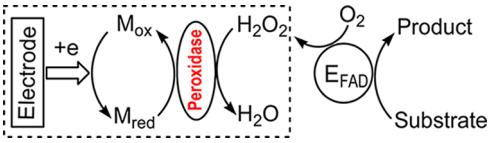
ABSTRACT: The ferric TAML catalysts $[Fe\{C_6H_2\text{-}1,2\text{-}(NCOCMe_2NCO)_2CMe_2\}(OH_2)]^-$ (**1**) with counterions Na^+ (**a**) and PPh_4^+ (**b**) function similar to horseradish peroxidase in the mediated electron transfer relays, which constitute a basis for amperometric biosensors. The mediators are mono- and bis-cyclometalated Ru and Os compounds of the type of $[M(C\sim N)_x(N\sim N)_{3-x}]^{m+}$ with x = 1 and 2 (N∼N = 2,2′-bipyridine, $^-$C∼N = 2-phenylpyridinato). Cyclic voltammograms of the Ru and Os compounds are not affected by **1a** though cathodic currents increase drastically in the presence of hydrogen peroxide. The reduction potentials of $[M(C\sim N)_x(N\sim N)_{3-x}]^{m+}$ complexes vary with both the nature of metal (Ru or Os) and the number of cyclometalated ligands x (1 or 2) and therefore the potential of working electrode can be set in the range of from −0.1 to +0.6 V versus the normal hydrogen electrode (NHE). A prototype of a biosensor for H_2O_2 is described, in which the **1b** catalyst and $[Os(C\sim N)_2(N\sim N)]^+$ mediator were coimmobilized on the surface of the glassy carbon electrode using a polymeric coating.

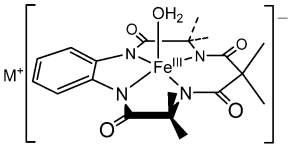


Peroxidases are heme-containing enzymes that catalyze the oxidation of, inter alia, a variety of aromatic amines and phenols by hydrogen peroxides.[1] Peroxidases efficiently recognize and activate H_2O_2 converting the chemical information of its presence into an electrochemical signal as shown in Scheme 1.[2] Peroxidases are key components of a myriad of amperometric biosensors.[3−7] Synthetic TAML activators (**1**) are ferric complexes of tetra-deprotonated tetraamide macrocyclic ligands (Figure 1) that effectively replicate peroxidase behavior in a variety of environmentally important oxidation processes.[8−10] In fact, TAML activators are at least competitive with peroxidase enzymes both in intimate mechanistic details of the corresponding catalytic cycles and in the functional reactivity in diverse applications[11−13] including colorimetric determination of H_2O_2 and glucose.[14] The relatively small peroxidase enzyme, horseradish peroxidase (HRP), enjoys extensive use in electrocatalytic sensing assemblies involving mediated electron transfer from an electrode to H_2O_2 as presented in Scheme 1. Here we show that TAML activators in combination with cyclometalated

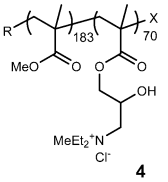
Scheme 1. Conceptual Scheme for Typical Peroxidase-Based Amperometric Biosensors with Mediated Electron Transfer[a]



[a]The box contains the H_2O_2 sensitive system where M_{ox} and M_{red} are the oxidized and reduced forms of the mediator, respectively. The entire scheme explains the sequence of events in any device that responds to a substrate in the presence of an effective oxidizing enzyme, commonly a FAD-dependent oxidase (E_{FAD}).



1, M = Na (**a**), PPh_4 (**b**)



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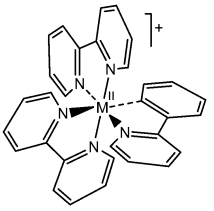
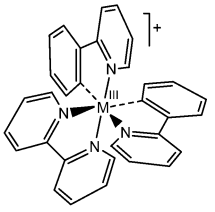


2, M = Os (**c**), Ru (**d**)



3, M = Os (**c**), Ru (**d**)

Figure 1. TAML activators (**1**), cyclometalated osmium (**2c**, **3c**) and ruthenium (**2d**, **3d**) mediators, and the cationic polymer (**4**) used in this work.

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ruthenium[15,16] and recently reported osmium[17] mediators provide effective behavioral substitutes for HRP-based amperometric sensors (Scheme 1).

