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Fe^{III}–TAML activator: A potent peroxidase mimic for chemiluminescent determination of hydrogen peroxide

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ABSTRACT

Efforts to replace native peroxidase with its low molecular weight alternatives have stimulated a search for peroxidase mimetics. Herein we describe the oxidation of luminol with hydrogen peroxide catalyzed by commercially available Fe^{III}–TAML activator 1a, which was shown to be a more active catalyst than hemin. At Fe^{III}–TAML activator 1a use in chemiluminescent assay for H_2O_2 determination the detection limit value (3σ) of 5×10^{-8} M was similar to the detection limit obtained with horseradish peroxidase (1×10^{-7} M) and significantly lower than that obtained in the presence of hemin (6×10^{-7} M). The linear ranges ($R^2=0.98$) of the assay were 6×10^{-8}–1×10^{-6} M and 6×10^{-7}–1×10^{-6} M H_2O_2 for Fe^{III}–TAML 1a and hemin, respectively. The CV values for Fe^{III}–TAML 1a-based assay measured within the working range varied from 1.0% to 3.7% ($n=4$), whereas in the case of hemin −5.0% to 9.7% ($n=4$). Moreover, the sensitivity of Fe^{III}–TAML 1a-based method was 56 and 5 times higher than that of hemin- and HRP-based methods, respectively. The obtained results open good perspectives to apply Fe^{III}–TAML activator 1a in CL analytical methods instead of hemin, a traditionally used peroxidase mimetic.

1. Introduction

Chemiluminescent (CL) reactions attract an increasing attention of researchers working in the field of analytical chemistry. The main peculiarity of CL techniques is their high sensitivity: as the light signal is generated by a chemical reaction "in the dark," a low or negligible nonspecific signal is produced, thus resulting in a high signal-to-noise ratio.

The most applied CL reaction is the catalytic or non-catalytic oxidation of luminol with peroxides [1–4]. Luminol oxidation leads to the formation of a 3-aminophthalate ion in an excited state, which emits light when returning to the ground state. The emission spectrum shows a maximum at 425 nm [5]. Nowadays, this CL reaction is extensively used in various analytical techniques such as HPLC, enzyme immunoassay, CL resonance energy transfer assays, capillary electrophoresis, etc. [6–9].

Plant peroxidase isolated from different sources effectively catalyzes the luminol oxidation [10–12]. But the high cost of the native enzymes has stimulated a search for its alternatives. Some peroxidase mimetics [13–21] such as hemin, metal-containing porphyrins and phthalocyanines and Fe_3O_4, CuO, TiO_2 and Ag nanoparticles showed a peroxidase-like activity in this CL reaction. Moreover, peroxidase mimetics have higher thermal stability than the native enzyme.

Previously Fe^{III}–TAML activators, a family of low-molecular weight potent catalysts for the oxidation of a wide spectrum of organic compounds have been reported [22]. These iron catalysts contain central ferric iron coordinated to the cavity of a tetraamidomacrocyclic ligand (TAML). Some physico-chemical and catalytic properties of Fe^{III}–TAML activators were already studied [23–25]. Presently one of Fe^{III}–TAML activators named Fe^{III}–TAML activator 1a (**1a**, Fig. 1) is commercially available. This catalyst has shown an extremely high peroxidase-like activity in degradation of polychlorophenols, thiophosphate pesticides and nitrophenols, azo dyes, dibenzothiophenes, as well as an anthrax surrogate and natural or synthetic estrogens [26–31]. Recently TAML activators have been successfully applied to construct amperometric biosensors [32].

Herein we describe the oxidation of luminol with hydrogen peroxide catalyzed by commercially available **1a**. The determination of the kinetic constants of luminol oxidation allowed a comparison of the catalytic abilities of **1a** with hemin, traditionally used peroxidase mimetic. Using **1a**-catalyzed oxidation of luminol a sensitive CL method for the determination of hydrogen peroxide was developed.

