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Comparative study of different fluorescent dyes for the detection of proteins on membranes using the peroxyoxalate chemiluminescent reaction

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Abstract

We have previously shown that the *bis*(2,4,6-trichlorophenyl)oxalate (TCPO)–H_2O_2 chemiluminescent reaction in acetone can be used for the detection of proteins labeled with the fluorescent reagent 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF) on polyvinylidene difluoride (PVDF) membranes. To improve this method, in this work we have designed and constructed a cell that allows us to perform this chemiluminescent reaction on PVDF membranes with a homogeneous distribution of the reagents. Using this cell we have examined the analytical properties of several recently developed fluorescent protein dyes chemically different from MDPF. We have found that the metal chelate dye SYPRO Ruby can also be excited by the high-energy intermediate produced in the TCPO–H_2O_2 reaction.
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1. Introduction

In the peroxyoxalate chemiluminescent reaction, an oxalate ester reacts with H_2O_2 giving rise to a high-energy intermediate that is able to excite different fluorophores [1,2]. Various structures have been proposed for the high-energy intermediate and it has been suggested that the excitation of the fluorophore is produced through a charge transfer mechanism [3,4]. The resulting chemiexcited fluorophore emits light of the same spectral properties observed after its photoexcitation in typical fluorescence experiments [5]. Several oxalate esters have been developed, but *bis*(2,4,6-trichlorophenyl)oxalate (TCPO) is the most used. In organic media the TCPO–H_2O_2 chemiluminescent reaction is very efficient, having a quantum yield higher than that observed for most of the chemiluminescent and bioluminescent reactions discovered to date [4,5].

These interesting photochemical properties of the peroxyoxalate reaction have been used to detect different fluorescent compounds in liquid chromatography, thin-layer chromatography and capillary electrophoresis [2,6–10]. Unfortunately, the analytical applications of TCPO and other peroxyoxalate reagents have been very limited because in aqueous media they are unstable and have a low solubility [11–13]. Micellar systems and water-soluble peroxyoxalate reagents, such as the *N,N′*-di(trifluoromethanesulfonyl) - *N, N′* - di(4 - sulfobenzylmethyl) - oxamide [14], have been developed but they do not

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show high chemiluminescence efficiency in analytical applications [11,15].

We have previously shown that the TCPO–H_2O_2 reaction in acetone can be used for the detection of proteins on polyvinylidene difluoride (PVDF) membranes [16], and DNA on nylon membranes [17]. After blotting, the proteins on the membrane were labeled with the fluorescent reagent 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF) and finally detected with the TCPO–H_2O_2 reaction. The main problem encountered with this method is the high and irregular background that is often observed in the resulting chemiluminescent images [16]. In this work, we have constructed a specially designed cell that allows us to carry out the TCPO–H_2O_2 reaction for the detection proteins on PVDF membranes with a significant reduction of the background. Furthermore, we have used this reaction cell to investigate the capability of various recently developed fluorescent dyes, different from MDPF, to become chemically excited by the peroxyoxalate chemiluminescent reaction.

2. Experimental

2.1. Chemicals and proteins

The chemiluminescent reagent TCPO was obtained from Sigma (St. Louis, MO, USA). Atto Label 590 NHS ester, MDPF and H_2O_2 (analytical grade) were obtained from Fluka (Buchs, Switzerland). The fluorescent dye BODIPY TR-X and SYPRO Ruby Protein Blot Stain solution were from Molecular Probes (Eugene, OR, USA). The proteins BSA, ovalbumin (chicken) and trypsinogen (bovine pancreas) were purchased from Sigma. Lysozyme (chicken egg white) was from Merck (Darmstadt, Germany). Organic solvents and other chemicals were analytical or HPLC-grade.

