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L-Tyrosine-based biocompatible low-toxic substrate of peroxyoxalate chemiluminescent reaction

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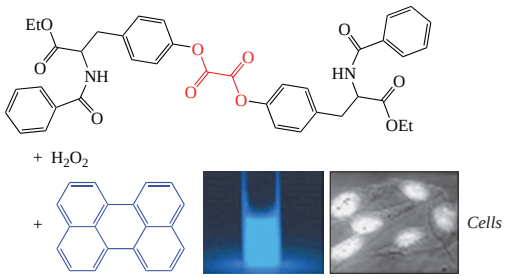
We synthesized a biocompatible substrate of the peroxyoxalate chemiluminescent reaction on the basis of *N*-benzoyl-L-tyrosine ethyl ester. Its ability to interact with hydrogen peroxide was evaluated by light emission in the presence of a perylene activator. Its cytotoxicity is three times less than that of bis[2,4,5-trichloro-6-(pentyloxycarbonyl)phenyl] oxalate (CPPO).



Keywords: peroxyoxalate chemiluminescent reaction, poly-L-lactide–PEG amphiphilic copolymers, L-tyrosine, bis[2,4,5-trichloro-6-(pentyloxycarbonyl)phenyl] oxalate, chemo-induced PDT, micelles.

Peroxyoxalate chemiluminescent (POCL) reaction of activated oxalic acid derivatives with hydrogen peroxide in the presence of a polyaromatic compound was first discovered by Chandross in 1964.[1] The reaction proceeds *via* the nucleophilic attack of hydrogen peroxide toward the oxalic ester moiety with a release of two phenol molecules and the formation of four-membered 1,2-dioxetanedione (Figure 1). Decomposition of the latter into two CO_2 molecules is catalyzed by a polyaromatic compound (activator) through the chemically induced electron exchange luminescence mechanism.[2] The excessive energy is released as photons. The quantum efficiency of the reaction is mainly determined by the reductive properties of the activator.

As early as 1986, Philip *et al*. proposed using the energy of this reaction to excite photosensitizer molecules, thus replacing the external excitation source in photodynamic therapy (PDT), which would greatly expand the potential of PDT in treatment of deep-seated tumors.[3,4] Noteworthy, exogenic hydrogen peroxide was administered in these works. Several scientific groups demonstrated the POCL reaction in cells or animal tissues without addition of H_2O_2, but it occurred in the inflammation area with elevated concentrations of endogenous hydrogen peroxide released by leucocytes,[5–8] or was induced under oxidative stress.[9,10] The POCL reaction represents a development of the well-known method of photodynamic therapy and therefore can be designated as chemo-induced PDT. The approach is of prime interest in relation to cancer cells characterized by elevated production of hydrogen peroxide.[11,12]

In vivo application of the POCL reaction requires components of low toxicity. However, byproducts of this reaction are phenol derivatives (see Figure 1) which are toxic in most cases due to uncoupling of oxidative phosphorylation, neurotoxicity or influence on the endocrine status of the patient.[13,14] In contrast to synthetic phenols, L-tyrosine cannot penetrate into mitochondria or affect endocrine and neuronal pathways. Therefore, use of tyrosine derivatives as building blocks for oxalate synthesis seemed quite reasonable.

Herein, we suggest *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as a low-toxic biocompatible tyrosine derivative, which was cross-linked with oxalyl chloride in a pyridine/THF mixture [Figure 2(*a*) and Online Supplementary Materials, Table S1]. Oxalyl chloride was added to BTEE excess to avoid the formation of the oxalic acid monoester. Any chromatography procedures for BTEE-oxalate (BTEE-ox) purification were avoided because water or silica gel could promote oxalate hydrolysis.[15] The product was analyzed by ^{1}H NMR. The signal at 6.53 ppm corresponding to the phenolic hydroxyl in the original BTEE