2. Experimental

2.1. Materials

Fe^{III}–TAML activator **1a** was purchased from GreenOx Catalysts (USA). Luminol, hemin, horseradish peroxidase (HRP, RZ 3.0) and Trizma (Tris) were from Sigma Chemical Co. (USA), H_2O_2 (30%) – from

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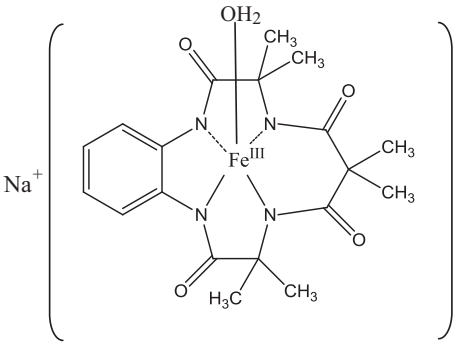

Fig. 1. Chemical structure of Fe^{III}–TAML activator 1a.

ChemMed (Russia). The concentration of H_2O_2 was estimated by measuring absorbance using $\varepsilon_{240}=43.6$ [33].

2.2. pH-dependence of Fe^{III}–TAML activator ***1a***-catalyzed oxidation of luminol

pH-dependence of **1a**-catalyzed oxidation of luminol was assayed as follows: 275 μl of 20 mM carbonate-Tris buffer, pH 9.0–10.5, containing 0.11 mM hydrogen peroxide and 3.3 μM luminol, were mixed with 25 μl of **1a** solution (1.2×10^{-7} M) in wells of black polystyrene plates (MaxiSorp, NUNC, Denmark) for CL enzyme immunoassay. CL kinetics was measured at room temperature on a luminometer Zenyth 3100 (Anthos, Austria). The chemiluminescent signal in the absence of **1a** was taken as background value. The light intensity was expressed in relative luminescence units (RLU).

2.3. Dependence of Tris concentration on ***1a***-catalyzed chemiluminescence

Effect of Tris concentration in the reaction medium on CL intensity formed upon the luminol oxidation in the presence and absence of Fe^{III}–TAML activator **1a** was assayed as follows: 275 μl of 21.8 mM carbonate buffer, pH 9.9, containing 0–200 mM Tris, 0.11 mM hydrogen peroxide and 3.3 μM luminol, were mixed with 25 μl of **1a** solution (1.2×10^{-7} M) in wells of black polystyrene plates.

2.4. Determination of kinetic constants of mimetics-catalyzed oxidation of luminol

All kinetic measurements were performed at 25 °C using a Shimadzu UV-2401PC spectrophotometer equipped with a thermostatted cell holder. Stock solutions of **1a** (1.5 mM) and luminol (100 mM) were prepared in acetonitrile and 0.4 M NaOH, respectively. The catalytic luminol oxidation reactions were carried out in 20 mM carbonate, pH 9.9 with 50 mM Tris. Initial rates of luminol oxidation were calculated from the linear absorbance versus time plots using the differential coefficient of extinction (349 nm) for luminol of 5900 M^{-1} cm^{-1}, when the conversion of the luminol did not exceed 10%. A typical kinetic run was performed as follows: appropriate amounts of the stock solutions of **1a** and luminol were added in a cuvette with the carbonate buffer. To initiate the oxidation a required amount of the stock solution of H_2O_2 was added to a 1 ml reaction mixture in a cuvette. Calculations of the rate constants were carried out using a SigmaPlot 8.0.

2.5. Spectral study of the catalytic oxidation of luminol

Spectral changes upon the luminol oxidation catalyzed by **1a** were studied in 20 mM carbonate buffer, pH 9.9 containing 50 mM Tris, 25 μM luminol, 100 μM H_2O_2, 0.1 μM **1a**; the spectra were recorded with 1 min at 25 °C.

2.6. Chemiluminescent assay for the determination of hydrogen peroxide

Chemiluminescent determination of hydrogen peroxide was carried out as follows: 100 μl of 81 μM luminol was added to 100 μl of 0.018–18 μM H_2O_2 in wells of black polystyrene plates. Then the catalytic reaction was initiated by adding 100 μl of **1a** solution. The final concentration of the catalyst in plate wells was 10^{-8} M. All solutions were prepared in 20 mM carbonate buffer, containing 50 mM Tris, pH 9.9. CL determination of H_2O_2 using hemin as a catalyst was carried out under the same conditions. CL intensity was measured at room temperature on a luminometer SpectraMax L (USA).

Peroxidase-based determination of H_2O_2 was carried out as follows: to initiate the indicator reaction HRP (1.8×10^{-10} M) was added to 60 mM Tris buffer, pH 8.3 containing 1.5 mM luminol and 0.01–1 μM H_2O_2. The experimental conditions in chemiluminescent HRP assay were optimized using full (2^4) factorial design.

