Peroxyoxalate Chemiluminescent Reaction as a Tool for Elimination of Tumour Cells Under Oxidative Stress

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The overproduction of hydrogen peroxide is an inherent feature of some tumour cells and inflamed tissues. We took advantage of this peculiarity to eliminate cells using chemiluminescent peroxyoxalate reaction. We designed dispersions containing polyoxalate and tetramethylhematoporhyrin (TMHP) in dimethylphthalate droplets stabilized with Pluronic L64. The porphyrin plays the dual role. On the one hand, it serves as an activator of the peroxyoxalate reaction of polyoxalate with intracellular hydrogen peroxide and experiences excitation as a result of the reaction. The light emitted in the reaction in the model system without cells was used to optimize the dispersion’s composition. On the other hand, TMHP acts as a photosensitizer (PS) causing cell damage. The formation of singlet oxygen led to cell elimination if the dispersions were used in combination with inducers of oxidative stress: hydrogen peroxide, paraquat, antitumour drug doxorubicin, or a nutritional additive menadione. The PS-induced cytotoxicity correlated with the level of intracellular ROS. The developed approach targeted to endogenous ROS is orthogonal to the classical chemotherapy and can be applied to increase its efficiency.

Photodynamic therapy (PDT) implies photochemical interaction of three components: light, photosensitizer (PS), and oxygen. Molecules of a PS, localizing into or in proximity to cells and exposed to external light source, turn into excited state and then transfer energy to ambient molecules, including molecular oxygen. This two-step process leads to generation of singlet oxygen, an extremely strong oxidizing agent that destroys cellular components thus causing toxic effects⁴. Excitation of PS molecule is the key step, which defines the effectiveness of the approach.

Despite significant progress in technical development of PDT, it is not free from limitations. One of them is the opacity of tissues that interferes with application of PDT for treatment of visceral and metastatic tumours²–⁵. In order to overcome this limitation, chemiluminescent reactions as a source of light have been proposed. Application of luciferase catalyzed oxidation of luciferin resulted in the development of “molecular flashlight” sufficient for PS excitation without external light source⁶,⁷.

Phillip et al. were the first who employed peroxyoxalate chemiluminescent (PO-CL) reaction as an internal light source⁸–⁹. The reaction proceeds between active derivatives of oxalic acid and hydrogen peroxide (Fig. 1a). The first step of the reaction is the nucleophilic attack of an oxalate by a molecule of peroxide-anion which results in formation of high-energy intermediate (HEI) 1,2-dioxetanedione (Fig. 1a, I). In the presence of activators (ACT) (different polyaromatic compounds), 1,2-dioxetanedione is able to transfer excess energy to these molecules while experiencing decomposition into two CO₂ molecules (Fig. 1a, II)¹⁰–¹².

Phillip et al. used PO-CL reaction for elimination of tumour cells⁸–º. Transplanted tumours were treated with a mixture of an active water-soluble oxamide, 1% solution of hydrogen peroxide and disulfurubrene served as an ACT. Chemiluminescence emitted by rubrene was absorbed by PS preliminary accumulated in the tumour thus creating conditions for intracellular generation of singlet oxygen.

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In this work the exogeneous hydrogen peroxide was used to trigger the chemiluminescent reaction. However large amounts of endogenous peroxide are often found in malignant cells and inflamed tissues. This peculiarity provides the possibility to run PO-CL reaction without addition of exogeneous peroxide and provoked a number of approaches to visualization and elimination of cells with increased ROS production. The first chemiluminescent system triggered by endogenous peroxide was presented by Laptev et al. In this work, oxidation of luminol with endogeneous H2O2 resulted in light emission. The emitted chemiluminescence was absorbed by the PS attached to the transferrin molecule thus inducing selective elimination of cells with elevated amounts of transferrin receptors.

Further, Lee et al. designed polyoxalate-containing nanoparticles responsive to endogenous hydrogen peroxide for in vivo imaging of inflamed tissues in mice. Later, peroxyoxalate chemiluminescent systems combined with the effect of aggregation-enhanced fluorescence and semiconducting polymers were applied for detection of hydrogen peroxide associated with lipopolysaccharide-induced inflammation.

The goal of the present study was to construct polyoxalate-containing dispersions capable of elimination of tumour cells through PO-CL reaction with endogenous hydrogen peroxide in the presence of tetramethylhematoporphyrin (TMHP) (Fig. 1d). The latter participated as an activator in PO-CL reaction as well as an effective PS for singlet oxygen generation, thus avoiding a superfluous step of ACT to PS energy transfer. POX and TMHP were formulated into dispersion droplets of dimethyl phthalate stabilized with Pluronic L64 as a surfactant (Fig. 1c). Cytotoxicity of these formulations toward human breast adenocarcinoma MCF-7/ADR cells without external light illumination was studied.