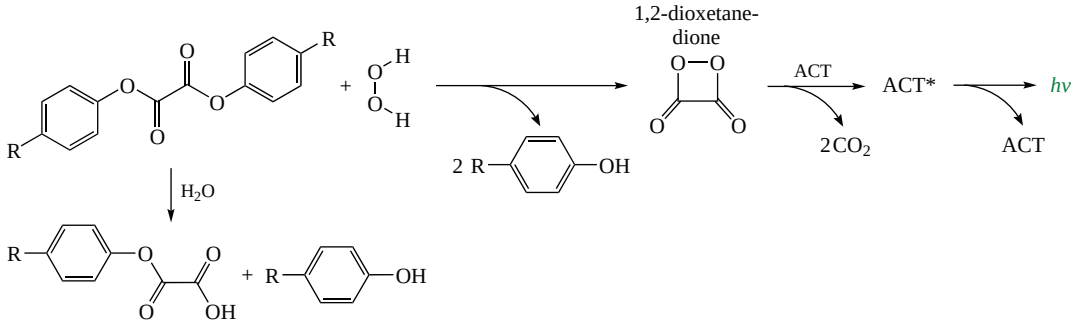


Figure 1 Peroxyoxalate chemiluminescent reaction and oxalate hydrolysis (ACT is an activator molecule, perylene, in the present work).

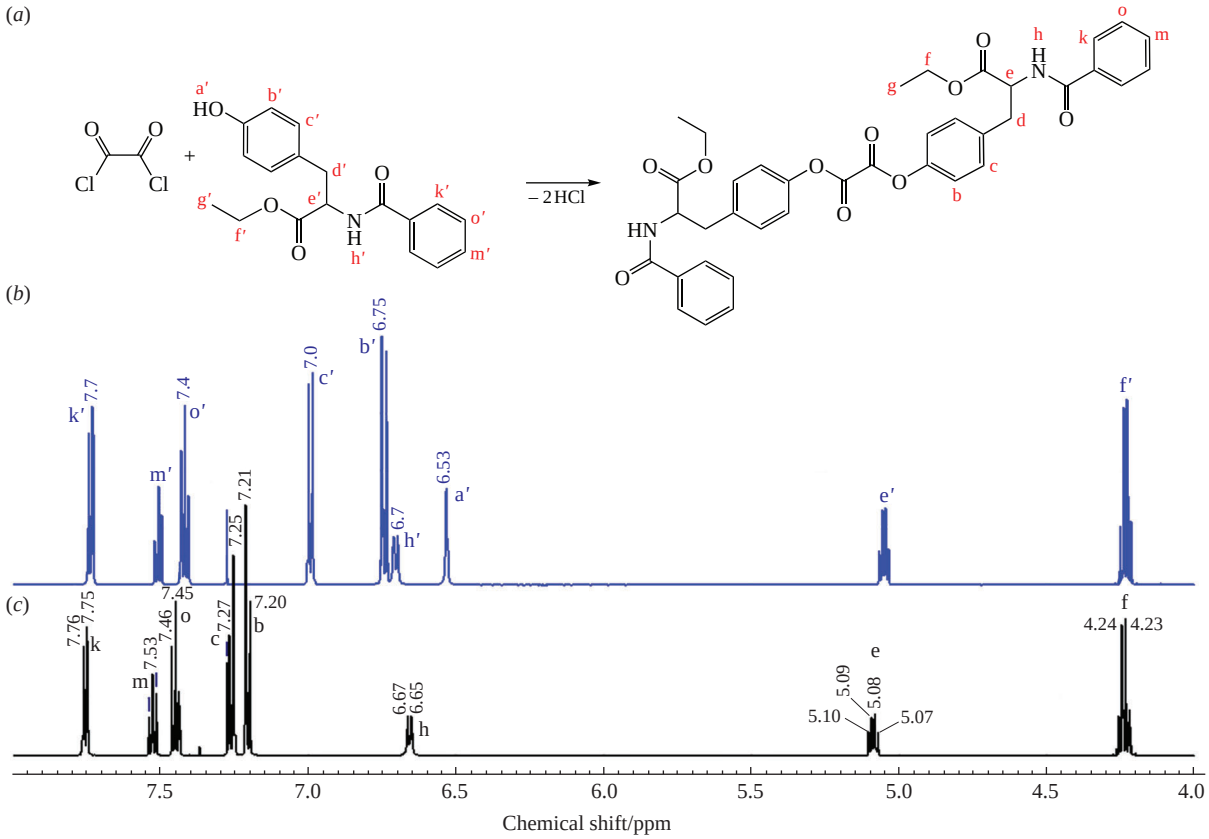


Figure 2 (*a*) Synthetic scheme for BTEE-ox; (*b*) ^{1}H NMR ($CDCl_3$) for starting BTEE and (*c*) for purified BTEE-ox.

[see Figure 2(*b*)] disappeared in the oxalate spectrum indicating the absence of the monoester in the final product [see Figure 2(*c*)].

