SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
**10** (2004)

# OPTIMIZATION OF A NEW CHEMILUMINESCENCE SENSOR BASED ON PEROXYOXALATE CHEMILUMINESCENCE

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Received September 30, 2004

*The optimization of the experimental conditions for using new single-shot sensors based on chemiluminescence (CL) with two oxalate esters is presented. The new sensors are suitable for the determination of hydrogen peroxide in different kinds of samples. Each sensor contains an oxalic acid ester, a fluorescer, and a polymer. Bis(2,4-dinitrophenyl) oxalate (DNPO), and bis(2,4,6-trichlorophenyl) oxalate (TCPO) are used as esters in chemiluminescent reagents. The fluorescer and the polymer in both systems was 9,10-diphenylanthracene and cellulose acetate. The polymeric membrane on a transparent glass support contained reactants and was placed over a photocell as detector; the sample was directly applied onto the membrane. The composition of the membrane was optimized. The detection limit (3σ) was 0.6 $\mu g\, l^{-1}$ $H_2O_2$ for TCPO, and 5.3 $\mu g\, l^{-1}$ for DNPO. The sensors will be tested on samples of commercially available washing powders.*

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## Introduction

A chemiluminescent (CL) sensor can be defined as an analytical device incorporating a chemiluminescence generating reagent in the recognition layer, with the purpose of detecting the concentration of a certain chemical in diverse samples [1]. Significant advances in design and applications of CL sensors and biosensors have been reported in the last few years.

Chemiluminescence is defined as the emission of electromagnetic radiation (usually in the visible or near-infrared region) produced by a chemical reaction. Such reactions generally yield one of the reaction products in an electronically excited state producing light when relaxing to the ground state. CL is often described as a dark-field technique[1]. In general, a chemiluminescent reaction can be generated by two basic mechanisms (Fig. 1).

*Direct chemiluminescence* proceeds *via* emission of light directly by the excited species.

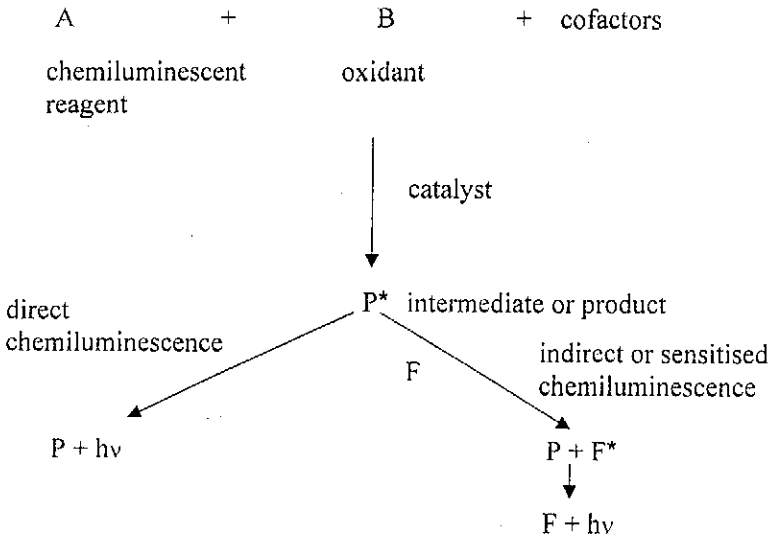


Fig.1 Mechanism of chemiluminescence reactions

*Indirect* or *sensitized CL* is based on a process of energy transfer of the excited species to a fluorescer. This process makes it possible for those molecules that would otherwise relax preferentially without radiation to transfer their excess of energy to a fluorescer that in turn is excited, relaxing to its ground state with photon emission [1].

Peroxyoxalate chemiluminescence (POCL) presents indirect or sensitized

type of chemiluminescence, as the intermediates or reaction products from the primary reaction do not emit a significant quantity of light. Instead, it transfers its energy to an energy accepting fluorescer, which becomes electronically excited and subsequently emits light. The intermediates generated in the POCL reaction are capable of exciting fluorescers that emit light from the near-ultraviolet to the near-infrared region; these characteristics constitute the usefulness of the reaction in analytical chemistry [1–5].