**Results**

**Synthesis of polymeric oxalate.** Polymeric oxalates have been reported to be comparatively resistant to hydrolysis and therefore can be used in PO-CL reaction in aqueous environment. POX (Fig. 1b) was synthesized through polycondensation of oxalyl chloride, bisphenol A and oligo(propylene glycol) (see Supplementary Fig. S1).

The polymer had a wide molecular weight distribution (see Supplementary Fig. S2). Molecular weight characteristics were estimated from calibration using polystyrene standards. Mw, Mn, and PDI values were found to be 4400, 1700, and 2.6, respectively. POX composition was determined with 1H-NMR and the molar ratio of monomers bisphenol A/oligopropylene oxide was found to be 0.85:0.15 (see Supplementary Fig. S3).

![Figure 1. Scheme of the designed approach.](image-url)
Preparation of polyoxalate dispersions. Since aromatic oxalates are susceptible to hydrolytic degradation, we endeavoured to diminish contact of POX with water in the course of dispersing procedure. To this end, POX was first dissolved in aprotic solvents. DMP and THF were used as the best solvents for POX among other tested. The solution was mixed with Pluronic L64 and then about 100-fold volume of aqueous buffer (PBS) was injected into the vial under intensive shaking at 37 °C resulting in spontaneous formation of dispersions. The surfactant forms a hydrophobic core and a hydrophilic corona which stabilizes the particles. Herein, the concentration of the dispersions is expressed in mg/mL of Pluronic L64 at an indicated weight ratio of other components.

The dynamic light scattering of the dispersions prepared from POX solutions in DMP and THF revealed two types of particles in both formulations. DMP led to formation of particles with the average hydrodynamic radii ($R_h$) about 105 ± 25 nm and 400 ± 100 nm (Fig. 2a), which remained practically unchanged during several hours (Fig. 2b). When THF was used instead of DMP, the particles were considerably smaller ($R_h$ about 23 ± 9 and 100 ± 30 nm).

Dilution of both dispersions resulted in a linear decrease in scattering intensity within the concentration range from 0.03 to 0.25 mg/mL of Pluronic L64 at a fixed Pluronic/POX/DMP weight ratio (1:2.7:0.3 by mass) (Fig. 2c, curve 1). It means that the particles retained even after 10-fold dilution of the dispersion. Similar results were obtained for the dispersions prepared from POX solution in THF (Fig. 2c, curve 2). Scattering intensity in this case was considerably less due to lower size of the particles.

The substantial difference between the dispersions prepared from POX solutions in DMP and THF revealed differences in solubilization of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). This technique is based on the increase in fluorescence of DPH upon its partitioning into hydrophobic microphase (Fig. S4 in Supplementary Information). High sensitivity of this approach affords an opportunity to determine the critical concentration of the dispersions of various compositions corresponding to the onset of a hydrophobic microphase.

Formation of a hydrophobic core in the solutions of Pluronic L64 was observed at concentrations above 0.35 mg/mL in complete agreement with the previously published data. Pluronic L64/DMP or L64/DMP/POX dispersions formed a hydrophobic microphase at 1.5 and 10-fold less concentrations (Fig. 2d), indicating that the solubilization of DMP and even more POX/DMP in Pluronic micelles stabilized the particles upon their dilution. In the latter case the critical concentration was as low as 0.03 mg/mL in full agreement with light scattering data (cf. Fig. 2d, curve 1 and 3c). In contrast, POX solution in THF caused only marginal effect on the critical concentration of the dispersions (Fig. 2d). Neither DMP, nor POX formed a hydrophobic phase in the absence of Pluronic L64.
These results show that THF used as a solvent for POX most probably undergoes partitioning into the continuous phase resulting in the formation of the dispersion particles made only of Pluronic and POX. On the contrary, if the dispersions were prepared from POX solution in DMP, the hydrophobic core contained not only Pluronic and POX but also DMP.

Efficiency of PO-CL reaction in buffer solution as a function of dispersion’s composition. PO-CL reaction between POX and hydrogen peroxide was performed in the presence of TMHP which participated as an activator\(^20\) and an effective PS\(^1\), combining both properties. The rate of the chemiluminescence reaction was very slow (Fig. 3a, curve 1). It was accelerated by more than an order of magnitude by addition of imidazole, an established catalyst of this reaction\(^24\) (Fig. 3a, curve 2). Previously\(^25\)–\(^28\), it was demonstrated that it possesses both base and nucleophilic catalytic activity and it accelerates the hydrogen peroxide attack on oxalate moiety and the formation of high-energy intermediates which is the rate-determining step of the whole process (Fig. 1a). This resulted in faster decay of chemiluminescence intensity and allowed reducing the time of light measurement to more convenient intervals (about half an hour).

Concentrations of imidazole below 30 mM did not affect the integral intensity of light (Q) emitted during the chemiluminescent reaction, while further increase in the concentration of the catalyst caused a slight decrease in chemiluminescence efficiency (Fig. 3b) in full agreement with the previous reports\(^24\), \(^29\). So, we performed all further experiments in the presence of 26 mM imidazole.

