Journal of Fluorescence, Vol. 15, No. 5, September 2005 (© 2005)
DOI: 10.1007/s10895-005-2984-6

Nonenzymatic Direct Assay of Hydrogen Peroxide at Neutral pH Using the Eu_3Tc Fluorescent Probe

Axel Dürkop[1,2] and Otto S. Wolfbeis[1]

Received June 13, 2005; accepted July 17, 2005

A detailed study is presented on the use of an easily accessible probe (the europium–tetracycline 3:1 complex; referred to as Eu_3Tc) for determination of hydrogen peroxide (HP). Eu_3Tc undergoes a 15-fold increase in luminescence intensity on exposure to an excess of HP. Data are given on the time dependence of the reaction, on the pH dependence of the absorption and emission spectra of both the probe and its complex with HP, and on the effect of stoichiometry between Eu^{3+} and Tc on selectivity and signal change. HP can be quantified in aqueous solution of pH 6.9 over a 2–400 μM concentration range with a limit of detection of 960 nM. The assay is validated using standard additions, and mean recoveries are found to be between 97.0 and 101.8%. Species that interfere in concentrations below 1 mM include phosphate, copper(II), fluoride and citrate. The addition of detergents causes the response curves towards HP to shift to higher HP concentrations. The method is critically assessed with respect to other common optical methods for determination of HP.

KEY WORDS: Hydrogen peroxide; europium; tetracycline; luminescence assay.

INTRODUCTION

Methods for determination of hydrogen peroxide (HP) are very often applied to analyze environmental, biochemical or clinical samples [1,2]. HP also is widely used in industry in fairly large (>1%) concentrations. In addition, HP is the product of the activity of oxidative enzymes [3]. These, but also their substrates, inhibitors and activators may be assayed *via* measurement of the amount of HP formed [4]. Optical methods for the detection of HP include chemiluminescence (CL) [5,6], electroluminescence [6] and the formation of fluorescent dimers in presence of peroxidase [7,8]. The fluorescent dimers are produced by reacting HP with certain phenols when peroxidase is present. Among those, the terbium-based detection scheme reported by Karst *et al*. [9] is particularly intriguing since it displays the unique optical features of a luminescent lanthanide probe, namely large Stokes' shift and long decay time.

Complexes of the antibiotic tetracycline (Tc) with metal ions (including lanthanides) have been subject of extensive work [10–13] related to the quantitation of Tc and its derivatives in body fluids, to their toxicity and complexation constants. Complexes of Tc with Eu^{3+} have been used for extremely sensitive fluorescent detection of Tc in various matrices. Prototropic dissociation constants and formation constants of the complexes are mostly known and strongly depend on pH [14–17]. We were stimulated by a recent report by Rakicioglu and Schulman [16] who showed that the addition of HP to EuTc causes a large increase of the Tc-sensitized luminescence intensity of the $^5D_0 \rightarrow {}^7F_2$ transition (at 616 nm) of the Eu^{3+} ion. The effect was ascribed to an oxidation process and used to determine tetracycline with a 10-fold lower limit of detection than obtained with previous methods. Luminescence enhancement of complexes of EuTc on going from a 1:1 to a >10:1 stoichiometry of Eu^{3+}/Tc was reported also to occur on addition of surfactants [17].

We have shown that this effect is not due to a redox reaction, but rather to a reversible binding process in

[1] Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Universitätsstraße 31, 93040 Regensburg, Germany.
[2] To whom correspondence should be addressed. E-Mail: axel.duerkop@chemie.uni-regensburg.de

1053-0509/05/0900-0755/0 © 2005 Springer Science+Business Media, Inc.

which HP probably replaces water molecules from the inner coordination sphere around the Eu^{3+} central ion [18]. The decrease in luminescence quenching upon coordination of H_2O_2 occurs at near-neutral pH. Thus, EuTc can act as a luminescent probe for HP at neutral pH without the need for using an oxidative enzyme such as a peroxidase (which is mandatory in practically all other assays working at near-neutral pHs). Following our preliminary communication [18], we now give a full account on the potential of the method for determination of HP, its kinetics, and on the effects of interferents and detergents.

MATERIALS AND METHODS

Chemicals and Solvents

Europium trichloride hexahydrate ($EuCl_3 \cdot 6H_2O$) was purchased from Alfa (Karlsruhe, Germany), 3-morpholino-propanesulfonic acid (MOPS) from Roth (Karlsruhe, Germany), HP from Merck (Darmstadt, Germany), and tetracycline hydrochloride from Serva (Heidelberg, Germany). All chemicals were of analytical grade and used without further purification. Water was doubly distilled.