Diaryl oxalate esters with strongly electron withdrawing substituents provide the highest quantum yields, whereas diarlyl oxalate esters with electron-donating or weak electron-withdrawing groups were found to result in either inefficiency or poor production of chemiluminescence. The most efficient diaryl oxalate ester reported was bis(2,4-dinitrophenyl) oxalate (DNPO), and although this reagent finds occasional use, analytically it has been surpassed in popularity by bis(2,4,6-trichlorophenyl) oxalate (TCPO), which is not quite as efficient but has the advantage of being more conveniently handled [6–9].

Optical sensors belong to the most important types of chemical sensors that have been extensively studied in recent years for real-time monitoring of analytes. Depending on the origin of the optical signals, these types of sensors may be roughly classified into absorbance and luminescence-based sensors, the latter mainly exploiting the principle of fluorescence and chemiluminescence. Although they offer many advantages, such as high sensitivity, good selectivity, and fast response time, fluorescence-based sensors require an excitation light source and spectral separation of exciting and emitted light leading to relatively sophisticated equipment producing high background signals [10–13].

For the work presented here, the Low-range Electric Luminescence Assay (LELA), designed and built in the laboratory, was used as a detection device for the new solid-phase single-shot chemiluminescent sensors [14]. The optimization of the experimental conditions for the developing of the new sensors will be described.

## Experimental

### Apparatus

All the measurements were performed with the instrument LELA, version 1.0.0. It consists of a reaction cell in a light tight housing, a photocell as the light detector, and an amplification, data acquisition, and signal-processing system. The upper inner part of the cell is designed to fix a Hamilton syringe (25 μl), by which the sample is injected directly onto the membrane. A membrane with the CL reagents (ester, fluorescer, and polymer) is placed in the measurement cell directly on the surface of the photodiode. One membrane can be used only once for the

producing reaction. The measuring device is microprocessor controlled, and the measured data are transferred serially to a personal computer; the data were processed by means of a program written in Visual Basic®.

### Reagents

DNPO and TCPO, ethyl acetate, cellulose acetate, were purchased from Fluka. Hydrogen peroxide (30 %), was obtained from Merck, 9,10-diphenylanthracene from Sigma-Aldrich. Microscope cover glasses (18×18×04 mm) were purchased from Menzel-Gläser, Germany.

### Preparation of Membranes

The reagents for preparing membranes with TCPO comprised TCPO, 9,10-diphenylanthracene, and cellulose acetate. Different quantities of ester (1 mg; 1.5 mg; 2 mg; 2.5 mg; 3 mg), fluorescer (3 mg; 3.5 mg; 6 mg; 7.5 mg; 9 mg) and polymer (2.5 mg; 3.5 mg; 5 mg; 7.5 mg; 10 mg) were used during the optimization process. The optimized casting solution contained 2 mg of TCPO, 6 mg of 9,10-diphenylanthracene and 5 mg of cellulose acetate dissolved in ethyl acetate (2.5 ml ). A 10 µl sample was dropped on a glass slide (18×18 mm). The membranes were dried in an empty dessicator for a couple of minutes. The dried membranes were used for determination.

The reagents for preparing membranes with DNPO comprised DNPO, 9,10-diphenylanthracene, and cellulose acetate. Different quantities of ester (1 mg; 3 mg; 5 mg; 7 mg), fluorescer (3 mg; 9 mg; 15 mg; 21 mg) and polymer (5 mg; 10 mg; 12 mg; 16 mg; 19 mg) were used during the optimization process. The optimized casting solution contained 3 mg of DNPO, 9 mg of 9,10-diphenylanthracene and 12 mg of cellulose acetate dissolved in ethyl acetate (2.5 ml). A 10 µl sample was dropped on a glass slide. The membranes were dried in an empty dessicator for a couple of minutes. The dried membranes were used for determination.