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The integral intensity of light emitted by DMP-containing dispersions was two orders of magnitude higher than that obtained with THF, indicating a striking difference between the arrangement of components in L64/DMP/POX and L64/THF/POX dispersions.

L64/DMP/POX dispersions capable of chemiluminescence generation in PO-CL reaction will be referred to as chemiluminescence dispersions (CLD). Their composition was optimized to achieve the maximum efficiency of PO-CL reaction.

The increase in the concentration of Pluronic L64 at a constant concentration of the hydrophobic components (2.7 mg/mL of DMP and 0.3 mg/mL of POX) led to a steep growth in integral intensity of chemiluminescence that reached a smooth maximum at the weight fraction of Pluronic being 0.34 (Fig. 4a). According to the classical concept of dispersion stability\(^30\) this amount of Pluronic corresponds to its minimum sufficient for coating the total surface of the particles. The PO-CL efficiency slowly receded at higher content of Pluronic.

The chemiluminescence intensity increased linearly with the concentration of the activator up to 20 μM TMHP (Fig. 4b) in complete agreement with the mechanism of PO-CL reaction (Fig. 1a). However, above this value the chemiluminescence intensity levelled off obviously due to the self-quenching of porphyrin luminescence.

Variation of POX concentration in DMP solution at a fixed concentration of TMHP was found to be of primary importance for the efficiency of PO-CL reaction. The integral intensity normalized to the amount of POX decreased with the growth of POX concentration in DMP (Fig. 4c, curve 1). Low POX concentrations (2.5 and 5.0 mass % in DMP) form the plateau on the plot and provide the highest chemiluminescence yield. However, these two points are characterized by relatively low absolute intensities (Fig. 4c, curve 2, right axis). At the same time, the dispersions prepared from 10% and 30% of POX in DMP produced nearly similar integral intensities of chemiluminescence (Fig. 4c, curve 2). Therefore, we accepted 10 mass % to be the optimum POX content in DMP since it provides high integral intensity (Q, right axis) as well as a suitable chemiluminescence efficiency (Q/C\(_{POX}\)).

Based on these results, we determined the optimum L64/DMP/POX/TMHP weight ratio as equal to 1:2:7:0.3:0.05.
Stability of POX toward hydrolysis in the dispersions prepared from 10 mass % of POX in DMP in the presence of 1.0 mg/mL of Pluronic L64 was evaluated by incubation of the dispersions in aqueous buffer at 37 °C during various time intervals before injection of hydrogen peroxide and imidazole. Half-life period of POX in the dispersions was found to be about 1.4 h (Fig. 4d) that is appropriate for cell culture experiments. Further we studied the capacity of CLD to generate singlet oxygen due to PO-CL reaction.

Generation of singlet oxygen during PO-CL reaction in the cell-free system. To obtain experimental evidence for singlet oxygen generation in PO-CL reaction, we used the commercially available probe Singlet Oxygen Sensor Green (SOSG, Fig. 4e). Fluorescent properties of fluorescein are suppressed in this

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**Figure 4.** Relative integral intensity of PO-CL reaction ($Q_{rel} = Q/Q_{max}$) as a function of the composition of the dispersions and evidence for singlet oxygen generation in PO-CL reaction in cell-free system. (a) Variation of Pluronic L64 content expressed as its weight ratio toward the organic phase (DMP + POX). C(DMP) = 2.7 mg/mL, C(POX) = 0.3 mg/mL, C(TMHP) = 7.6 μM; (b) Variation in TMHP concentration. C(L64) = 1.0 mg/mL, C(DMP) = 2.7 mg/mL, C(POX) = 0.3 mg/mL. (c) Variation in POX content: (1) normalized PO-CL efficiency per POX amount (left axis, $Q/C_{POX}$) and (2) the relative integral intensity (right axis) on the overall POX concentration (top axis) and POX fraction in DMP solution (bottom axis). C(L64) = 1.0 mg/mL, C(DMP + POX) = 3.0 mg/mL, C(TMHP) = 7.6 μM; (d) The kinetics of hydrolysis of POX in the dispersions of L64/DMP/POX (1:2.7:0.3 weight ratio). In all experiments $C(\text{imidazole}) = 26$ mM, $C(\text{H}_2\text{O}_2) = 15.0$ mM. (e) Structure of Singlet Oxygen Sensor Green fluorescent probe and the reaction of its oxidation by singlet oxygen. (f) Kinetics of SOSG oxidation in the presence of 30 μM H₂O₂ and complete CLD (1), the dispersion without TMHP (2), or POX (3).
molecule due to intramolecular electron transfer. Oxidation of anthracene moiety in SOSG by singlet oxygen destroys the conjugation system and restores bright fluorescence of chlorofluorescein fragment.