The signals corresponding to the phenolic protons in the *ortho* position with respect to the hydroxyl gorup are shifted from 6.75 ppm [see Figure 2(*b*), signal b′] to 7.21 ppm [see Figure 2(*c*), signal b]. The signals corresponding to the protons in the *meta* positions are shifted from 7.00 ppm [see Figure 2(*b*), signal c′] to 7.27 ppm [see Figure 2(*c*), signal c], which is obviously caused by the electron withdrawing properties of the oxalic carbonyl group. Other proton signals were shifted insignificantly (see Table S1).

The BTEE-ox interaction with hydrogen peroxide was tested in the presence of perylene as an activator in a mixture of THF and water–carbonate buffer, pH 7. Light emission was observed within a minute and faded due to oxalate depletion [Figure 3(*a*), curve *1*]. When POCL was performed in an organic-water mixture, oxalate hydrolysis contributed significantly to the efficiency of the chemiluminescence reaction. Previously, we have shown that placing oxalates in the micelles of Pluronic or dimethylsiloxane, *i.e.*, polyethylene glycol copolymers could significantly slow down hydrolysis and thereby increase chemiluminescence efficiency.[16] In the present work, we used a similar approach and formulated BTEE-ox into the micelles of poly(L-lactic acid) and polyethylene glycol block copolymers (PLLA–PEG) to protect the oxalate from hydrolysis. The hydrophobic core of such micelles is much less hydrated than those of Pluronics and has a semi-crystalline structure, which could additionally contribute to BTEE-ox protection. The PLLA–PEG block copolymer was synthesized by initiating L-lactide polymerization on the monomethoxy-PEG initiator using tin(II) octanoate as a catalyst as described previously.[17–19] The molecular weight characteristics and composition of the block copolymer were determined by exclusion chromatography and ^{1}H-NMR methods (see Online Supplementary Materials, Figure S1 and Table S2).

The PLLA61–PEG87 block copolymer was dissolved in THF and injected in the aqueous buffer (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4), which caused the formation of micelles with a diameter of 40 ± 15 nm (Figure S2). To incorporate the components of the POCL reaction into the micelles, the oxalate and perylene solutions were added to the polymer dissolved in THF, and the mixture was injected into the aqueous buffer. The POCL light emission was recorded after injection of H_2O_2. It turned out that incorporation of BTEE-ox into PLLA–PEG block copolymer micelles considerably increased the run time of the reaction between BTEE-ox and H_2O_2 [see Figure 3(*a*), *cf.* curves *1* and *2*]. The efficiency of the POCL reaction was evaluated by the integral intensity of chemiluminescence from the beginning of the light emission to the time corresponding to the signal decay to 10% of the maximum [the shaded areas in Figure 3(*a*),(*b*)]. Chemiluminescence efficiency of BTEE-ox was compared with that of the most effective oxalate, bis[2,4,5-trichloro-6-(pentyloxycarbonyl)phenyl] oxalate (CPPO).[20] Compound CPPO in PLLA–PEG micelles produced a prolonged

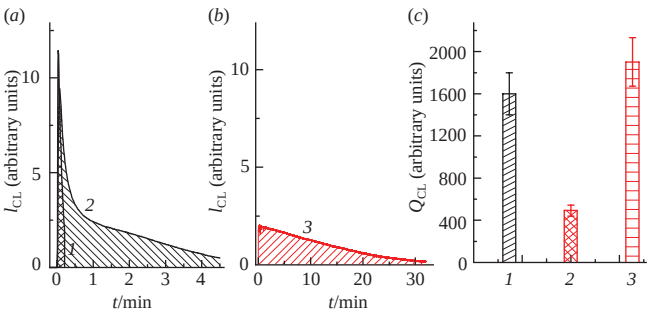


Figure 3 Light emission during the POCL reaction with (*a*) BTEE-ox and (*b*) CPPO in the 50 mM bicarbonate pH 7.5/THF (2 : 8) mixture (curve *1*) and in PLLA61-PEG87 micelles (curves *2*, *3*). (*c*) Quantum yields of the POCL reaction with CPPO (*1*) and BTEE-ox in the absence (*2*) and presence (*3*) of 25 mM imidazole. $C_{oxalate}$ = 0.5 mM, $C_{perylene}$ = 0.1 mM, $C_{PLLA-PEG}$ = 0.75%, and $C(H_2O_2)$ = 3 mM. The reactions proceeded at 37 °C.