3. Results and discussion

3.1. Choice of the optimal conditions to perform ***1a***-catalyzed oxidation of luminol

As described previously, **1a** shows its catalytic activity under alkaline conditions [34]. To optimize the conditions of **1a**-catalyzed luminol oxidation a dependence of CL intensity as a function of pH was determined. It should be noted that the composition of the buffer containing both Tris and sodium carbonate was picked out to widen its pH range (9–10.5). As seen in Fig. 2, the highest catalytic activity of **1a** was at pH 9.9.

Since the introduction of Tris having a pK_a of 8.07 at 25 °C does not affect the buffer capacity at pH 9.9, we decided to remove this compound from buffer composition. Unexpectedly, such change in the buffer composition resulted in a decrease of the activity of Fe^{III}–TAML activator **1a** (Fig. 3). At varying the Tris concentration it was shown that **1a** possessed the maximum activity in carbonate buffer, pH 9.9 containing 50 mM Tris.

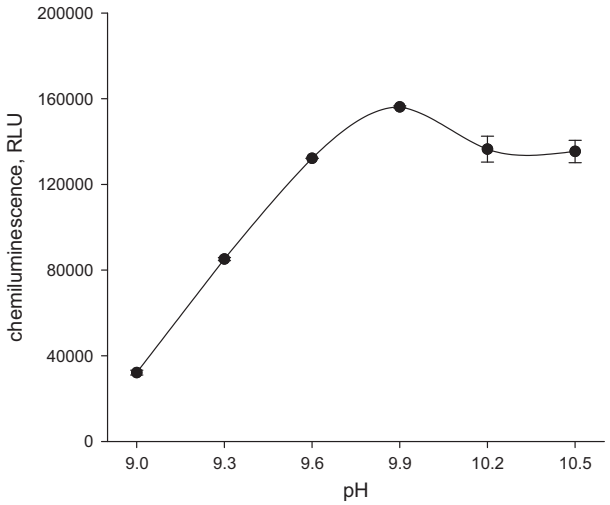

Fig. 2. pH-dependence of chemiluminescence intensity formed upon the luminol oxidation in the presence of Fe^{III}–TAML activator **1a**. Conditions: 20 mM Tris-carbonate buffer, [**1a**]=10 nM, [luminol]=3 μM, [H_2O_2]=100 μM.

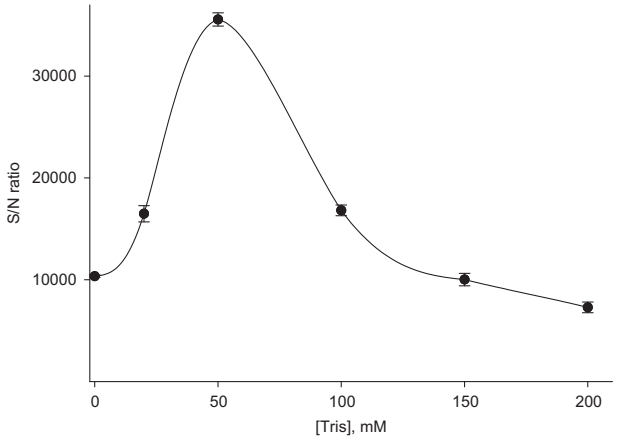

Fig. 3. Effect of Tris concentration in the reaction medium on a ratio of CL intensity formed upon the luminol oxidation in the presence and absence of Fe^{III}–TAML activator 1a (S/N). Conditions: 20 mM carbonate buffer, pH 9.9, [**1a**]=10 nM, [luminol]=3 μM, [H_2O_2]=100 μM.