2.2. Preparation of slot blots and fluorescent labeling of proteins

PVDF membranes were obtained from Bio-Rad (Hercules, CA, USA). Slot blots were carried out in a 24-Slot Blotting Manifold (Hoefer Scientific Instruments, San Francisco, CA, USA). In some experiments the fluorescent staining with MDPF was performed with wet PVDF membranes following the procedure described elsewhere [16,18]. The membranes (3.7×11.4 cm) were wetted with about 15 ml of methanol (about 5 s) and then equilibrated for 10 min in 100 ml of 10 m*M* sodium borate buffer (pH 9.5). Slots were loaded with decreasing amounts of proteins in 100 μl of borate buffer (pH 9.5). The protein solutions were filtered (vacuum set at 130–250 mm Hg) and then each slot was rinsed with 0.5 ml of the same buffer. Finally the membrane was incubated at room temperature for 20 min with a solution containing 80 ml of borate buffer (pH 9.5) and 400 μl of 35 m*M* MDPF in DMSO. After staining the membrane was briefly rinsed (about 10 s) with deionized water. The wet membrane was placed on a UV transilluminator and photographed with the Fluor-S MultiImager system (Bio-Rad) using the 520 long pass filter.

For SYPRO Ruby staining, the PVDF membrane was wetted with methanol and equilibrated for 10 min in deionized water. Slots were loaded with the indicated amounts of protein dissolved in deionized water, rinsed with deionized water and then, according to the procedure described by Berggren et al. [19], the membrane was allowed to dry completely. The dry membrane was floated face down in 7% (v/v) acetic acid–10% (v/v) methanol for 15 min at room temperature and then washed (face down) four times (for 5 min each time) with deionized water. Next, the membrane was floated face down in 10 ml of the SYPRO Ruby Protein Blot Stain solution for 20 min at room temperature and, finally, washed (face down) three times (for 1 min each time) with deionized water to remove excess dye. The stained dry membrane was epi-illuminated with UV light and photographed with the Fluor-S MultiImager system with a 610 long pass filter.

The staining procedure of dry PVDF membranes described for SYPRO Ruby was used with minor modifications for the staining with BODIPY and Atto Label. In the case of BODIPY, the membrane was floated face down, for 20 min at room temperature, in a solution containing 40 ml of 10 m*M* sodium borate buffer (pH 8.5) and 0.5 ml of a 0.79 m*M* solution of this reagent in DMSO. With the Atto Label the staining was performed under the same conditions in a solution containing 10 ml of borate buffer (pH 8.5) and 5 μl of a concentrated solution of this dye (2.4 m*M* in DMSO). In some experi-

ments we have also used the dry membrane protocol to stain proteins with MDPF. In this case the membrane was floated face down, for 20 min at room temperature, in a solution containing 40 ml of borate buffer (pH 9.5) and 200 μl of 35 m*M* MDPF in DMSO. The resulting stained dry membranes were epi-illuminated with UV light and photographed with the Fluor-S MultiImager system. The 610 long pass filter was used for BODIPY and Atto Label, and the 520 long pass filter for MDPF. In all the procedures described in this section, the washings and incubations of PVDF membranes were carried out on a rotary shaker at about 70 rpm. The staining solutions containing BODIPY, Atto Label and MDPF were prepared immediately before use from the concentrated stock solutions of these dyes in DMSO.

2.3. Construction of the TCPO–H_2O_2 reaction cell for membranes and chemiluminescent detection of proteins

The home-built cell for the chemiexcitation with the peroxyoxalate reaction of the fluorescent labeled protein on membranes is shown schematically in Fig. 1. The glass plates (8×17 cm) together with the Teflon spacer (internal dimensions 5×13 cm; 0.7 mm thick) form a reaction chamber that avoids the evaporation of the TCPO solvent (acetone) and produces a close contact between the Whatman 3 MM filter papers and the PVDF membrane. The transparency of the glass plates (2 mm thick) in the visible region is high; the upper glass plate does not absorb significant amounts of the light produced in the different chemiluminescent reactions investigated in this work.