## Procedure

The sensor was inserted into the reaction cell of the device. The Hamilton syringe (25 µl) was inserted in the upper part of the housing and mounted on the cell. The system was completely light tight. The solution of hydrogen peroxide (10 µl) was applied with the syringe onto the membrane surface. At the same time, recording of the response curves was started. The sample output curves (four differently

amplified signals) were registered on the personal computer with the corresponding software. The reaction was finished after a few seconds.

### Evaluation of Signals

The peak shaped response obtained with the chemiluminescence of DNPO and TCPO systems were evaluated by original software written in Visual Basic®. It allows to set the measurement parameters as well as data handling and evaluation (smoothing, integration of the peak area, determination of the peak height etc.).

The photocurrent of the photodiode is converted into 4 different amplified voltages (4 channels) by 4 operational amplifiers. The amplification ratios between the channels were around 10 from one channel to the next.

Each channel of the amplified signals is read and stored separately. The number of points over the entire measurement period can be chosen. The software also allows determining the number of repetitive measurements of the microprocessor over which each data point is averaged. A waiting period between the measurements increments can also be adjusted which is particularly useful with broad or glow-like chemiluminescence signals [14].

## Results and Discussion

### General design

The new single-shot sensor based on peroxyoxalate chemiluminescence presents a new device for the on-site quantification of different kinds of samples.

In general, the device consists of four parts (A, B, C, D – Fig. 2) of which part C is made of black PVC and other parts are made of aluminum. Part A, which served as a holder for the microsyringe, is mounted on part B facilitating the exchange of the sensor S. The part has a centric drilling which is tapering at its end, and guarantees that the tip of the syringe is positioned centrically and in a defined distance over the sensor. Part B together with part C form a Faraday cage shielding the photodiode as well as the electronic board which is screwed on part C. This design minimizes the distance between the actual detector (photodiode) and the amplification circuit; thus, the signal noise due to electrostatic interferences should be minimized.

Rubber sealings ($S_1$, $S_2$, $S_3$) were used to achieve light tightness and mechanical stability [14].