Measurements of pH were performed with a WTW 196 pH meter with temperature compensation. Emission spectra were recorded with a luminescence spectrometer (Aminco Bowman, Series 2, from SLM) with a 150-W xenon lamp as the excitation source. The slit width was typically 4 nm for both excitation and emission. Quantum yields (QYs) were determined in aqueous solution against the reference dye $Ru(bpy)_3Cl_2$ whose QY is reported to be 0.028 [19] in air-saturated water.

MOPS buffer solution: 2.96 g of MOPS sodium salt is dissolved in 490 mL of doubly distilled water. The solution is adjusted to pH 6.9 with 70% perchloric acid and filled up to 500 mL to give a 26 mM buffer.

Solution A (the Eu_3Tc working solution): 4.0 mg of tetracycline hydrochloride and 9.6 mg of $EuCl_3 \cdot 6H_2O$ (99.9%) are dissolved in 200 mL of MOPS buffer solution.

Solution B (a 400 μM HP solution): 1 mL of 30% HP is dissolved in 10 mL of distilled water. This stock solution is stable for 4 weeks in a refrigerator. Dilute 50 μL of this stock solution to 100 mL with distilled water to obtain solution B. This solution should be prepared fresh daily.

Calibration Plot

Mix 1.00 mL of solution A and 1.00 mL of solutions containing 400, 200, 100, 50, 20, and 10 μM concentrations of HP, respectively. These were obtained by mixing distilled water and solution B in volumes of $10 + 0$, $8 + 2$, $5 + 5$, $2.5 + 7.5$, $1 + 9$, and $0.5 + 9.5$ mL in a 10-mL volumetric flask. Thermostat to 20 °C (or other temperature if required). Measure, after 10 min, the luminescence intensity at 616 nm following excitation at a wavelength between 400 and 405 nm. Emission intensity is strongly temperature dependent.

Assay Protocol

Mix 1.00 mL of solution A and 1.00 mL of a sample containing between 2 and 400 μM concentrations of HP. Measure, after 10 min, the emission intensity at 616 nm. The concentration of HP can be calculated from the equation given later. For detection in microplates use one-tenth of the volumes given.

RESULTS

Effect of Hydrogen Peroxide on Spectral Properties

The probe EuTc has a longwave absorption band centered at around 400 nm which is rather longwave for a europium–ligand complex. This longwave band makes it compatible with violet LEDs and the 405-nm violet diode laser which are highly attractive solid-state light sources. The absorption spectra of Eu_3Tc in absence and presence of HP at different Eu/Tc stoichiometries have been presented elsewhere [14,15,18].

The luminescence emission spectra of Eu_3Tc (*solution A*) were recorded in absence and presence of increasing concentrations of HP and are presented in Fig. 1. In contrast to the intensity of the 400-nm absorption band, the intensity of the 616-nm line-like emission strongly depends on the concentration of HP in rising by a factor of 15 on addition of excess HP (see Fig. 1). If a spectrofluorimeter with a resolution of better than 1 nm is used, the band splits into a doublet with maxima at 613 and 619 nm of about the same intensity. This is particularly the case if the concentration of Eu_3Tc exceeds 10 μM. On addition of HP, the emission intensity of the other (smaller) bands also increases (see Fig. 1). The quantum yield of Eu_3Tc in MOPS buffer solution of pH 6.9 is 0.2 ($\pm$0.1)% at 22°C and increases to 2.7 ($\pm$0.1)% on addition of HP.

Time Dependence of the Reaction Between Eu_3Tc and Hydrogen Peroxide

The enhancement of the luminescence of Eu_3Tc is time dependent and reaches a constant value only after

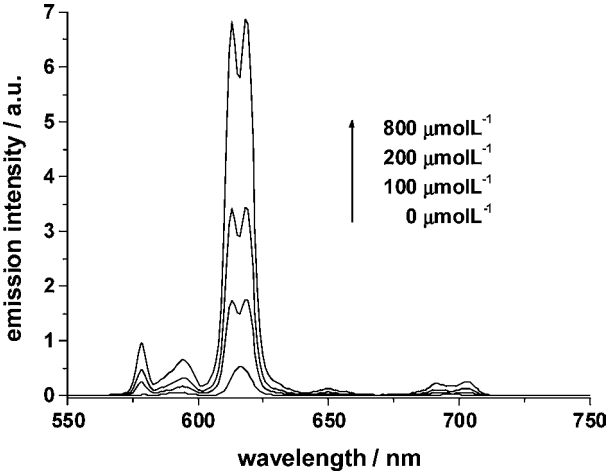

Fig. 1. Emission spectra of Eu_3Tc in the presence of different concentrations of HP in MOPS buffer of pH 6.9.