The manufacturer of SOSG reports extremely high responsiveness of SOSG to singlet oxygen. Unexpectedly, we observed a fast oxidation of SOSG in the presence of 20 mM \( \text{H}_2\text{O}_2 \) without a PS (see Supplementary Fig. S5a), the reaction being occurred until exhaustion of all SOSG. However, the oxidation rate decreased in line with \( \text{H}_2\text{O}_2 \) concentration and became negligible at 100 \( \mu \text{M} \) and 30 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (see Supplementary Fig. S5a). This fact clearly showed that SOSG could be used for monitoring \(^1\text{O}_2\) formation during PO-CL reaction in the presence of low \( \text{H}_2\text{O}_2 \) concentrations.

In the presence of complete CLD, the kinetics of oxidation of SOSG in the presence of 30 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) obeyed the monoeponential law with characteristic time about 20 min that corresponded to the kinetics of PO-CL reaction (Fig. 4f, curve 1). Further incubation of the reaction mixture after completion of PO-CL reaction did not result in an additional oxidation of SOSG. It means that SOSG oxidation in this case occurred due to the reaction with singlet oxygen generated by TMHP during PO-CL reaction, rather than direct oxidation by \( \text{H}_2\text{O}_2 \) or by molecular (atmospheric) oxygen. Importantly, the omission of either POX or TMHP in CLD formulation completely prevented SOSG from oxidation at low concentrations of \( \text{H}_2\text{O}_2 \) (Fig. 4f, curves 2 and 3).

It is known, that porphyrins triplet state lifetime is considerably enhanced in deuterated solvents. We observed that the replacement of water by \( \text{D}_2\text{O} \) resulted in a two-fold increase in the amount of SOSG endoperoxide produced during PO-CL reaction in the presence of the complete CLD (see Supplementary Fig. S5b). This result gave additional argument in favour formation of singlet oxygen during PO-CL reaction in the cell-free system.

**Interaction of chemiluminescent dispersions with cells.**

Cytotoxicity of dispersions. CLD formulations were tested on multidrug resistant human breast adenocarcinoma MCF-7/ADR cells as an example of refractory cell line. The cytotoxicity of the CLD formulations (Fig. 5a, curve 1) was compared to that of the reference dispersion deprived of TMHP (Fig. 5a, curve 2). Their IC50 values were nearly similar within the experimental error (0.21 ± 0.03 and 0.26 ± 0.02 mg/mL respectively). We suggested that inability of CLD to eliminate their exposure to complete medium results in the decay of peroxide concentration in the medium nearly to zero due to the presence of serum catalase (see Supplementary Fig. S7c).

At the same time, the level of intracellular fluorescence of DCF in the same samples increased after 1 h incubation of the cells in the presence of 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) and remained constant during at least 1.5 h incubation in the serum-containing medium (see Supplementary Fig. S7d).

**Figure 5.** Interaction of CLD with MCF-7/ADR cells. Dose-response survival curves of MCF-7/ADR cells either untreated (a), or pretreated with 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (b) in the presence of complete CLD (1) and reference dispersion deprived of TMHP (2).
Thus, a significant PS-mediated cytotoxicity was observed in the cells under oxidative stress. Of special note is that the intracellular PO-CL reaction occurred with hydrogen peroxide biosynthesized in response to stimulation.

Chemiluminescence in MCF-7/ADR cells under oxidative stress conditions. Use of CLD formulations did not allow detecting light emission in cells using LCSM technique. Obviously this was due to special features of hematoporphyrin, which is supposed to be a very efficient photosensitizer (quantum yield of singlet oxygen generation is 0.7440), but rather poor fluorophore (fluorescence quantum yield in mitochondria was found to be 0.11–0.1340). To enhance the rate of light emission we modified CLD composition. TMHP was replaced for perylene (Fig. 6a) that does not exhibit photosensitizing activity, but manifests extremely high fluorescence quantum yield (about 0.9241). This fluorophore emits blue light which is most effectively detected by the majority of photomultipliers. Another apparent factor capable of increasing the rate of light emission in PO-CL reaction is addition of a nucleophilic catalyst. Imidazole effectively used in cell-free system (Fig. 3) is highly toxic for cells in culture. In addition, it poorly accumulates in the cells owing its hydrophilicity. Instead, we used benzimidazole (Fig. 6a) that readily partitions into the organic phase of CLD and therefore can be used at much lower concentrations. So, the modified CLD formulation contained 1.0 mg/ml Pluronic L64, 2.5 mg/ml DMP, 2.0 mM POX, 0.4 mM perylene and 1 mM benzimidazole.

The designed dispersion was considerably more sensitive to H₂O₂ than that based on TMHP/imidazole (Fig. 6b). Addition of CLD containing perylene and benzimidazole to the monolayer of cells under oxidative stress conditions (about 0.45 millions per sample) resulted in a detectable emission of light (Fig. 6c). These results confirmed that PO-CL reaction can be triggered by intracellular hydrogen peroxide.