RESULTS AND DISCUSSION

Key Observations. A cyclometalated analogue of **2d** with a 1,10-phenanthroline instead of a 2,2′-bipyridine ligand has previously been used as a chromophoric sensor of the activation of H_2O_2 by **1a**. Importantly, the **1a**-catalyzed rate of $Ru^{II} \rightarrow Ru^{III}$ oxidation by H_2O_2 does not depend on the concentration of the ruthenium electron donor over a broad pH range.[11] Thus, we were aware that the intermediates produced from **1** and H_2O_2 should oxidize complex **2d** fast. Moreover, even faster oxidations of the osmium(II) analogues (e.g., **2c** and **3c**) were anticipated based on the larger driving force (see values of reduction potentials in Table 1). Thus, the previous findings have driven us to the conclusion that compounds **1** could be efficient replicas of peroxidases in amperometric analytical assemblies. The results reported below confirmed the hypothesis.

Table 1. Rate Constants for the Reductions of HRP Compounds I (k_2) and II (k_3) and the Corresponding Fe^V and Fe^{IV} TAML Derivatives by Os^{II} and Ru^{II} Species 2 and 3[a]

		HR peroxidase		TAML **1a**	
complex	$E^{0\prime}$/V (NHE)	$k_2/M^{-1}s^{-1}$	$k_3/M^{-1}s^{-1}$	$k_2/M^{-1}s^{-1}$	$k_3/M^{-1}s^{-1}$
2c	0.23	2.0×10^6	1.1×10^6	1×10^4	7×10^3
3c	−0.095	6×10^8	4×10^7	1×10^8	1×10^6
2d	0.50	3.4×10^6	2.1×10^6	6×10^5	5×10^5
3d	−0.059	3×10^8	2.5×10^7	4×10^7	9×10^6

[a]For conditions for TAML and HRP experiments, see the legends to Figures 1 and 2, respectively.

The cyclic voltammogram of complex **3c** obtained in aqueous solution is shown in Figure 2. The pH-dependent rate of reaction between **1a** and H_2O_2 is at its maximum around pH 10,[11] so this pH was chosen for data collection. The cyclic voltammogram of **3c** was not altered by the addition of **1a** but the current increased dramatically when H_2O_2 (0.001 M) was also added. The data in Figure 2 confirms that **1a** behaves in this situation as an outstanding peroxidase mimic. This behavior can be rationalized by assuming that **1a** participates in the sequence of electrocatalytic events shown in the box of Scheme 1 with **1a** instead of peroxidase.

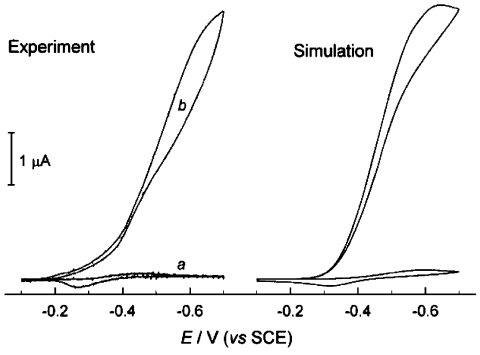

Figure 2. Experimental and simulated cyclic voltammograms of **3c** (1×10^{-4} M) and the TAML activator **1a** (1×10^{-6} M) in the absence (a) and in the presence (b) of H_2O_2 (1×10^{-3} M). Conditions: pH 10 (0.01 M phosphate), scan rate 2 mV s^{-1}, 21 ± 1 °C, background subtraction is applied.

The data in Figures 2 and 3 highlight the electrocatalytic mimicry by TAML activator **1a** of horseradish peroxidase. The cyclic voltammogram of **3c** in the presence of HRP/H_2O_2 (Figure 3) looks very similar to that measured in the presence of **1a**/H_2O_2 (Figure 2). A quantitative comparison of the abilities of **1a** and HRP to exchange electrons with **3c** was obtained by simulating these cyclic voltammograms, the simulations are also presented in Figures 2 and 3. The simulation of the HRP data was performed as described elsewhere[18] assuming the generally accepted mechanism of peroxidase electrocatalysis in the absence of complicating factors (Scheme 2).

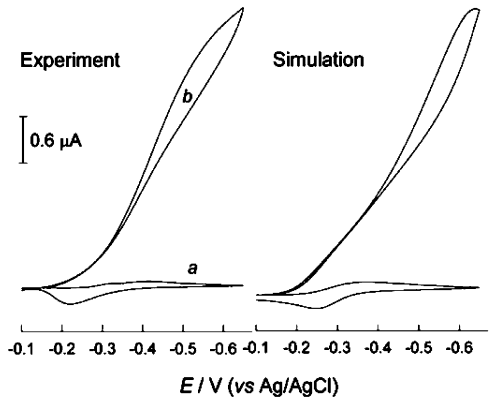

Figure 3. Experimental and simulated cyclic voltammograms of **3c** (0.2×10^{-4} M) obtained in the absence (a) and in the presence (b) of HRP/H_2O_2 (0.2 μM/1 mM): pH 7.6 (0.01 M phosphate); scan rate 10 mV s^{-1}; 21 ± 1 °C.