In all cases the PVDF membranes with the fluorescent labeled proteins should be dry before initiation of the chemiluminescent detection protocol. The dry fluorescent stained membrane was fully wetted with a fresh solution containing 20 μl of 30% (w/v) H_2O_2 in 2 ml of acetone. The membrane was allowed to air-dry and the deposition of H_2O_2 and drying process was repeated once. Immediately, two sheets of Whatman 3MM paper (cut to the same size as the PVDF membrane) were wetted with 4 ml of a fresh solution of 4.5 m*M* TCPO in acetone. A sheet of Whatman 3MM paper of the same size but without TCPO was placed quickly on top of these

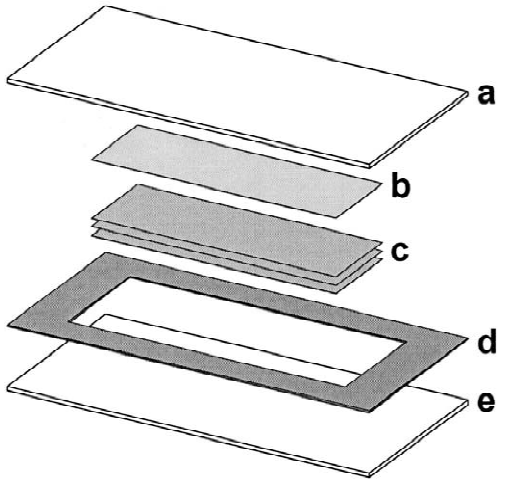

Fig. 1. Schematic drawing of the TCPO–H_2O_2 reaction cell for the chemiluminescent detection of proteins on PVDF membranes. Two sheets of Whatman 3MM filter paper (c, bottom) were wetted with TCPO in acetone, a sheet of Whatman paper (c, top) without TCPO and acetone was placed on top of these filters, and the dry PVDF membrane (b) previously treated with H_2O_2 in acetone was quickly laid over them. The reaction chamber was immediately sealed by the Teflon spacer (d) sandwiched between the glass plates (a and e). The assembly was held together with four metal spring clips and placed quickly in the Fluor-S MultiImager system for image acquisition. Additional experimental details are given in Section 2.3.

two sheets and the PVDF membrane treated with H_2O_2 was placed on the top of the set of filters. Finally, and very quickly, the reaction cell was sealed (see legend of Fig. 1) and placed on the Fluor-S MultiImager system with the PVDF membrane facing the CCD camera. Image acquisition time was varied in different experiments using the Quantity One (Bio-Rad) software. In all chemiluminescence experiments the reaction cell and all the solvents and solutions were equilibrated with the temperature of the room (22–24 °C) before the initiation of the detection protocol.

3. Results and discussion

3.1. Comparison of different fluorescent staining methods for proteins on membranes

In Fig. 2 the results obtained in the fluorescent staining with MDPF can be compared with the results obtained with other dyes. The MDPF staining