Intensity of light depended on cell density. The integral of emitted light decreased with diminution in the amount of cells (Fig. 6d) confirming that hydrogen peroxide in the sample was originated from the cells and not from the contamination with H₂O₂ used for oxidative stress stimulation.

The linear character of this dependence gave an opportunity to evaluate the amount of hydrogen peroxide in MCF-7/ADR cells in oxidative stress state. The ratio of the slopes of lines in Fig. 6d and line 2 in Fig. 6b gave about 0.9 nmoles per million of cells. Noteworthy, this concentration was detected in the cells stimulated with the maximum tolerable concentration of H₂O₂.
The effect of CLD composition on the PS-mediated cytotoxicity. Thus, we have found that the CLD composition optimized by means of chemiluminescence measurements in cell-free systems appeared to be optimum to cause PS-mediated cytotoxicity in vitro (see Fig. S8 and the corresponding explanations in Supplementary Information online). This fact implies that the CLD cytotoxicity was mainly determined by PO-CL reaction resulting in production of toxic species.

Accumulation of the dispersions in the cells and generation of singlet oxygen due to PO-CL reaction. Cells treatment with CLD resulted in an uptake of the TMHP-containing particles and their distribution all over the cytoplasm (red fluorescence) except for nuclei (blue fluorescence) (Fig. 7a). To obtain evidence for singlet oxygen generation in the cells due to PO-CL reaction we used SOSG. This probe is considered by the manufacturer to be cell-impermeable. However, it was shown that in the absence of serum it is accumulated in cells and can be oxidized by singlet oxygen produced by different photosensitizers. Addition of SOSG to the intact cells (Fig. 7b) or the cells stimulated with H$_2$O$_2$ (Fig. 7c) resulted in a subtle staining of cytosol. However, addition of complete CLD with SOSG to the stimulated cells resulted in a considerable staining of all cellular compartments including nuclei (Fig. 7d). In contrast, the CLD without TMHP caused much lesser staining than complete CLD (Fig. 7e). However, some cells in this sample were considerably brighter than untreated cells (Fig. 7b and c).

Distributions of cells by the fluorescence intensity in the samples (a-d) show that the mean fluorescence of the cells in the sample treated with CLD is twice of that treated with the dispersion without TMHP (Fig. 7f). Similar results were obtained in the independent experiment, where the cell monolayers were treated with different compositions and SOSG-endoperoxide fluorescence was measured using plate fluorometer (Fig. 7g).

Figure 7. Intracellular localization of TMHP and oxidized SOSG in the cells treated with CLD. (a) LCSM image of the cells treated with complete CLD in the serum-free culture medium for 1 h at 37 °C and fixed with 4% formaldehyde in PBS. Nuclei were stained with 0.3 μM DAPI. The left panel shows merged blue and red channels, the right panel shows differential interference contrast image. (b–g) Evidence for singlet oxygen generation due to PO-CL reaction in the cells. Confocal images of (b) intact cells, (c) the cells in the oxidative stress state, (d) similarly prepared cells treated with the complete and (e) reference CLD without TMHP. All samples were stained with 10 mM of SOSG (f) Distributions of the cells by the intensity of their fluorescence calculated from the analysis of at least 10 different images containing about 300 cells. 1 – intact cells, 2 – the cells under oxidative stress conditions, 3 – the cells prepared as in the sample (2) and treated with the complete CLD or 4 – with the reference dispersion without TMHP. (g) Measurement of SOSG endoperoxide fluorescence in the similarly prepared samples using plate fluorometer. Each sample contained about 0.2 millions of cells.
It seems probable that a considerable amount (about 15%) of brightly stained cells in the sample treated with the control dispersion (Fig. 7e) is caused by the ability of SOSG to serve not only as a photosensitizer that has been shown previously 46, but also as an activator of PO-CL reaction. This feature is peculiar for condensed polyarenes including anthracene and diphenylanthracene 47 and therefore the ability of anthracene part of SOSG to be an activator for peroxyoxalate chemiluminescence appears to be expectable. This may be a reason for the comparatively high level of SOSG endoperoxide fluorescence in the samples with omitted TMHP.

So, the obtained results give evidence for the formation of singlet oxygen due to PO-CL reaction both in aqueous solution and in cells.

**Level of oxidative stress and PS-mediated CLD cytotoxicity.** Cell treatment with hydrogen peroxide in the concentration range up to 200 μM resulted in an increase of DCF fluorescence (Fig. 8a, shaded bars) indicating the induction of oxidative stress by H₂O₂. The relative PS-mediated cytotoxicity of CLD measured as IC₅₀/IC₅₀CLD ratio increased in line with augmentation of H₂O₂ concentration (Fig. 8a, filled bars). This fact proves that the level of ROS determines the PS-mediated CLD cytotoxicity.