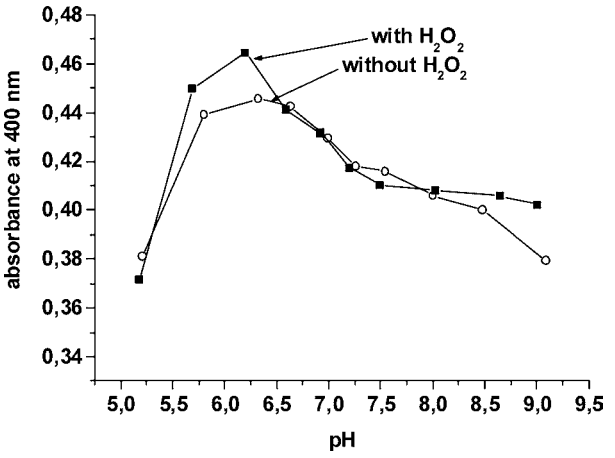

Fig. 3. Effect of pH on the absorbance at 400 nm of Eu_3Tc in absence (circles) and in presence (squares) of 400 μM of HP after a 10-min incubation time.

30 min (see Fig. 2). We attribute this to the slow displacement of the water ligand at the Eu^{3+} ion (with its 8–9 binding sites) by the HP ligand. In fact, Eu_3Tc–HP can be reconverted into Eu_3Tc by addition of thiosulfate which is known to reduce HP. The enhancement reaction is completed by more than 90% after 10 min, and the data are highly reproducible, then. After a reaction time of 30 min, luminescence intensity remains constant over time, thereby also ruling out the possibility of chemiluminescence. We used a 10-min incubation time in the subsequent experiments because this is the best compromise between acquiring reliable data and a reasonably fast analytical procedure.

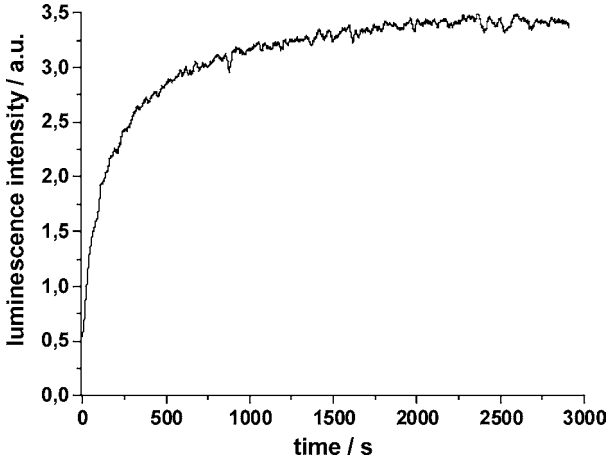

Fig. 2. Time-dependent enhancement of the luminescence intensity at 616 nm of a solution of Eu_3Tc in MOPS buffer pH 6.9 after addition of hydrogen peroxide (λ_{exc} = 405 nm, c_{HP} = 800 μmol/L).

Effects of pH

The effect of pH on the absorbance at 400 nm of Eu_3Tc in absence and in presence of 400 μM of HP after a 10-min incubation time is shown in Fig. 3. Absorbance increases by 20% between pH 5.2 and 6.2 (both in absence and presence of HP), but decreases at higher pHs. The absorbance remains unaffected by HP at pHs above 6.5. Therefore, 400 nm is a good choice for a kind of isosbestic excitation wavelength for luminescence assays at pH 6.5–7.0.