Scheme 2. Mechanism of Electrocatalysis by Horseradish Peroxidase or a TAML Activator Replacement[a]

$$L + H_2O_2 \rightarrow Fe^{IV}\text{-}(L^{\cdot+}) \text{ (C-I) or } Fe^{V}\text{-L } (k_1)$$

$$Fe^{IV}\text{-}(L^{\cdot+}) \text{ or } Fe^{V}\text{-L} + M^{II} \rightarrow Fe^{IV}\text{-L (C-II)} + M^{III} \ (k_2)$$

$$Fe^{IV}\text{-L} + M^{II} \rightarrow L + M^{III} \ (k_3)$$

$$M^{III} + e \rightarrow M^{II} \text{ (electrode)}$$

[a]L represent the resting states of the catalysts; C-I and C-II are the intermediate compounds I and II of HRP; M is Os or Ru. See text for details.

Figure 3 shows both voltammogram simulations using the DigiElch package of the experimental CVs measured either in the absence or presence of HRP. The rate constants k_2 and k_3 that were obtained by seeking the best visual match as described in the Experimental Section are presented in Table 1. The mechanism shown in Scheme 2 was also applied for simulation of the CV for **1a** (Figure 2). The mechanism involves reactive Fe^V and Fe^{IV} intermediates, which are reduced by the M^{II} metalacycles **2** and **3**. The formation of Fe^V and Fe^{IV} species from **1** and either H_2O_2 or ^{t}BuOOH has been discussed in detail in a recent mechanistic publication.[19] It is worth noting here that *m*-chloroperbenzoic acid converts **1b** into the

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monomeric iron(V)oxo species in organic nitriles[20] which performs a clean and very fast 2e oxidation of thioanisoles.[21] Mössbauer and EXAFS spectroscopies support the case that **1a** reacts with ^{t}BuOOH in water to give Fe^{IV} observable species,[22] a 1e oxidation performed by a 2-electron oxidant with the likely intermediacy of an iron(V)oxo complex that comproportionates rapidly with additional **1a**.[21] The chosen rate constants k_2 and k_3 that produce the best-looking facsimile of the experimental CV (Figure 2) are included in Table 1. Other reaction sequences were also tested for **1a** in the search for an optimal simulation of the CV in Figure 2. These included, for example, processes that assumed only a single reactive intermediate, which could be either Fe^{IV} or Fe^{V} (the software cannot distinguish these). None of the schemes explored were competent to deliver an acceptable match between the experimental and simulated voltammograms.

Comparison of the rate constants k_2 and k_3 for HRP and the peroxidase mimic **1a** obtained under the most favorable for each catalyst conditions confirms quantitatively that TAMLs are capable of competing with the natural enzyme. The rate constant k_2 for **3c** and HRP is 1 order of magnitude higher than that for **1a** if expressed in M^{-1} s^{-1}. The units are important because HRP is ~100 times heavier[1] than the synthetic catalyst. Therefore, the activity of **1a** in terms of k_2 exceeds that of HRP if the rate constants are recalculated on a per gram of catalyst basis. For k_3, the reactivity gap between HRP and **1a** in the case of **3c** is by a factor of 40 higher. Nevertheless, TAML still holds a significant lead over the enzyme based on weight.

Complexes **2** and **3** exhibit limited solubility in water including at pH 10 where the majority of experiments were conducted. Advantageously, **3c** or **3d** were found to absorb onto the surface of a glassy carbon electrode. The resulting surface-modified electrode exhibited a minimal electrochemical response when the potential was swept from −0.3 to −0.7 V vs SCE. However, when **1a** and H_2O_2 were both present in the electrolytic solution, a profound catalytic cathodic current was observed (Figure 4).

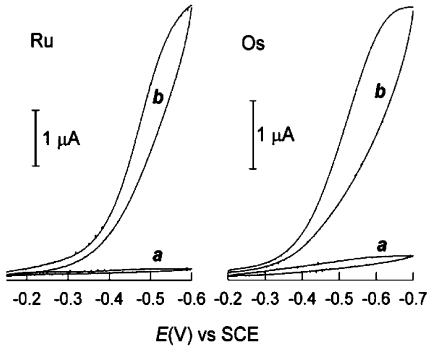

Figure 4. Cyclic voltammograms of structurally similar cyclometalated Ru (**3d**) and Os (**3c**) complexes absorbed on a surface of a glassy carbon electrode in the absence (a) and in the presence (b) of TAML activator **1a** (1×10^{-6} M) and H_2O_2 (1 mM). Conditions: pH 10 (0.01 M phosphate), scan rate 2 mV s^{-1}, 21 ± 1 °C.