This conclusion was supported in the experiments with doxorubicin, menadione and paraquat, well-known inducers of oxidative stress 48–50. All of them favoured ROS production as shown by the augment in DCF fluorescence (Fig. 8b, shaded bars) and enhanced the PS-mediated cytotoxicity of CLD (Fig. 8b, filled bars). A direct correlation between the relative increase in ROS production and cytotoxicity expressed as IC₅₀/IC₅₀CLD values clearly show that the efficiency of PO-CL reaction in the dispersion captured by cells endowed their elimination.

**Discussion**

Oxidative stress plays an important role in cancerogenesis 51, 52 and proliferation of malignant cells 53–55. As it has been deduced by López-Lázaro, this is due to the fundamental changes in cancer cells metabolism, which are expressed in the shift of the O₂ metabolism “from the pathway that generates ATP to the pathway that generates ROS” 14. Therefore prooxidant nanoparticles targeted to the cells with increased ROS production might be powerful for treatment of a broad spectrum of tumours irrespectively of their type. As hydrogen peroxide is the most stable representative of ROS family, it accumulates in the cells experiencing oxidative stress. We employed this peculiarity to eliminate tumour cells via PO-CL reaction between a synthetic polymeric oxalate and endogenous hydrogen peroxide.

The polymeric oxalate as a substrate of PO-CL reaction was synthesized via polycondensation of bisphenol A, oxalyl chloride, and oligomeric polypropylene oxide. The choice of building blocks for polyoxalate synthesis was guided by low hydrolytic stability of oxalic esters containing electron-withdrawing substituents in phenyl fragment 42, 46. This consideration restricted our choice to biphenols with low acidities (high pKᵃ values). Our attempt to synthesize polyoxalate using 4,4'-dihydroxybiphenyl resulted in the copolymer practically insoluble in common solvents, pointing to the importance of flexibility of the biphenyl fragment. Therefore, we turned attention to bisphenol A containing aliphatic joint carbon between two hydroxyphenyl groups (Fig. 1) and therefore manifesting higher flexibility in comparison to 4,4'-dihydroxybiphenyl. In addition, bisphenol A exhibits prooxidant activity in the form of reactive phenoxyl-radicals when oxidized 57. These properties favour biological activity of the resulting formulation. In this respect bisphenol A gains advantage over 4-hydroxybenzyl alcohol exerting antioxidant properties 17, 38 and thus defending cells from the destructive action of singlet oxygen. For these reasons we have chosen bisphenol A for POX synthesis. Short oligo(propylene oxide) with M = 425 was used as an additive to further enhance solubility of the resulting product in organic solvents. Thus prepared polymer exhibited high solubility in DMP and THF.

The polymer was formulated into nanosized dispersions stabilized by Pluronic L64 as a biocompatible surfactant. Among the family of polymeric surfactants, Pluronic L64 exhibits relatively low cytotoxicity 23 and high efficacy in solubilization and emulsification of aromatic substances 30. It was reported to form stable xylene 59 and tributylphosphate 40 emulsions. So, Pluronic L64 was selected for stabilization of POX dispersions in water solution.
DMP and THF are good solvents for aromatic oxalates according to Rauhut. They both can be used for preparation of rather concentrated solutions of POX and display negligible cytotoxicity up to 0.4%. However, a striking difference in the properties of the dispersions prepared with these solvents was observed (Fig. 2d and S8a). It seems reasonable to suggest that this distinction results from different hydrophobicity of THF and DMP. Hydrophilic THF partitions from the particles into bulk water, while POX remaining in the micelles obviously appears to be exposed to hydrolysis. Therefore THF-based dispersions were inactive both in chemiluminescence and cytotoxicity measurements.

On the contrary, more hydrophobic DMP is poorly miscible with water and partitions into the hydrophobic core of Pluronic micelles resulting in the shift of its CMC (Fig. 3d). So, DMP forms hydrophobic core of the particles, preventing POX from hydrolysis within at least 2 h and the broad range of concentrations, even upon dilution to 0.03 mg/ml that was essential for cell experiments.

Colocalization of POX and TMHP dissolved in DMP favours efficiency of PO-CL reaction as follows from the fact that the PO-CL efficiency was reduced in excess of Pluronic L64 (see Fig. 4a and Supplementary Fig. S8b). The fact that the efficiency of peroxoxyxalate chemiluminescence and the cytotoxicity both increased with the content of TMHP in the dispersions (see Fig. 4b and Supplementary Fig. S8d) proves our supposition that porphyrin fulfills the functions of an activator and a PS all in one. The resemblance of the dependences of the chemiluminescence efficiency and the PS-mediated cytotoxicity on the composition of the dispersions gives evidence that PO-CL reaction underlies toxic effects in cell culture.