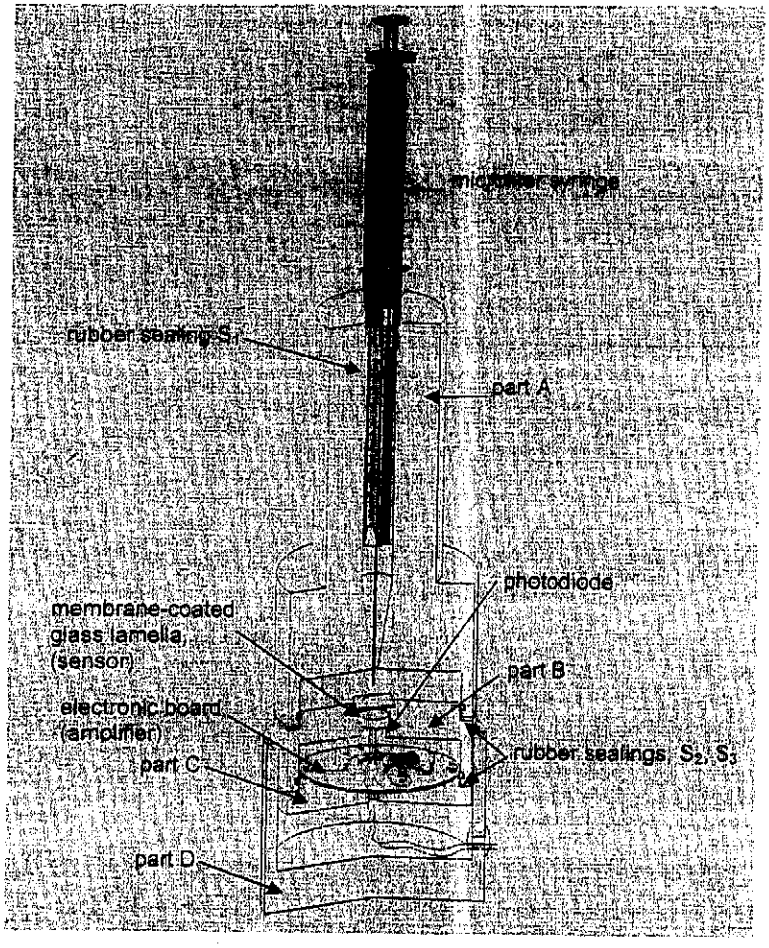


Fig. 2 Sketch of the measurement cell designed

## Preparation of Membranes

The preparation of membranes presents the most important task of preparing the sensors. A stable and homogenous membrane is the main factor for obtaining reproducible and reliable results. The first step for fabricating such membranes is to dissolve all chemiluminescence reagents in one solvent.

The most efficient solvent for both types of sensors, i.e., with TCPO and with DNPO as chemiluminescent reagents, was ethyl acetate. After casting the membrane-forming solution onto the glass support and after drying the obtained membranes were ready for determination. Cover glasses for microscopic purposes were conveniently used as glass supports. The membranes were stable over two

days. The reproducibility of the membranes obtained with this method is satisfying but it probably could still be increased by replacing the manual preparation of membranes by an automated process; this is the subject of current research. In general, the standard deviations as obtained with DNPO were higher than those obtained with TCPO.

## Optimizations

### *The Content of Ester and Fluorescer in the Membrane*

The optimization of the content of ester (TCPO or DNPO) and fluorescer (9,10-diphenylanthracene) was done in two steps; first, the ratio between ester and fluorescer was optimized, and afterwards their absolute concentration in the membrane with the optimum ratio.

The dependence of the signal on the ratio between ester and fluorescer for both systems is shown in Figs 3 and 4.

For both types of sensors an optimum mass ratio of 1:3 was found between ester and diphenylanthracene. From the ratio 1:3 to ratio 1:4, a decrease in the signal can be observed. Increasing ratios from 1:4 to any higher values will not have effect on chemiluminescent signal. Thus, it is evident that the maximum of the chemiluminescent light is obtained with ester/fluorescer ratio 1:3. Lower and higher ratios yield smaller signals, differing only in the course of the corresponding graph. Therefore, the composition where the maximum signal was

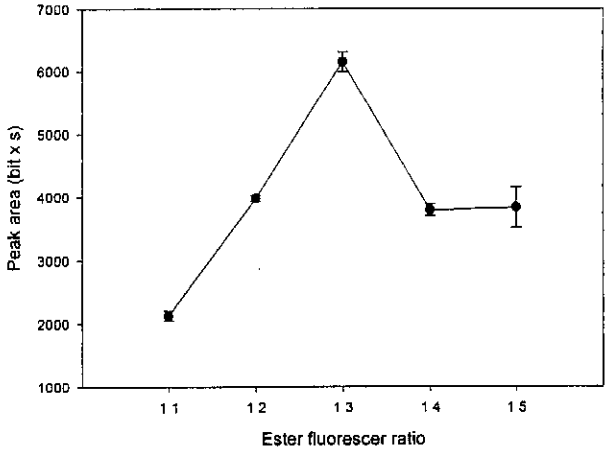


Fig. 3 Optimization of the ester-to-fluorescer mass ratio for sensors with TCPO; concentration of hydrogen peroxide: 100 $\mu g\, l^{-1}$; TCPO mass: 1mg; 9,10-diphenylanthracene masses: 1 mg, 2 mg, 3 mg, 4 mg, 5 mg; solvent volume: 2.5 ml