Similar behavior is observed when the hydrophobic TAML **1b**, which is practically insoluble in water,[23] is first adsorbed on the electrode surface (data not shown) and the potential is swept with both a mediator (**2** or **3**) and H_2O_2 present in the electrolytic medium. The experiments could also be performed at pH 8 where the catalytic currents were found to be lower by ~30%, which agrees with the lower activity of the **1** TAML activators toward H_2O_2 at pH 8 vs pH 10.[11]

Biosensor Prototype. Both components **3c** and **1a** could be coimmobilized on a glassy carbon electrode surface in a positively charged polymer **4** as described in the Experimental Section. The polymer was selected in anticipation that its tetralkylammonium units would electrostatically hold within the matrix the anionic TAML catalyst **1a**, which exists as the bis-aqua $[FeL(H_2O)_2]^-$ species at pH below 10.[24] First **3c** was adsorbed from a solution in dichloromethane on the polished glassy carbon electrode surface as above. Then an ethanol/water solution of **4** and **1a** was dropped on the surface and allowed to evaporate to dryness. The experiments at different H_2O_2 concentrations were then performed using this surface modified electrode at pH 8 rather than at pH 10 in order to have a better comparison with a HRP biosensor (pH 7.4).[3] The data in Figure 5 show that the catalytic current at the modified

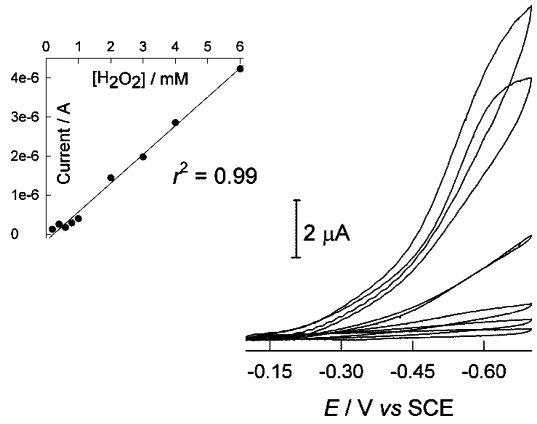

Figure 5. Cyclic voltammograms obtained using a modified polymer (**4**) glassy carbon electrode with immobilized **1a** and **3c** (see experimental details for preparation) with varying $[H_2O_2]$ 0.4, 1, 2, 4, and 6 mM and pH 8 (0.01 M phosphate) and 21 ± 1 °C. Inset shows the correlation between $[H_2O_2]$ and catalytic current at −0.7 V vs SCE.

electrode increases monotonically with increasing hydrogen peroxide concentrations. A linear relationship is observed between the catalytic current and $[H_2O_2]$ in the range of at least 0.4−6.0 mM with r^2 = 0.99 (inset to Figure 5). Multiple measurements revealed a good reproducibility in current readings. The same modified electrode can be reused after washing with HPLC grade water if kept dry. Unfortunately the polymer starts to decompose if kept in water or in the pH 8 buffer. The surface of fresh-prepared electrode is always plane and smooth though distinct pores are developed on the surface on storage. The limited hydrolytic stability of structurally similar polymers on solid supports has been reported.[25]

The electrode stability with time was evaluated by measuring catalytic currents at a constant H_2O_2 concentration. The electrode could be used for up to 20 h after depositing the polymer without any loss of activity. However, a 30−40% current decrease was observed after 24 h and degradation of the polymer was evident upon optical inspection. There was no evidence for the leakage of the **3c** mediator from the surface. Although the voltammograms become "noisy", there is no significant and systematic decrease in current measured in the absence of H_2O_2. Correspondingly, the noise and current decrease are likely due to a worsened electrical communication

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of participants within the damaged polymer though the leakage of TAML cannot be completely ruled out at a moment.

Compounds **1** are not oxidized by dioxygen in the aqueous medium,[23] and therefore the sensor is insensitive to the presence of O_2. Though organic hydroperoxides such as tertiary butyl and cumyl hydroperoxide react with **1** to afford reactive species, they are by factors of 65 and 130, respectively, less active than H_2O_2[26] and cannot be interfering factors particularly because they are not constituents of biological liquids and are not generated via catalysis by H_2O_2-producing oxidases.