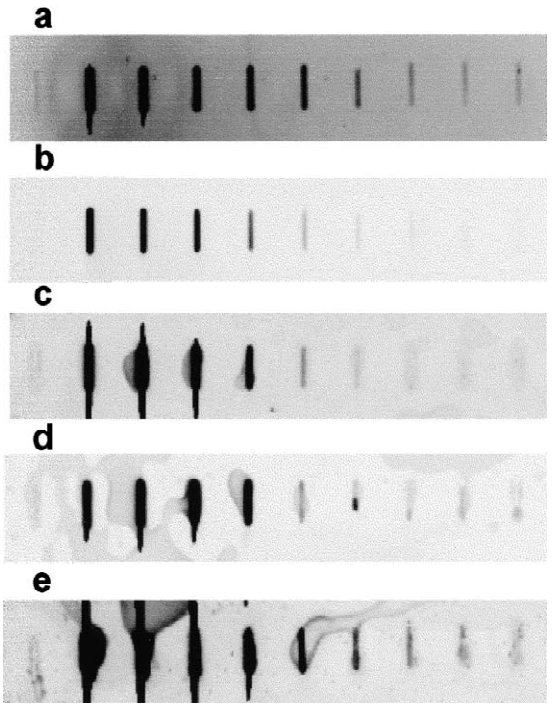

Fig. 2. Fluorescence of protein slot blots labeled with different dyes. The labeling with MDPF was carried out using the wet (a) and dry (b) membrane protocols (see Section 2.2). The reactions with BODIPY (c), Atto Label (d) and SYPRO Ruby (e) were performed on dry membranes. In all cases, the amount of BSA blotted was, from left to right, 0, 10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.013, 0.0046, and 0.0015 μg. In several blots the bands containing high amounts of protein produce distorted images due to a local saturation of the Fluor-S CCD camera. In all blots the acquisition time was 3 s.

was carried out with wet membranes (blot a) according to the procedure previously developed in our laboratory [16,18]. For the staining with SYPRO Ruby (blot e) and BODIPY (blot c) we followed the procedures described by Berggren et al. [19] and Pretty On Top et al. [20], respectively, which require dry membranes (see Section 2.2). We have also been able to extend this solid-phase method to stain proteins on dry membranes with Atto Label (blot d). Our results also demonstrate that even MDPF can react with blotted proteins on dry membranes (blot b), but the resulting fluorescence intensity is lower than that obtained with the original procedure performed with wet membranes. On the contrary, we have been unable to stain proteins on wet membranes with SYPRO Ruby. The best results are obtained with the original MDPF procedure and with SYPRO Ruby on wet and dry membranes, respectively. Less than 5 ng of protein per slot can be detected with these fluorescent staining methods.

3.2. Characteristics of the TCPO–H_2O_2 chemiluminescent reaction performed in the specially designed cell

As indicated in the Introduction, in our previous studies about the application of the peroxyoxalate chemiluminescent reaction for the detection of fluorescent proteins on membranes we frequently observed a nonhomogeneous background [16]. This problem is presumably due to the irregular distribution of the reagents in the transparent plastic bag that contained the PVDF membrane and the filter papers used in our original procedure. In contrast, in the reaction cell described in Section 2.3 and Fig. 1, the reaction chamber formed by the glass plates and the thin spacer produces a close contact between the sheets of filter paper and the membrane, thus avoiding the nonhomogeneous accumulation of the chemiluminescent reagents.

Furthermore, considering that an additional cause of background generation is the intrinsic chemiluminescence produced by the TCPO–H_2O_2 reaction in the absence of fluorophores [16,21], this reaction is performed inside the cell under conditions adjusted to reduce this second cause of background. Note that in the procedure described in Section 2.3, the reagents TCPO and H_2O_2 are initially separated. Since after the addition of the H_2O_2 solution in acetone, the membrane is allowed to air-dry for a few minutes to evaporate completely the acetone, the membrane becomes essentially dry but retains enough H_2O_2 to make possible the chemiluminescent reaction. Once the Whatman filters wetted with the solution of TCPO in acetone become in contact with the dry PVDF membrane inside the reaction chamber, there is a spontaneous transfer of the TCPO solution from the wet filters to the dry membrane. This causes the initiation of the peroxyoxalate reaction almost exclusively in the PVDF membrane, thus favoring the efficient chemiexcitation of the fluorescent labeled proteins by the intermediate produced in the TCPO–H_2O_2 reaction

and decreasing the background chemiluminescence. Examples of the low and homogeneous background obtained using this system are presented in Figs. 3 and 4. The background intensity is only significant around the bands that contain large amounts of protein and produce very intense chemiluminescence emission.

Finally, the third improvement is related with the kinetics of the reaction. In order to have enough time to seal the reaction cell and to place it in the Fluor-S MultiImager without losing any significant amount of chemiluminescence we have placed an additional sheet of Whatman filter between the two sheets containing TCPO and the PVDF membrane (see Section 2.3). This extra filter without reagents reduces the rate of TCPO transfer to the membrane and consequently delays the initiation of the chemiluminescent reaction. The kinetic results presented in Fig. 5 show that, when the TCPO–H_2O_2 reaction is performed in the specially designed cell, the fluorescent-labeled protein bands emit light for about 60 min.