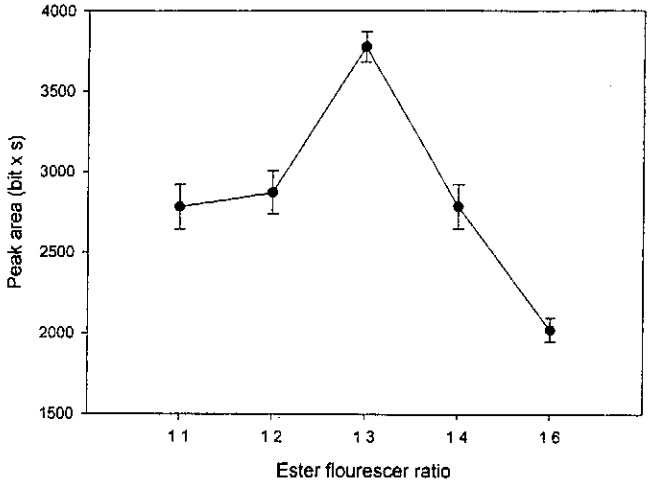


Fig. 4 Optimization of the ester-to-fluorescer mass ratio for sensors with DNPO; concentration of hydrogen peroxide: 100 $\mu g\ l^{-1}$; DNPO mass: 1 mg; 9,10-diphenylanthracene masses: 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg; solvent volume: 2.5 ml.

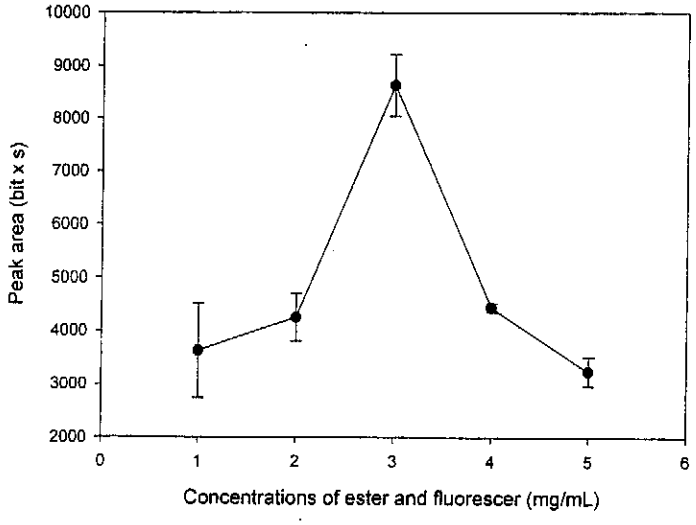


Fig. 5 Optimization of the absolute amounts at the ester-to-fluorescer mass ratio 1:3 for the sensor with TCPO; concentration of hydrogen peroxide: 100 $\mu g\ l^{-1}$; TCPO concentrations: 0.4 mg $ml^{-1}$; 0.6 mg $ml^{-1}$; 0.8 mg $ml^{-1}$; 1 mg $ml^{-1}$; 1.2 mg $ml^{-1}$; 9,10-diphenylantracene concentrations: 1.2 mg $ml^{-1}$; 1.4 mg $ml^{-1}$; 2.4 mg $ml^{-1}$; 3 mg $ml^{-1}$; 3.6 mg $ml^{-1}$; solvent volume: 2.5 ml