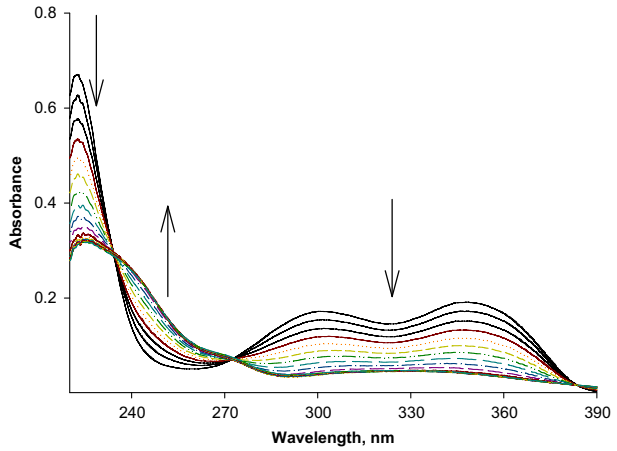

Fig. 4. Spectral changes upon the luminol oxidation catalyzed by Fe^{III}–TAML activator 1a. Conditions: 20 mM carbonate buffer, pH 9.9 with 50 mM Tris, [luminol]=25 μM, [H_2O_2]=100 μM, [**1a**]=0.1 μM, 25 °C, spectra recorded with 1 min. Arrows indicate a change of time upon spectra recording.

3.2. Catalytic activity of *1a* in luminol oxidation

It should be noted that under the favorable conditions even at relatively low concentrations of **1a** the high CL intensities were observed. To monitor this reaction an electronic spectroscopy was used. Luminol soluble in the used buffer has absorption bands with maxima at 223, 300 and 349 nm (Fig. 4). The main oxidation trend is a disappearance of peak at 349 nm. The complete oxidation of luminol allowed a calculation of differential coefficient of extinction value (ε_{349}), which was 5900 M^{-1} cm^{-1}.

Hydrogen peroxide itself, in the absence of the catalyst, oxidizes luminol very slowly; Fe^{III}–TAML activator **1a** increases the rate immensely. The spectral changes observed during **1a**-catalyzed oxidation of luminol are shown in Fig. 4. Upon the catalytic oxidation of luminol two isosbestic points at 235 and 272 nm appeared. This assumes that in the reaction solution there are just two absorbing species: luminol and its oxidized derivative (3-aminophthalate).

To evaluate the catalytic efficiency of **1a** we studied the effect of the concentrations of luminol and H_2O_2 on an initial rate of luminol oxidation. The data obtained under the favorable conditions were consistent with the catalysis mechanism proposed

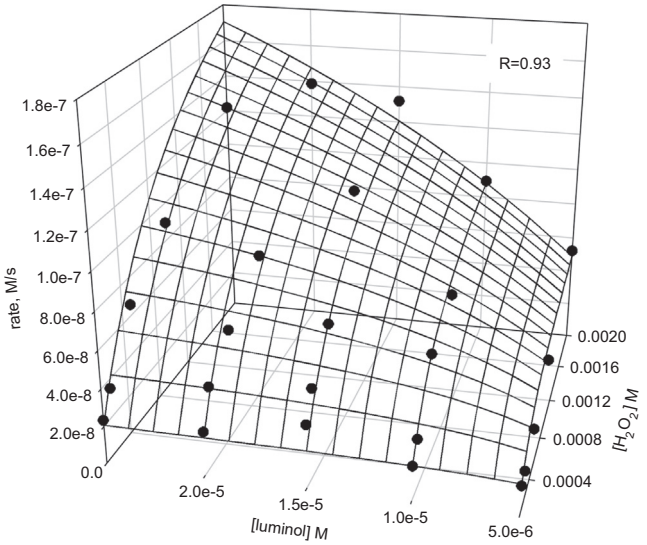

Fig. 5. Effect of concentrations of luminol and hydrogen peroxide in the reaction solution on initial rate of Fe^{III}–TAML activator 1a-catalyzed oxidation of luminol. Conditions: 20 mM carbonate buffer, pH 9.9, [**1a**]=10 nM.

previously by Collins et al. [28]

$$Fe^{III}-TAML+H_2O_2 \xrightarrow{k_I} \text{oxidized TAML}$$

$$\text{oxidized TAML}+\text{luminol} \xrightarrow{k_{II}} Fe^{III}-TAML+\text{product}$$

Under steady state conditions it leads to

$$-\frac{d[\mathbf{luminol}]}{dt}=\frac{kIkII[\mathbf{1a}][\mathbf{peroxide}][\mathbf{luminol}]}{kI[\mathbf{peroxide}]+kII[\mathbf{luminol}]} \quad (1)$$

The initial rate values have been fitted to Eq. (1) (Fig. 5) to calculate the reaction rate constants k_I and k_{II} of $(2.2\pm0.3)\times10^3$ and $(1.1\pm0.3)\times10^5$ M^{-1} s^{-1}, respectively. The k_I value obtained in this work was similar to that determined previously (1.9×10^3 M^{-1} s^{-1}) [28]. Comparison of the k_{II} value calculated for the catalytic oxidation of luminol and the k_{II} values for Orange II [28] and pinacyanol chloride [34] equal to 3.8×10^4 and 1×10^5 M^{-1} s^{-1}, respectively, demonstrated that **1a** catalyzes the oxidation of luminol with the same efficiency as the oxidation of the compounds studied previously.