The effect of pH on the luminescence emission of Eu_3Tc at 616 nm (under 400-nm excitation) in absence and presence of HP is shown in Fig. 4. The concentration of Eu_3Tc is the same as given for *solution A* in the experimental part, and a 13 mM MOPS buffer is used. The concentration of HP was adjusted by adding microliter quantities of a concentrated HP solution to 100 mL of the stock solution of Eu_3Tc. The data points presented are an average of five measurements at each pH value, and are corrected for pH-induced changes in absorbance (as displayed in Fig. 3). The data points for Eu_3Tc in absence and in presence of HP were normalized for better comparison. The luminescence at 616 nm (λ_{exc} 400 nm) of both species initially rises with pH (see Fig. 4), approaches a maximum at near-neutral pH, and drops quickly once the pH exceeds 7.5 (in case of Eu_3Tc) or 8 (Eu_3Tc-HP). A pH of 6.9 was chosen for the following experiments because the luminescence of the probe is quite intense in this range and because most bioassays require near-neutral pHs. Furthermore, this pH minimizes the risk of precipitation of $Eu(OH)_3$ [15]. In fact, at concentrations of Eu^{3+} like in *solution A* precipitation of $Eu(OH)_3$ is the

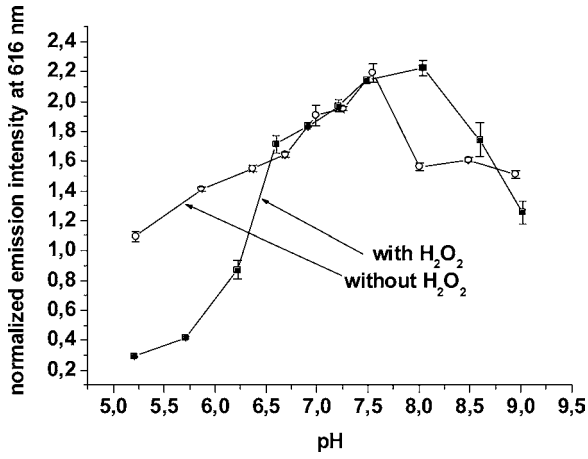

Fig. 4. Effect of pH on the luminescence at 616 nm (at $\lambda_{exc} = 400$ nm) in the absence (circles) and in presence (squares) of 400 μM hydrogen peroxide after a 10-min incubation time.

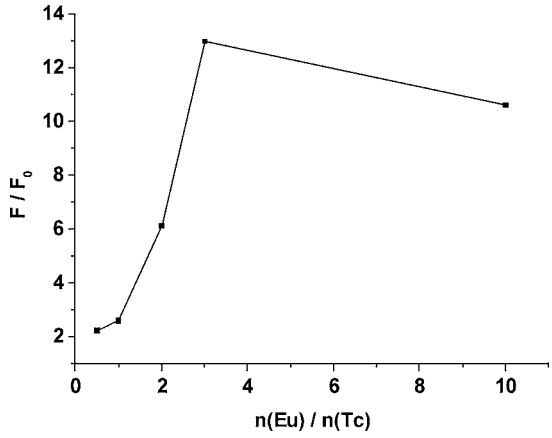

Fig. 5. Effect of molar ratio of $[Eu^{3+}]/[Tc]$ on the ratio of the luminescence intensity at 616 nm in the absence (F_0) and presence (F), respectively, of 400 μmol/L hydrogen peroxide ($\lambda_{exc} = 405$ nm).

cause for the drop of luminescence at pHs >7.5. A further welcome feature of pH 6.9 is that the absorbance at 400 nm ($= \lambda_{exc}$) remains unchanged (see Fig. 3).

Effect of the Ratio Between Eu^{3+} and Tc

It has been reported that the molar ratio between Eu^{3+} and Tc affects the enhancement of the luminescence of Eu_3Tc by surfactants [17]. Therefore, we have tested whether the molar ratio between Eu^{3+} and Tc may have an effect on the determination of HP. Samples were prepared containing $EuCl_3 \cdot 6H_2O$ and tetracycline hydrochloride in molar ratios of 10:1, 3:1, 2:1, 1:1, and 1:2 in 13 mM buffer of pH 6.9. The concentration of Eu^{3+} was kept constant as given for *solution A* (see the 'Experimental' section). Then, HP was added to give a final concentration of 400 μM. The effect of the ratio $[Eu^{3+}]/[Tc]$ on the ratio of the luminescence at 616 nm before and after addition of HP is plotted in Fig. 5. It can be seen that the most significant enhancement with HP occurs if Eu^{3+} is present in a threefold or higher molar excess.