3.3. Compatibility of the different fluorescent labels with the TCPO–H_2O_2 chemiluminescent system

We have used the reaction cell to examine the emission efficiency in the TCPO–H_2O_2 reaction of the fluorescent dyes considered above. Unfortunately, the background observed with membranes stained with BODIPY and Atto Label (results not shown) is higher that that produced in membranes excited with UV-light (Fig. 2) and precludes the detection of slots with low amounts of protein. This is presumably due to the unspecific binding of these fluorescent dyes to the hydrophobic PVDF membrane during staining and to the enhancement of emission caused by the acetone present in the chemiluminescent system. We have been unable to eliminate the background even using organic solvents (methanol and acetone) to wash the membrane after staining. In contrast, MDPF allows the visualization of proteins with the TCPO–H_2O_2 reaction without the problems found with these dyes (Fig. 3, blots a and c). These differences can be explained taking into account that whereas BODIPY and Atto Label are fluorescent before the reaction with proteins, neither MDPF itself nor the hydrolysis products produced during the labeling reaction are fluorescent [22].

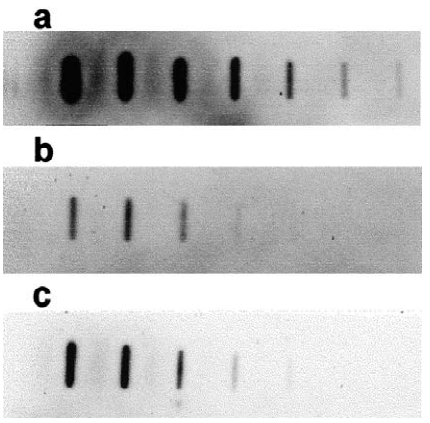

Fig. 3. Chemiluminescence of the TCPO–H_2O_2 reaction on protein slot blots labeled with MDPF and SYPRO Ruby. The labeling with MDPF was performed using the wet (a) and dry (c) membrane protocols; SYPRO Ruby labeling (b) was performed on a dry membrane (see Section 2.2). In all cases, the amount of BSA blotted was, from left to right, 0, 10, 3.3, 1.1, 0.37, 0.12, 0.041, and 0.013 μg. In all blots the acquisition time with the Fluor-S CCD camera was 60 min.

SYPRO Ruby is a ruthenium-based metal chelate

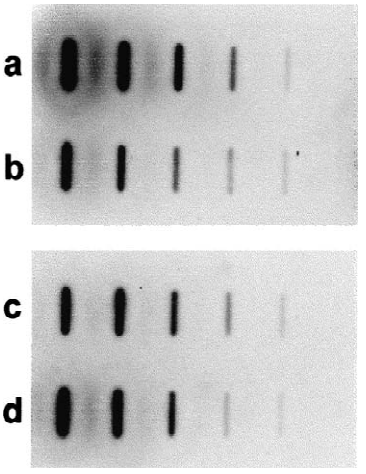

Fig. 4. Chemiluminescent detection of different proteins labeled with MDPF on slot blots. (a) BSA; (b) trypsinogen; (c) ovalbumin; (d) lysozyme. The labeling of proteins with MDPF was performed using the wet membrane protocol described in Section 2.2. In all cases, the amount of protein blotted was, from left to right, 10, 2.5, 0.63, 0.16, 0.039, and 0 μg. In all blots the acquisition time with the Fluor-S CCD camera was 60 min.