CONCLUSIONS

This study shows that, at least with the cyclometalated Ru and Os mediators **2** and **3**, the TAML activators behave comparably to horseradish peroxidase to give a highly effective, non-optimized amperometric prototype of a sensor for hydrogen peroxide. The advantages of **1** over HRP are obviously those of cost and of practically indefinite storage stability under ambient conditions. The inorganic compound **1** does not have a surrounding protein globule and the well-known two-step inactivation mechanism of peroxidases, which involves protein unfolding and the loss of the heme group,[27] is not a limitation of **1**. Successful utilization of the TAML is a next important step in advancing the concept of minizymes, much smaller molecules than redox enzymes which efficiently function instead of the biocatalysts.[28] Note that the chemistry of TAML activators and HRP is mechanistically very similar, and replacement of the enzyme by the TAML activator could be performed preserving all other features of the design of the entire biosensor. In this respect, TAML activators have an obvious advantage compared to devices involving alternative minizymes such as Prussian Blue or Fe_3O_4 magnetic nanoparticles.[29−31]

EXPERIMENTAL SECTION

All reagents, components of buffer solutions, and solvents were at least ACS reagent grade and were used as received. TAML activator **1a** was obtained from GreenOx Catalysts, Inc. Compound **1b** was obtained as described elsewhere.[32] Cyclometalated ruthenium mediators **2d**[15] and **3d**[16] were obtained as previously reported. The osmium species **2c** and **3c** were prepared as described elsewhere.[17] The block copolymer **4** of molecular weight 20 kDa (obtained from ATRP Solutions, Inc.) was comprised of a larger hydrophobic poly(methyl methacrylate) segment and a shorter hydrophilic segment containing an ammonium group in each repeat unit. The block copolymer was prepared by sequential atom transfer radical polymerization (ATRP)[33] of methyl methacrylate and glycidyl methacrylate[34] followed by chemical modification of the repeat units derived from the latter monomer.[35] Electrochemical measurements were performed on a PC-interfaced potentiostat-galvanostat AUTOLAB PGSTAT 12. A three-electrode setup was used with a working glassy carbon electrode, with SCE or Ag/AgCl as a reference electrode, and with an auxiliary platinum electrode. Before each measurement, the working electrode was polished with diamond paste and rinsed with water and then with MeOH. The solution was purged with argon. Stock solutions of the catalyst were prepared in HPLC grade H_2O or MeCN. Hydrogen peroxide was standardized daily.[36] Electrodes with adsorbed species (either **1** or **2**/**3**) were prepared as follows. A drop of solution of a cyclometalated complex in CH_2Cl_2 (1 mM) was deposited on the previously polished electrode surface and the solvent was left to evaporate. TAML activator **1b** (1 mM in CH_2Cl_2) was then deposited similarly. Electrodes with both **1a** and **3c** incorporated into the polymeric matrix of **4** were made as follows. TAML activator (2.63 mg, 0.005 mmol) was dissolved in ethanol/water (90:10 v/v, 5 mL), and the solution was added to the polymer (100 mg, 0.005 mmol). The resulting solution was stirred for 2 h at 22 °C. If kept in the refrigerator, the solution can be used for up to 3 days. A drop of a CH_2Cl_2 solution of **3c** (1 mM) was deposited on the polished electrode surface, and after the solvent evaporated, a drop of the polymer/TAML mixture was deposited and the electrode was allowed to dry for 10−15 min. The modified electrode was kept dry in air. All simulations were carried out using a DigiElch 4.0 package from ElchSoft Simulation Software & Experience (Kleinromstedt, Germany).[37] The diffusion coefficients were taken as 1.6×10^{-6} cm^2 s^{-1} for **1a** (assumed value), 1.98×10^{-6} cm^2 s^{-1} for **3c**[17], and 1.6×10^{-5} cm^2 s^{-1} for H_2O_2.[38] The rate constant for the reaction between H_2O_2 and TAML (k_1) of 1.3×10^4 M^{-1} s^{-1} was used.[11] The electrode surface was taken as 0.0707 cm^2. The rate constant k_s = 0.002 cm s^{-1} and the transfer coefficient α = 0.15 were first found in simulation of separate cyclic voltammograms of **2** or **3** in the absence of H_2O_2 and **1a** and then were further used to simulate the cyclic voltammograms of **2** and **3** in the presence of HRP/H_2O_2 or **1a**/H_2O_2 by changing the rate constants k_2 and k_3 under inspection until the best visual match with the experimental CVs were achieved.

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Notes

The authors declare no competing financial interest.

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