The Eu^{3+} ion is known to weakly bind to Tc [15]. The resulting complexes are rather labile. On the other side, Tc has three en-dione centers which are capable of binding bi- or trivalent ions. This suggests two possible environments for the HP complex at two different coordination sites for europium at the Tc Ligand, if not three. Currently, theoretical calculations are being made whether even triple coordination of Eu^{3+} is possible or impossible due to steric hindrance. The assumption of two possible environments for the HP complex is further supported by the fact that band splitting (compare Fig. 1) only appears at molar ratios of Eu/Tc between 10:1 and 2:1 but not at 1:1. We have been unable to isolate the complex in solid form in order to perform an X-ray analysis. Moreover, NMR studies (presently going on) are complicated by the fact that Eu^{3+} is a paramagnetic ion that causes substantial signal shifts.

Calibration Graph

Solutions were prepared according to the calibration protocol given in the materials and methods section. Six samples of each concentration of HP were made by mixing 1 mL of the sample with 1 mL of *solution A*, incubating for 10 min at 20°C while stirring, and measuring luminescence intensity at 616 nm. The resulting plot (Fig. 6) is linear between 2 and 400 μM HP. The plot can be described by a linear equation of type $y = A + Bx$, with $A = (0.3995 \pm 0.0741)$, $B = (0.0141 \pm 0.0004)$, and $r = 0.9978$. This small error, especially in the slope B, reveals the high precision of the whole assay [20]. The limit of detection (defined as 3σ/slope) is 960 nM. It is important to note that both the calibration solutions and the samples also can be measured after a rather long incubation time (up to 24 h tested) without loss of the linearity of the calibration graph.

Standard Additions

Samples were prepared by standard additions to contain 20, 40, and 80 μM of HP, respectively ($n = 3$). Then, luminescence intensity was measured at 616 nm after a

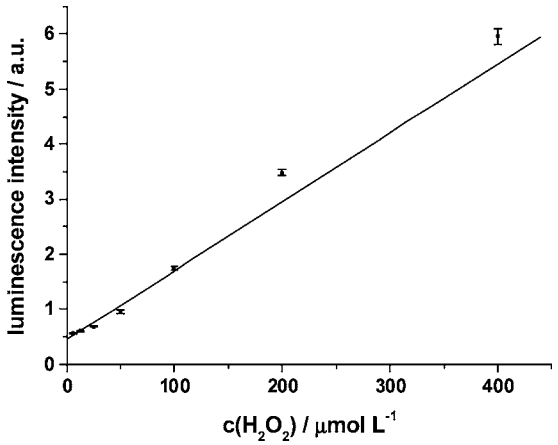

Fig. 6. Calibration curve for hydrogen peroxide including error bars ($n = 6$). Luminescence intensity was measured at 616 nm after a 10 min incubation at 20°C.

10 min incubation period at 20°C. The resulting data gave concentrations of 19.4, 40.7, and 79.8 μM, which corresponds to a mean recovery of 97.0, 101.8, and 99.7%, respectively. This again demonstrates the reliability of the method.

Interferents

As described before [18], alkali and earth alkaline ions have no effect in up to 100 mM concentrations. Many transition metals (Co^{2+}, Fe^{3+}, Zn^{2+}) quench in 0.1–1 mM concentrations, and Cu^{2+} is found to be a particularly strong quencher even in <0.1 μM concentration. Ag^{+} slightly increases luminescence intensity (<20 μM) and decreases emission more pronounced at concentrations exceeding 100 μM. Chloride, sulfate, acetate and nitrate are inert, but phosphate (>1 μM) gives an increase in intensity (provided the stoichiometry Eu/Tc is 3:1). This interference implies that, in practice, phosphate buffers cannot be used, and samples containing unknown levels of phosphate cannot be analyzed directly. On the other side, this may be used for quantitation of phosphate (in the absence of HP). Fluoride quenches significantly at >25 μM concentrations, bromide and iodide do not interfere up to 1 mM. Carbonate displays quenching at >200 μM. Among the organic species, citrate and malate interfere in giving a strong increase in the luminescence intensity of Eu_3Tc (and a decrease in the intensity of the Eu_3Tc–HP complex) [33]. Furthermore, the fluorescence of the EuTc–HP system is weakly quenched by molecular oxygen. An 11% decrease in fluorescence intensity was observed on going from nitrogen-saturated solutions to air-saturated solutions.