chemiluminescence [see Figure 3(*b*)]. As the result, its integral chemiluminescence efficiency was thrice more than that of BTEE-ox [see Figure 3(*c*)]. The effect may be explained by higher hydrophobicity of CPPO leading to stronger interaction with the PLLA micellar core. However, when the POCL reaction was catalyzed by imidazole, the integral chemiluminescence intensity with BTEE-ox strongly increased [see Figure 3(*c*)]. These results confirm the applicability of the synthesized BTEE-ox as a substrate of the POCL reaction. At the same time, the use of imidazole in cell culture experiments or in pharmacological formulations is impossible due to its high lysosome disrupting activity.[21] Nevertheless, the results obtained show that despite the lower activity of BTEE-ox in chemiluminescence experiments, its use as a POCL substrate in chemo-induced PDT is reasonable not only because of its lower cytotoxicity, but also because its enzymatic degradation releases the natural amino acid L-tyrosine rather than trichlorosalicylic acid released during the enzymatic degradation of CPPO.

To assess its applicability for biological experiments on cells in culture, we analyzed cytotoxicity of BTEE-ox and CPPO. For this purpose, both substrates were solubilized in PLLA61–mPEG87 micelles and examined on ovary cancer cells NCI-ADR/RES. In the absence of oxalates, PLLA61–mPEG87 micelles were not toxic to cells up to 2.2 mg ml^{-1} (Figure 4, curve *1*). Their cytotoxicity increased significantly if loaded with oxalates (curves *2* and *3*). The number of survived cells decreased to 50% (IC_{50}) after incubation with 0.055 ± 0.005 mM CPPO (curve *3*). Three-fold higher concentration of BTEE-ox was needed to produce the same effect (IC_{50} = 0.155 ± 0.032 mM). It means that BTEE-ox based on the natural amino acid L-tyrosine was 3-fold less toxic than CPPO for cells in culture.

The cytotoxicity of oxalates was not due to the POCL reaction because the samples lacked any photosensitizer. It cannot be also ascribed to the oxalic acid because it is not cytotoxic at concentrations below 3 mM.[22] Therefore, we suggested that cytotoxicity of the tested oxalates was caused by phenols released due to hydrolysis. Hydrophobic phenols are known to be able to penetrate the cell membrane. Dissociation of the phenolic hydroxyl in mitochondria can reduce the pH-gradient at their membranes leading to a decrease in the ATP content and cell survival.[23,24]

This consideration was supported by comparison of phenols pK_a in BTEE-ox and CPPO molecules (see Online Supplementary Materials, Figure S3). The pK_a values of CPPO phenolic groups calculated with ACDLabs 6.0 were 4.56 ± 0.45, while the same analysis of BTEE-ox resulted in pK_a = 9.75 ± 0.15. A similar value (pK_a = 10.00 ± 0.03) was determined by spectrophotometric titration. The lower acidity of BTEE phenolic hydroxyls corresponds to the lower cytotoxicity of BTEE-oxalate (Table S3).

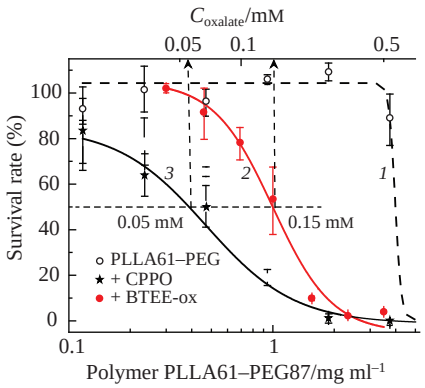


Figure 4 Cytotoxicity of free micelles of PLLA61–mPEG87 block copolymer (*1*), and the micelles loaded with BTEE-ox (*2*) or CPPO (*3*) toward human NCI/ADR-RES ovary cancer cells. The $C_{PLLA61-PEG87}/C_{oxalate}$ molar ratio was about 0.85 : 1 in all samples.

In conclusion, a new substrate of the POCL reaction synthesized from the natural L-tyrosine appeared to be less toxic for cells than the commercial CPPO prepared from synthetic phenols. Being biocompatible, BTEE-oxalate may be used in pharmacological assemblies aimed at the implementation of the principles of chemo-induced PDT. In addition, its combination with highly effective activators may allow the creation of chemiluminescent constructs designed for the detection of foci of the increased hydrogen peroxide content.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.10.018.

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