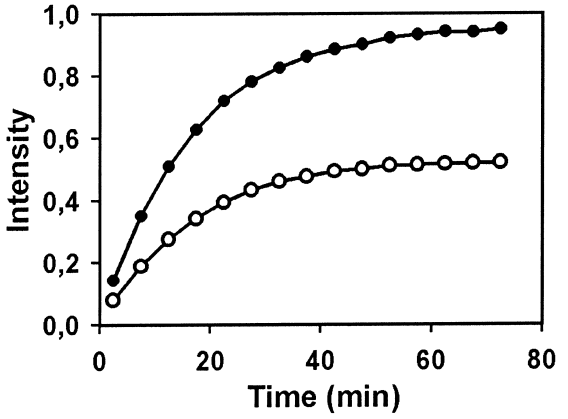

Fig. 5. Kinetics of the chemiluminescence produced by the TCPO–H_2O_2 system performed using the reaction cell described in Fig. 1 and Section 2.3. The bands analyzed contained 0.37 (●) and 0.12 (○) μg of BSA labeled with MDPF. Each point corresponds to the chemiluminescence (in arbitrary units) accumulated since the initiation of the reaction by the Fluor-S MultiImager system.

stain that interacts noncovalently with proteins [19]. As can be seen in Fig. 3 (slot b) this dye is compatible with the TCPO–H_2O_2 chemiluminescent system. However, the best results (detection limit of about 10 ng of protein per slot) have been obtained with MDPF staining following the procedure for wet membranes described above (blot a). When this dye is used to stain dry membranes (blot c), it produces similar results to those found with SYPRO Ruby. In both cases, however, the sensitivity obtained is significantly lower than that observed for MDPF-stained wet membranes. Furthermore, the results presented in Fig. 4 demonstrate that the TCPO–H_2O_2 reaction performed in the cell designed in this work permits the visualization of different proteins labeled with MDPF.

The relatively low chemiluminescence obtained with SYPRO Ruby is surprising because, as described in Section 3.1, the fluorescence of SYPRO Ruby-labeled membranes is roughly the same as that obtained with MDPF (Fig. 2, blots a and e). In addition, the excitation spectrum of SYPRO Ruby (maximum at about 450 nm [19]) compared with that of MDPF bound to proteins (maximum at about 390 nm [22]) indicates that the former dye requires less energy to be electronically excited. Since this should in principle facilitate the chemiexcitation of SYPRO Ruby by the high-energy intermediate of the peroxyoxalate reaction, it can be suggested that the relatively low yield of chemiluminescence emission observed with this dye is due to specific physicochemical requirements of this reaction that are better accomplished by the MDPF label.

4. Concluding remarks

As indicated in the Introduction, the TCPO–H_2O_2 chemiluminescent reaction in organic solvents has a very high quantum yield. In principle, this property makes this reaction very interesting from an analytical point of view. Unfortunately, however, the aqueous solutions currently used in analytical biochemistry reduce dramatically the efficiency of this reaction. Thus, in order to take advantage of the chemiluminescent properties of the peroxyoxalatereaction it is necessary to develop new analytical methods in organic media. In this work we have improved a procedure previously developed in our laboratory [16], in which the TCPO–H_2O_2 reaction in acetone was used for the chemiluminescent detection of proteins on PVDF membranes. We have designed a reaction cell that (i) avoids the nonhomogeneous accumulation of reagents, (ii) reduces the background due to the intrinsic chemiluminescence produced by the TCPO–H_2O_2 reaction in the absence of fluorophores, and (iii) extends the time of light emission. Furthermore our results show that, in addition to the covalent fluorescent reagent MDPF used in our previous study, the recently developed noncovalent dye SYPRO Ruby can also be used as energy acceptor in the peroxyoxalate reaction. The sensitivity obtained with this chemiluminescent method is relatively high (about 10 ng of protein labeled with MDPF), but it is lower than that obtained by the direct excitation of the fluorescent dyes with UV-light. Considering the remarkable chemiluminescent properties of the peroxyoxalate system, it can be expected that further improvements of the system presented in this work will permit to obtain higher sensitivities.

Acknowledgements

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