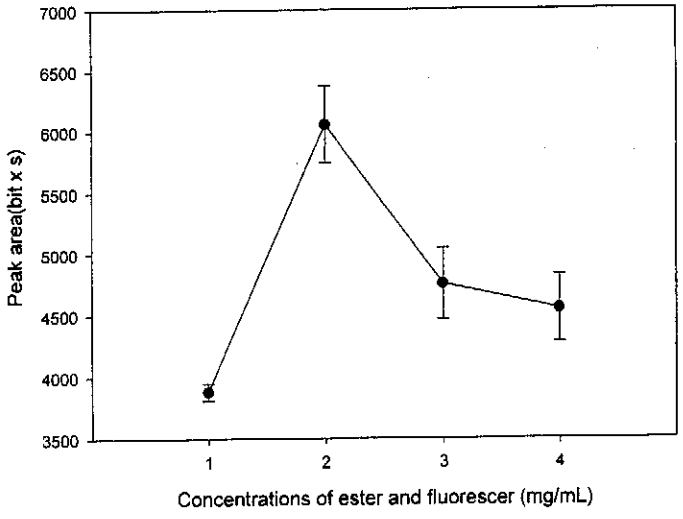


Fig. 6 Optimization of the absolute amounts at the ester-to-fluorescer mass ratio 1:3 for the sensor with DNPO; concentration of hydrogen peroxide: 100 $\mu g\ l^{-1}$; DNPO concentrations: 0.4 $mg\ ml^{-1}$; 1.2 $mg\ ml^{-1}$; 2 $mg\ ml^{-1}$; 2.8 $mg\ ml^{-1}$; 9,10-diphenylantracene concentrations: 1.2 $mg\ ml^{-1}$; 3.6 $mg\ ml^{-1}$; 6 $mg\ ml^{-1}$; 8.4 $mg\ ml^{-1}$; solvent volume: 2.5 ml

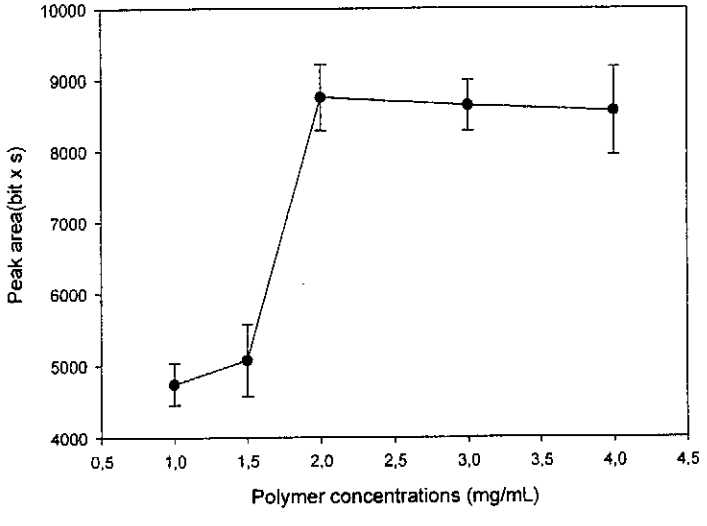


Fig. 7 Influence of different polymer concentrations on the chemiluminescent signal for the sensor with TCPO; concentration of hydrogen peroxide: 100 $\mu g\ l^{-1}$; TCPO concentration: 0.8 $mg\ ml^{-1}$; 9,10-diphenylanthracene concentration: 2.4 $mg\ ml^{-1}$; polymer concentrations; 1 $mg\ ml^{-1}$; 1.4 $mg\ ml^{-1}$; 2 $mg\ ml^{-1}$; 3 $mg\ ml^{-1}$; 4 $mg\ ml^{-1}$; solvent volume: 2.5 ml

obtained was taken as the optimum mass ratio between the two reagents.

The second step of the optimization process was to optimize the concentration of the ester and fluorescer in the solution for casting the membranes. For this the ratio between ester and fluorescer was kept constant (1:3), and the amount of both substances in the solution for preparing the membrane was varied. The results are shown in Figs 5 and 6.

The peak area of the signal is increasing from ester concentration of 0.4 mg $ml^{-1}$ and fluorescer concentration 1.2 mg $ml^{-1}$ (point 1 in Fig. 5) to the ester concentration of 0.8 mg $ml^{-1}$ and fluorescer concentration of 2.4 mg $ml^{-1}$ (point 3 in Fig. 5). Any higher values of both the concentrations will decrease signal. The ester concentration of 0.8 mg $ml^{-1}$ and the fluorescer concentration of 2.4 mg $ml^{-1}$ are the optimum concentrations which correspond to the ester/fluorescer ratio 1:3 and which produce the maximum of the CL light in the sensors where TCPO is used as the ester. The optimum concentrations were used for further optimization.