We found that the obtained dispersions are efficient for production of singlet oxygen both in cell-free system (Fig. 4f) and in cells (Fig. 7). So, the designed approach can be applied for elimination of the cells which intensively produce ROS. Hydrogen peroxide is the most long-living and the least toxic form of ROS family. Therefore, the elevation of the level of oxidative stress resulted in the increase in CLD cytotoxicity (Fig. 8a). This effect was independent on the way of induction of oxidative stress in the cells (Fig. 8b). All tested inducers of oxidative stress such as paraquat, the conventional antitumour drug doxorubicin, and even nutrient menadione were beneficial in PO-induced cytotoxicity. The latter is vitamin K3 and so can be regarded as safe for combined therapy. Combination with doxorubicin is of particular importance since it opens up the possibility to increase the efficiency of chemotherapy on the basis of conventional anti tumour medications.

We are conscious that the approach reported in the present article does not pretend to be a design of a ready-to-use therapeutic formulation. However, we believe that further development of these systems will result in the appearance of novel anti-cancer treatment modalities capable in elimination of currently incurable tumours.

Methods

Synthesis of POX. The flame-dried reaction flask was charged with 10.0 mmol (1.268 g, 0.858 mL) and 10 mL of freshly distilled THF. The flask was immediately sealed and immersed into ice bath for 10 min. Then, the solution of the mixture of bisphenol A (2.057 g), PPO425 (0.412 g) and TEA (1.011 g, 1.389 mL) in 10 mL of THF was added dropwise. Additional 10–20 mL of THF were used to transfer the reagents quantitatively. The flask was kept in ice bath for additional half an hour and then the reaction was allowed to proceed at room temperature for 7 days. To isolate POX, the reaction mixture was passed through a Schott filter and the residue was repeatedly washed with freshly dried THF. The solvent was evaporated from the filtrate and the product was dispersed in anhydrous dioxane. The obtained suspension was centrifuged (12000 rpm, 15 min.). The supernatant was collected, and the pellet was washed twice with fresh portions of dioxane. Dioxane solutions were combined and the solvent was removed using rotary evaporator. Product yield varied from 50 to 62% in different experiments.

IR: 1750 cm⁻¹ (C=O), 1176 cm⁻¹ (C-O-C, ester and ether).

¹H NMR: 1.67 ppm (m, 6 H, C(CH₃)₃); 7.2 and 6.7 ppm (m, 8 H, Ar); 1.14, 1.32 ppm (m, 3 H, -O-CH₂-(CH(CH₃))₂-O-), 3.63 ppm (m, 3 H, -O-CH₂-(CH(CH₃))₂-O-). 15% (mol.) Bisphenol A/PPO = 85/15 (mol.).

Preparation of dispersions. Pluronic L64, POX/DMP solution and TMHP solution in acetonitrile were mixed thoroughly in a desirable weight ratio (the optimized composition L64/POX/DMP/TMHP was 1:2:7:0.3:0.05). Then appropriate amounts of PBS or DMEM preheated at 37°C were added and the dispersion was shaken intensively using VibroFix VF-1 test tube shaker (IKA, Germany). Concentration of the dispersion is presented hereafter as the mass concentration of Pluronic L64 at a defined weight ratio of components.

Chemiluminescent reaction. A sample of the dispersion was prepared in a thermostated quartz or optical glass cell (1 × 1 cm, 3.5 mL) and kept at 37°C. Hydrogen peroxide and imidazole water solution (pH 7.4) were diluted with PBS and thermostated separately. The reaction was initiated by quick injection of H₂O₂/imidazole combined solution into the cell, the cover of the cell chamber being closed. The total volume of the sample was 2 mL. The chemiluminescent reaction was carried out at 37°C in the thermostated cell holder. Chemiluminescence intensity was measured by Hitachi 650–10S (Hitachi, Japan) spectrofluorimeter with photomultiplier tube R928 (Hamamatsu Photonics, Japan) with closed shutter of excited beam. Due to relatively slow reaction kinetics even in the presence of imidazole as a catalyst, the moment of 10-fold decrease in light intensity (I) as compared to maximum was considered to be the end of the reaction. Integral intensity (Q) was calculated for each experimental curve of light emission. This treatment of experimental curves corresponds to approximately 90% conversion. In all the experiments we used imidazole at concentration of 26 mM providing reaction duration of 20–30 minutes unless otherwise mentioned. Each experimental point is the average of at least 3 independent measurements.