As mentioned above, the CL intensity formed upon **1a**-catalyzed luminol oxidation in the absence of Tris in the reaction solution was significantly lower than that in the presence of 50 mM Tris (Fig. 3). To understand the reasons of this phenomenon the kinetic constants of luminol oxidation in the absence of Tris were determined. In this case the rate constants k_I and k_{II} were $(2.5\pm0.3)\times10^2$ and $(1.3\pm0.3)\times10^5$ M^{-1} s^{-1}, respectively. Comparison of the k_I and k_{II} values estimated in the presence and absence of Tris showed that the introduction of Tris in the reaction solution did not affect the reaction rate of the oxidized **1a** and luminol, but significantly improved the efficiency of the reaction of **1a** and H_2O_2.

One of reasons for the k_I value change may be a nonspecific binding between luminol and **1a** in the absence of Tris which is accompanied by **1a** inhibition. Similar effect was reported previously upon **1a**-catalyzed degradation of pinacyanol chloride [33]. At the addition of Tris to the reaction solution it may replace luminol in **1a**-luminol complex and prevent the substrate inhibition. Another reason for the k_{II} value change may be connected with that at the introduction of Tris the aqua ligand of **1a** (Fig. 1) may be replaced with Tris, which nitrogen atom reacts with Fe^{III} of **1a**. The similar bond exists in horseradish peroxidase, where Fe^{III} of hemin interacts with a nitrogen atom of His170 [35,36]. Since

the activity of the peroxidase is significantly higher than that of hemin itself, we assume that the interaction of **1a** and Tris explains the observed effect of **1a** activation in the presence of Tris.

Using the same kinetic approach the reaction rate constants (k_I and k_{II}) for hemin-catalyzed luminol oxidation were also evaluated. In the case of hemin catalysis the values of k_I and k_{II} were equal to 2.6 ± 1.0 and $(7.0 \pm 0.3) \times 10^2$ M^{-1} s^{-1}, respectively. Comparison of the obtained constants showed that in aqueous medium hemin itself and its oxidized form had much less reactivity in the luminol oxidation as compared to **1a**.

3.3. Chemiluminescent assay for the determination of hydrogen peroxide

To demonstrate possibilities of **1a** use in analytical practice we developed a CL assay to determine H_2O_2 concentration based on the catalytic oxidation of luminol. The determination of hydrogen peroxide was carried out in the carbonate buffer containing 27 μM luminol. This concentration was chosen as optimal, because at lower luminol concentration the CL intensity was less, whereas further increasing concentration of luminol did not change CL intensity (Fig. 6).

The calibration curves for the quantification of hydrogen peroxide using CL assay with the peroxidase mimetics are shown in Fig. 7. The detection limit value (3σ) obtained at **1a** use (curve 1) was 5×10^{-8} M. At use of hemin (control mimetic, curve 2) the detection limit value was significantly higher and equal to 6×10^{-7} M. As seen in Fig. 7, the linear ranges ($R^2 = 0.98$) of the assay were 6×10^{-8}–1×10^{-6} M and 6×10^{-7}–1×10^{-6} M H_2O_2 for **1a** and hemin, respectively. The values of coefficient of variation (CV) for **1a**-based assay measured within the working range varied from 1.0% to 3.7% ($n = 4$), whereas in the case of hemin −5.0% to 9.7% ($n = 4$). Moreover, the sensitivity of **1a**-based method (a value of curve slope in linear range) was 56 times higher than that of hemin-based method.