For DNPO, the signal is increasing from the concentrations 0.4 mg $ml^{-1}$ of the ester and 1.2 mg $ml^{-1}$ of the fluorescer (point 1 in Fig. 6) to the ester concentration of 1.2 mg $ml^{-1}$ and fluorescer concentration of 3.6 mg $ml^{-1}$ (point 2 in Fig. 5). Higher concentrations will decrease the signal. The ester concentration of 1.2 mg $ml^{-1}$ and fluorescer concentration of 3.6 mg $ml^{-1}$ are the optimum concentrations for producing the maximum CL.

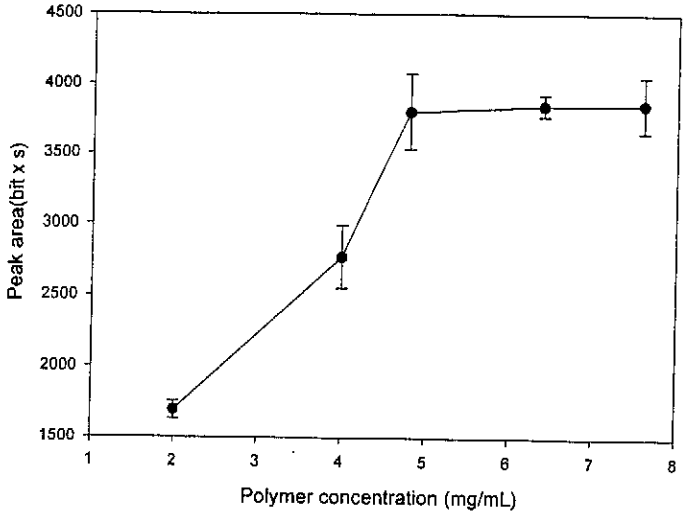


Fig. 8 Influence of different polymer concentrations on the chemiluminescent signal for the system with DNPO; concentration of hydrogen peroxide: 100 μg $l^{-1}$; DNPO concentration: 1.2 mg $ml^{-1}$; 9,10-diphenylanthracene concentration: 3.6 mg $ml^{-1}$; polymer concentrations; 2 mg $ml^{-1}$; 4 mg $ml^{-1}$; 4.8 mg $ml^{-1}$; 6.4 mg $ml^{-1}$; 7.6 mg $ml^{-1}$; solvent volume: 2.5 ml

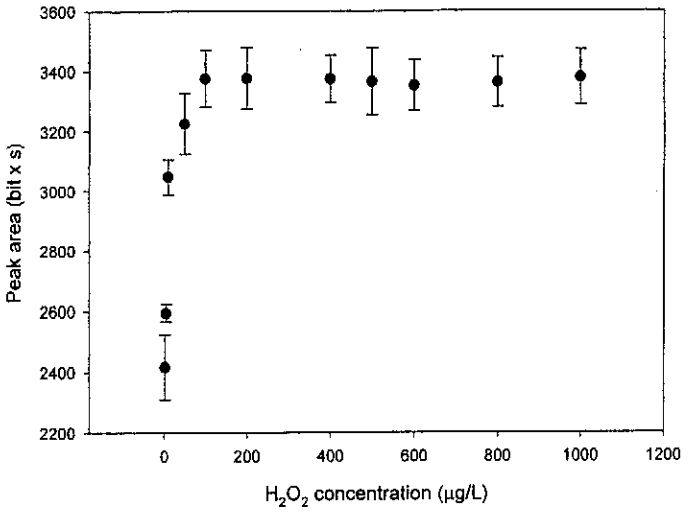


Fig. 9 Calibration curve for the sensor with TCPO; TCPO concentration: 0.8 mg $ml^{-1}$; 9,10-diphenylanthracene concentration: 2.4 mg $ml^{-1}$; polymer concentration; 2 mg $ml^{-1}$; solvent volume: 2.5 ml

*Polymer*

Influence of different polymer concentrations on the chemiluminescent signal in the new sensors was investigated. The results are shown in Figs 7 and 8. TCPO shows an increase in the signal up to concentrations of 2 mg $ml^{-1}$. Then the curve starts to level off and the chemiluminescent signal is slightly decreasing. For the further examination, polymer concentration of 2 mg $ml^{-1}$ was adopted.