DISCUSSION

The method for determination of HP presented here has several novel features. It is fast, simple, does not require elevated temperatures (as do certain Trinder reagents) nor the presence of a peroxidase or a respective enzyme mimic such as certain phthalocyanines [21,22], hemins [23,24], or other metalloporphyrins [25–27]. It is certainly not an undesired fact that the probe is easily accessible. Eu_3Tc also offers the typical advantages of luminescence assays over photometric assays including selectivity (due to a large Stokes' shift) and—in particular—a long decay time (30 μs at the average) which enables time resolved measurements [28]. Finally, the incubation time (10 min) is shorter than that needed for the widely used fluorescent probe Amplex Red which requires almost 30 min [32].

It should be noted that one of the typical advantages of certain fluorescent probes (namely measurement against a "black" background) is not provided by this method, since Eu_3Tc also displays intrinsic luminescence. Thus, the changes induced by HP cannot be measured against a black background, but only relative to the background luminescence of uncomplexed Eu_3Tc with its quantum yield of 0.2 %. Secondly, the interference by phosphate ion is annoying and must be kept in mind at any time, but—on the other hand—appears to pave the way for determination of phosphate in the absence of HP.

Photometric reagents for HP are based on changes in the absorbance at typically 400–440 nm, for example in case of the peroxytitanate ion or the Ti^{4+}/porphyrin probe [1,27]. In case of low concentrations of HP this decrease will result in hardly detectable changes in the absorbance. In addition, a correction is mandatory if other absorbers are present in the sample. In fact, this is the case for almost all absorptiometric reagents presented in Table I except for the Trinder reagent absorbing at 750 nm where background absorbance is often negligible. Luminescence-based methods are certainly advantageous over photometry, not the least because the concentration of reagent can be substantially lowered (compared to photometric reagents).

The Eu_3Tc-based method presented here works best in the near neutral pH range (6.9–7.4) in a 13 mM MOPS buffer. A neutral pH often is a prerequisite for measurements in samples containing proteins, enzymes, or other biological material. This is of particular significance if compared to common methods based on the use of Ti^{4+} ion [1] or titanium porphyrins [27] where a rather acidic pH (<3) is often necessary. Analysis of biological materials at such low pH values is often impossible and a change

Table I. Figures of Merits for Common Absorptiometric and Fluorimetric Methods for Determination of Hydrogen Peroxide

Reagent	Method	LOD (μM)	Remarks	References
Ti^{4+}	Photometry at 410 nm	8.0	Requires low pH (<4)	[1]
Ti^{4+}-porphyrin	Photometry at 432 nm	2.2	5 min incubation at RT, pH <1 required	[27]
Ti^{4+}-oxalate	Photometry at 420 nm	20	5 min incubation at RT, pH <1 required	[23]
Fe^{3+}-complex	Photometry at 575 nm	3	5 min incubation at RT, pH 3.5–7.5	[29]
Trinder reagent I	Photometry at 550 nm	>1	Enzymatic, 10 min incubation at 37°C, pH 4, poor water solubility	[30]
Trinder reagent II	Photometry at 750 nm	1	Enzymatic, 10 min incubation at 37°C, pH 4–8	[30]
Polyaniline	Photometry at >600 nm	15	Nonenzymatic; 10 min incubation; pH 5–9; water-insoluble film in microtiterplate	[31]
p-Hydroxyphenylacetic acid	Fluorescence at 380 nm	0.1–0.5	Enzymatic (POx)	[7,8]
Amplex Red	Fluorescence at 587 nm	0.1	Enzymatic, 30 min incubation at RT, pH 7.4; 24 nm Stokes' shift	[32]
Eu_3Tc reagent	Luminescence at 616 nm	1.0	Nonenzymatic, 10 min incubation RT, pH 6.9–7.4; 210 nm Stokes' shift	This work and [28]

in the pH during an analytical process is often tedious and time consuming, if not impossible.

The Eu_3Tc assay has a better LOD than most of the spectrophotometric methods. However, the Amplex Red assay and the Ti^{4+}-porphyrin method offer even better LODs. On the other hand, the Amplex Red method requires the presence of a peroxidase and suffers from oxidation of resorufin to nonfluorescent resazurin in presence of excess HP [32]. Therefore, the linear range of detection is limited to a concentration of 5 μM when using Amplex Red, whereas Eu_3Tc offers linearity over 2.4 decades (up to 400 μM). On the other side, one should not overemphasize such a wide analytical range since it is not necessarily needed in industrial and clinical practice.