As mentioned above, the main task of this work was to find a catalyst which could replace native peroxidase in the reaction of luminol oxidation and be applied in analytical methods based on this reaction. By this, we compared the calibration curves for H_2O_2 determination obtained in the presence of **1a** and HRP (Fig. 7, curves 1 and 3). The detection limit value obtained at HRP use was 1×10^{-7} M, i.e. similar to that for **1a**. At the same time, under used experimental conditions the replacement of the peroxidase with **1a** led to 5-fold increase of the assay sensitivity. However, it should be noted that HRP concentration used in the reaction solution was

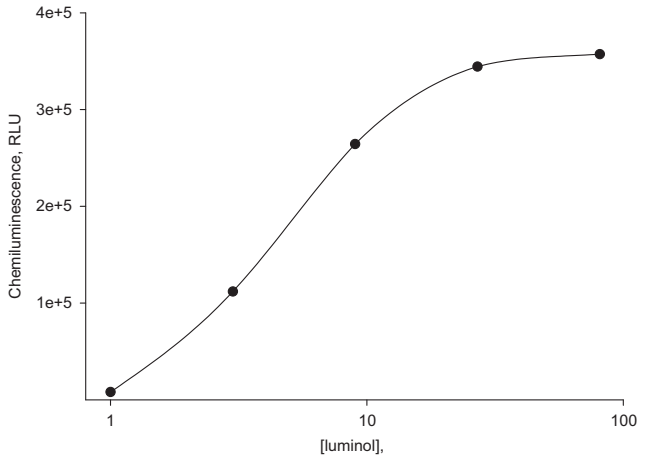

Fig. 6. Effect of luminol concentration in the reaction medium on CL intensity formed upon the luminol oxidation in the presence of Fe^{III}–TAML activator 1a. Conditions: 20 mM carbonate buffer, pH 9.9, [**1a**] = 10 nM, [H_2O_2] = 1.2 μM.

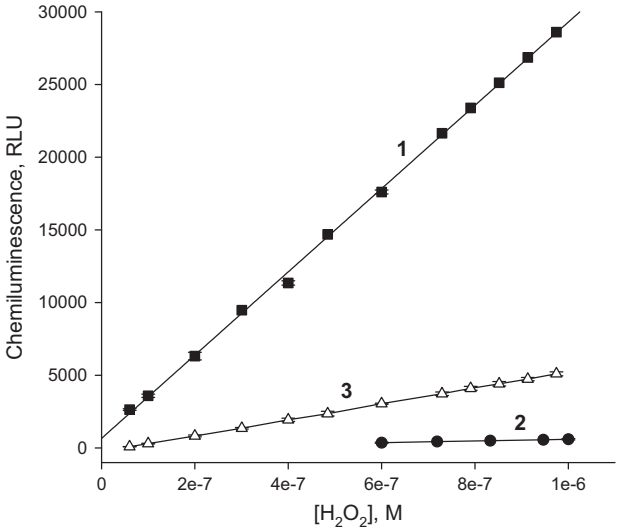

Fig. 7. Calibration curves for the chemiluminescent determination of hydrogen peroxide using the luminol oxidation catalyzed by Fe^{III}–TAML activator 1a (1), hemin (2) and horseradish peroxidase (3). Conditions for the assay with Fe^{III}–TAML activator 1a and hemin: 20 mM carbonate buffer, pH 9.9 with 50 mM Tris, [luminol] = 27 μM, [**1a**] = [hemin] = 10 nM. Conditions for HRP-based assay: 60 mM Tris buffer, pH 8.3, [luminol] = 1.5 mM, [HRP] = 0.18 nM.

55 times lower than that of **1a**. Therefore, the catalytic activity of HRP is 10-fold higher than that of **1a**. Taking into account that the cost of **1a** is 15 times lower than the HRP cost as well as its higher stability we can conclude that **1a** has good perspectives of use in CL analytical methods.

4. Conclusions

In order to replace native peroxidase in CL assays with its low molecular weight alternatives a commercially available Fe^{III}–TAML activator **1a** was first proposed. The obtained results demonstrated that **1a** is a potent catalyst in CL oxidation of luminol. The experimental conditions of the reaction of interest were optimized. The determination of the kinetic constants of luminol oxidation under favorable conditions allowed demonstrating advantages of Fe^{III}–TAML activator **1a** over hemin, a commonly used peroxidase mimetic. To demonstrate possibilities of **1a** use in analytical practice we developed CL assay based on the catalytic oxidation of luminol to determine hydrogen peroxide. The obtained results demonstrated that the assay with **1a** use has higher sensitivity and accuracy and lower detection limit than that with hemin use and may be successfully used in CL determination of hydrogen peroxide as HRP substitute.

Acknowledgments

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