With DNPO the CL signal is increasing from the polymer concentrations of 2 mg $ml^{-1}$ to 4.8 mg $ml^{-1}$. A further increase of the polymer concentration has no effect on the CL signal. Calibration curves for both sensors were made with optimized conditions (Figs 9 and 10)

With TCPO and hydrogen peroxide there is a small range of linear relation at low concentrations; at concentrations above 100 µg $l^{-1}$ the curve starts to level off. The lowest concentration which could be measured is 2.5 µg $l^{-1}$. The detection limit of the method calculated as 3σ value is 0.6 µg $l^{-1}$.

DNPO shows a lower response to hydrogen peroxide than TCPO; the shape of the curve is non-linear over the whole investigated concentration range and allows evaluation of concentrations up to 1000 µg $l^{-1}$ with external standards. The lowest concentration which could be measured was 10 µg $l^{-1}$. The detection limit of the method calculated as 3s value is 5.3 µg $l^{-1}$.

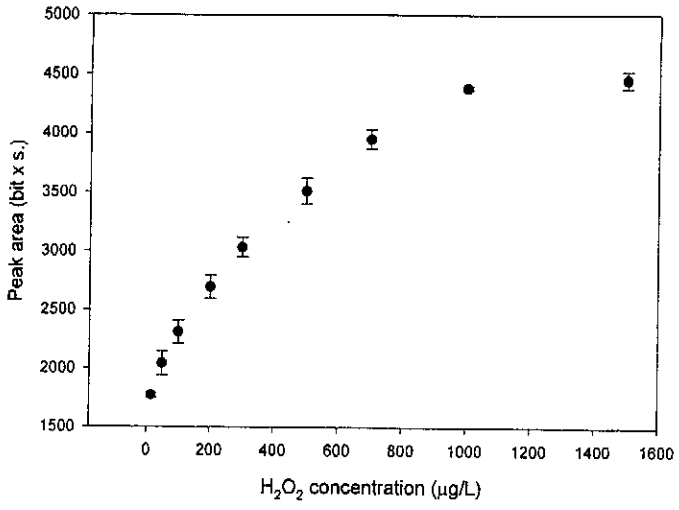


Fig. 10 Calibration curve for the sensor with DNPO; DNPO concentration: 1.2 mg $ml^{-1}$; 9,10-diphenylanthracene concentration: 3.6 mg $ml^{-1}$; polymer concentrations; 4.8 mg $ml^{-1}$; solvent volume: 2.5 ml

The results obtained show that for the preparation of the CL reagent for the TCPO system, required quantities are smaller than those in DNPO system. The detection limits, limit of quantitation and shape of calibration curve indicate that the sensor with TCPO is more stable and can be used with lower hydrogen peroxide concentrations.

The work presented here constitutes basic investigations on membrane sensors with oxalate complexes to determine hydrogen peroxide *via* chemiluminescence. The results of these fundamental studies are currently being applied to the determination of the target compound in samples such as washing powders, and to the development of biosensors.

## Conclusion

The new single-shot sensors based on peroxyoxalate chemiluminescence present a new device for on-site quantitation of hydrogen peroxide.

Bis(2,4-dinitrophenyl) oxalate (DNPO) and 2,4,6-trichlorophenyl oxalate (TCPO) have been found to be useful esters for this purpose, as they are the most efficient compounds to produce chemiluminescence *via* peroxyoxalates.

The system with TCPO is more sensitive and has better characteristics (lower quantities of reactants, lower detection limit and a better shape of

calibration curve) when compared to DNPO.

The sensors show highly promising features to develop single shot sensors for various types of analytes, but also biosensors based on oxidases which generate hydrogen peroxide as an intermediates.

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