A further feature of the Eu_3Tc assay is based on the fact that it is nonenzymatic. The use of enzymes often brings about restrictions in terms of pH, temperature and ionic strength. Moreover, storage conditions of reagents are more stringent and enzyme solutions suffer from poor stability at ambient temperature. This is true for the Trinder's reagents, for polyaniline [31], and Amplex Red. The assay presented here does not suffer from these shortcomings. We have shown in a previous work that Eu_3Tc can be incorporated into hydrogel membranes to give a solid state sensor membrane that responds to HP, *albeit* only at much higher concentrations [34].

By comparing the two luminescence-based methods it can be seen that the Eu_3Tc reagent displays a much larger Stokes' shift (210 nm) than Amplex Red with only 24 nm [31]. This is another advantage in case of analyses using optical filter-based microtiterplate readers, since the separation of excitation and emission light is accomplished much easier in case of large Stokes' shifts where straylight and Raman scatter are unlikely to interfere.

REFERENCES

1. L. C. Thomas (1980). *Colorimetric Chemical Analytical Methods*, Wiley, Chichester, New York, p. 195.
2. D. Harms, H. Luftmann, F. Mueller, B. Krebs, and U. Karst (2002). Selective determination of hydrogen peroxide by adduct formation with a dinuclear iron(III) complex and flow injection analysis/tandem mass spectrometry. *Analyst* **127**, 1410–1412.
3. T. E. Barman (1979). *The Enzyme Handbook*, Springer, New York, p. 92.
4. H. G. Bergmeyer (1984). *Methods of Enzymatic Analysis*, Verlag Chemie, Weinheim, p. 56.
5. W. R. Seitz (1978). In M. A. DeLuca (Ed.), *Bioluminescence & Chemiluminescence*, Academic Press, New York, pp. 445–462.
6. K. A. Fähnrich, M. Prawda, and G. G. Guilbault (2001). Recent applications of electrogenerated chemiluminescence in chemical analysis. *Talanta* **54**, 531–559.
7. G. G. Guilbault, P. Brignac P, and M. Zimmer (1968). Homovanillic acid as a fluorometric substrate for oxidative enzymes. Analytical applications of the peroxidase, glucose oxidase, and xanthine oxidase systems. *Anal. Chem.* **40**, 190–196.
8. G. G. Guilbault (1970). *Enzymatic Methods of Analysis*, Pergamon Press, Oxford, p. 128.
9. J. Meyer and U. Karst (2000). Peroxidase enhanced lanthanide luminescence—A new technique for the evaluation of bioassays. *Analyst*, **125**, 1537–1538.
10. J. L. Colaizzi, A. M. Knevel, and A. N. Martin (1965). Biophysical study of the mode of action of the tetracycline antibiotics. Inhibition of metalloflavoenzyme NADH cytochrome oxidoreductase. *J. Pharm. Sci.* **54**, 1425–1436.
11. A. Kaji and M. Royoji (1979). In F. E. Hahn (Ed.), *Antibiotics*, Vol. 1, Springer, Berlin, pp. 304–328.
12. M. D. Menachery and M. P. Cava (1984). Amino derivatives of anhydrotetracycline. *Can. J. Chem.* **62**, 2583–2585.
13. W. Dürckheimer (1975). Tetracyclines: Chemistry, biochemistry, and structure–activity relations. *Angew. Chem. Int. Ed.* **14**, 721–734.