Induction of oxidative stress and evaluation of intracellular level of ROS. The cells were seeded in 24-well plates at a density about 9000/cm² a day prior to the experiment. Oxidative stress inducers were added...
to the cells at a concentration close to the maximal tolerable concentration (MTC), i.e. the concentration ensuring viability of about 90% of the cells (C(H\textsubscript{2}O\textsubscript{2}) = 180\,\mu M, C(Doxorubicin) = 6\,\mu M, C(Menadione) = 20\,\mu M, C(Paraquat) = 200\,\mu M). The cells were incubated with oxidative stress inducers (hydrogen peroxide, doxorubicin, menadione or paraquat) in serum-free DMEM for 1 h at 37°C. Then the solutions were replaced for the complete medium. Further processing of cell cultures was performed after additional 1 h incubation to ensure complete decomposition of exogenous hydrogen peroxide by serum catalase\textsuperscript{38}. To evaluate the level of oxidative stress, the cells were incubated with 20\,\mu M of H\textsubscript{2}DCF-DA dissolved in indicator-free Hanks’ balanced salt solution for 30 min. The solution of H\textsubscript{2}DCF-DA was obtained by dilution of a freshly prepared 1.5\,mM solution of this compound in water-free DMF. The cell monolayers were washed with PBS, collected by scrapping in 50\,\mu L of PBS, transferred to the wells of black 96-well plates and the fluorescence was measured using Victor X5 2030 Multilabel Reader (Perkin Elmer, USA) at \lambda_{ex} = 490\,nm, \lambda_{em} = 535\,nm, exposition time 1.0 s/well. The obtained values were normalized to protein content which was measured after dissolution of the cells suspensions by addition of 0.1 mL of 1 N NaOH and further processing according to Lowry protocol\textsuperscript{42}.

**LCSM.** Confocal microscopy was performed using FluoView FV1000 microscope (Olympus Corp., Japan) equipped with spectral version scan unit and transmitted light detector. To observe TMHP fluorescence in the cells, the wavelength of excitation 405 nm (Diode laser) was used and the fluorescence was collected using an emission window set at 425–475 nm for acquisition of DAPI and 570–670 nm for TMHP fluorescence. Transmitted light Nomarski differential interference contrast (DIC) signal was detected simultaneously. Images were collected using the FV10 ASW 1.7 software (Olympus Corp., Japan).

To observe intracellular SOSG endoperoxide fluorescence, the cells were plated in glass bottom dishes at a density about 100,000 per sample a day before the experiment. One sample was kept untreated and the other samples were treated with H\textsubscript{2}O\textsubscript{2} as specified above. After 1 h incubation of the cells in the presence of complete medium either complete CLD of reference dispersion without TMHP both containing 10 mM of SOSG were added to the cells and incubated for an additional hour. The control samples without dispersions were treated with 10\,mM of SOSG in serum-free DMEM. All samples were prepared in DMEM without Phenol Red, sodium bicarbonate and fetal calf serum and supplemented with 10\,mM HEPES. CLSM images were obtained from these samples at the scanning rate 20\,\mu s/pixel. Each field was scanned no only once at excitation wavelength 488 nm (multiline Argon laser, the emission window 500–600 nm). All signals collected were adjusted to remain within the linear range of the detectors. The images were then treated by means of ImageJ 1.50i software (Wayne Rasband, National Institute of Health, USA) as follows. To measure mean pixel intensity, the cells were marked on microphotographs obtained via DIC channel and pixel intensity was measured on the fluorescence image. The data were averaged over about 300 cells on 10 images totally. Background on each image was averaged over 10 independent measurements and then the mean background value was subtracted from the mean value of cellular fluorescence in arbitrary fluorescence units.

**Evaluation of the dispersions cytotoxicity.** The cells were seeded in 96-well plates at a density about 9000/cm\textsuperscript{2} a day before the experiment. 2\,mL of serum-free DMEM were added to the mixture of 2 mg of Pluronic L64, 2.5\,\mu L of 10% POX solution in DMP and 2–25\,\mu L of TMHP solution in acetonitrile and thoroughly mixed with Vibrofix shaker. Thus prepared dispersions were sequentially diluted with serum-free DMEM and 100\,\mu L of these solutions were added to the cells. After 1 h incubation at 37 °C the solutions were replaced for 100\,\mu L of complete medium. Control samples contained no dispersions. The cells were allowed to grow for additional 3 days to reveal delayed toxic effects. The amount of living cells was determined by MTT test. 50\,\mu L of 1 mg/mL methyltetrazolium blue solution in DMEM was added to the wells. 3 h later the medium was removed, formazan crystals were dissolved in 100\,\mu L of DMSO, and the absorbance of the solutions at 550 nm was measured using Multiscan Plus Microplate Reader (Titertek, USA) plate spectrophotometer. Cell survival was calculated as the mean fraction of survived cells in the test samples in relation to the mean control. All measurements were performed at least in triplicates. The cytotoxicity was evaluated as the concentration of the sample corresponding to the survival of 30% of cells (IC\textsubscript{50}). This value was estimated by non-linear fitting of the experimental data using Logistic function.

\[
\text{Survival, } \% = \frac{100\%}{1 + \left(\frac{\text{IC}_{50}}{C}\right)^p},
\]

where C - is the concentration of the sample and p - is the parameter indicating the steepness of the curve.

**Statistical analysis.** Statistical analysis was performed according to t-test analysis using the Origin 7.5 software. Values of p < 0.05 were considered significant.

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**Author Contributions**

A.R. and N.M.-N. performed the main part of the experiments and wrote the manuscript, I.G. participated in the writing of the manuscript, A.E. performed CLSM experiments.

**Additional Information**

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