14. L. M. Hirschy, E. V. Dose, and J. D. Winefordner (1983). Lanthanide-sensitized luminescence for the detection of tetracyclines. *Anal. Chim. Acta* **147**, 311–316.
15. L. M. Hirschy, T. F. van Geel, J. D. Winefordner, R. N. Kelly, and S. G. Schulman (1985). Characteristics of the binding of europium(III) to tetracycline. *Anal. Chim. Acta* **166**, 207–219.
16. Y. Rakicioglu, J. H. Perrin, and S. G. Schulman (1999). Increased luminescence of the tetracycline–europium(III) system following oxidation by hydrogen peroxide. *J. Pharm. Biomed. Anal.* **20**, 397–399.
17. N. Arnaud and J. Georges (2001). Sensitive detection of tetracyclines using europium-sensitized fluorescence with EDTA as co-ligand and cetyltrimethylammonium chloride as surfactant. *Analyst* **126**, 694–697.
18. O. S. Wolfbeis, A. Dürkop, M. Wu, and Z. Lin (2002). Europium ion-based luminescent sensing probe for hydrogen peroxide. *Angew. Chem. Int. Ed.* **41**, 4495–4498.
19. K. Nakamaru (1982). Synthesis, luminescence quantum yields, and lifetimes of trischelated ruthenium (II) mixed-ligand complexes including 3,3′-dimethyl-2,2′-bipyridyl. *Bull. Chem. Soc. Jpn.* **55**, 2697–2705.
20. S. Kromidas (2001). *Handbuch der Validierung in der Analytik*, Wiley-VCH, Weinheim, pp. 179–185.
21. X.-L. Chen, H.-H. Yang, Q.-Z. Zhu, H. Zheng, J.-G. Xu, and D.-H. Li (2001). A new red-region substrate, tetra-substituted amino aluminium phthalocyanine, for the fluorimetric determination of H_2O_2 catalyzed by mimetic peroxidases. *Analyst* **126**, 523–527.
22. H. Yuan, R. Cai, and Z. Pan (2003). Catalytical kinetic determination of hydrogen peroxide using iron-tetrasulfonatophthalocyanine (with γ-CD) as a mimetic enzyme of peroxidase. *Anal. Lett.* **36**, 277–286.
23. C. Xu and Z. Zhang (2001). Fluorescence determination of hydrogen peroxide using hemoglobin as a mimetic enzyme of peroxidase. *Anal. Sci.* **17**, 1449–1451.
24. X. Wang, Y. Li, and W. Chang, (1999). Mimicry of peroxidase by co-immobilization of 1-allylimidazole and hemin on *N*-isopropylacrylamide-based hydrogel. *Anal. Chim. Acta* **400**, 135–142.
25. X. M. Huang, M. Zhu, and H. X. Shen (1998). *N*,*N*-Diethylaniline as hydrogen donor for determination of hydrogen peroxide catalyzed by metalloporphyrins as enzyme mimetic of peroxidase. *Microchim. Acta* **128**, 87–91.
26. Y. Ci, L. Chen, and S. Wei (1989). Fluorescence reaction of the system mimetic peroxidase [Mn-T(4-TAP)P]-homovanillic acid-hydrogen peroxide. Spectrofluorometric determination of hydrogen peroxide. *Fresenius Z. Anal. Chem.* **334**, 34–36.
27. C. Matsubara, N. Kawamoto, and K. Takamura (1992). Oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV): An ultra-high sensitivity spectrophotometric reagent for hydrogen peroxide. *Analyst* **117**, 1781–1784.
28. W. Lei, A. Duerkop, M. Wu, and O. S. Wolfbeis (2003). Determination of hydrogen peroxide in river water using a europium tetracycline microplate luminescence assay with time-resolved ("gated") detection. *Microchim. Acta* **143**, 269–274.
29. D. Harms, R. Than, U. Pinkernell, M. Schmidt, B. Krebs, and U. Karst (1998). Selective determination of hydrogen peroxide in household products using its molecular recognition by a dinuclear iron(III) complex. *Analyst* **123**, 2323–2327.
30. M. Mizoguchi, M. Ishiyama, M. Shiga, and K. Sasamoto (1998). Water-soluble chromogenic reagent for colorimetric detection of hydrogen peroxide—An alternative to 4-aminoantipyrine working at a long wavelength. *Anal. Comm.* **35**, 71–74.
31. S. A. Piletsky, T. L. Panasyuk, E. V. Piletskaya, T. A. Sergeeva, A. V. Elkaya, E. Pringsheim, and O. S. Wolfbeis (2000). Polyaniline-coated microtiter plates for use in longwave optical bioassays. *Fresenius J. Anal. Chem.* **366**, 807–810.
32. R. P. Haugland (Ed.) (2002). *Handbook of Molecular Probes*, 9th ed., Molecular Probes Inc., Eugene, p. 440.
33. Z. Lin, M. Wu, M. Schäferling, and O. S. Wolfbeis (2004). Fluorescent imaging of citrate and other intermediates in the citric acid cycle. *Angew. Chem. Int. Ed.* **43**, 1735–1738.
34. O. S. Wolfbeis, M. Schaeferling, and A. Duerkop (2003). Reversible optical sensor membrane for hydrogen peroxide using an immobilized fluorescent probe, and its application to a glucose biosensor. *Microchim. Acta* **143**, 221–227.