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Original Contribution

Scope and limitations of the TEMPO/EPR method for singlet oxygen detection: the misleading role of electron transfer

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ABSTRACT

For many biological and biomedical studies, it is essential to detect the production of $^1O_2$ and quantify its production yield. Among the available methods, detection of the characteristic 1270-nm phosphorescence of singlet oxygen by time-resolved near-infrared (TRNIR) emission constitutes the most direct and unambiguous approach. An alternative indirect method is electron paramagnetic resonance (EPR) in combination with a singlet oxygen probe. This is based on the detection of the TEMPO free radical formed after oxidation of TEMP (2,2,6,6-tetramethylpiperidine) by singlet oxygen. Although the TEMPO/EPR method has been widely employed, it can produce misleading data. This is demonstrated by the present study, in which the quantum yields of singlet oxygen formation obtained by TRNIR emission and by the TEMPO/EPR method are compared for a set of well-known photosensitizers. The results reveal that the TEMPO/EPR method leads to significant overestimation of singlet oxygen yield when the singlet or triplet excited state of the photosensitizer is efficiently quenched by TEMP, acting as electron donor. In such case, generation of the $TEMP^{+\bullet}$ radical cation, followed by deprotonation and reaction with molecular oxygen, gives rise to an EPR-detectable TEMPO signal that is not associated with singlet oxygen production. This knowledge is essential for an appropriate and error-free application of the TEMPO/EPR method in chemical, biological, and medical studies.

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Singlet oxygen (molecular oxygen in the $^1\Delta_g$ state, or $^1O_2$) is one of the most important "reactive oxygen species." Its reactions include oxidation of lipids [1,2], proteins [3–5], and nucleic acids [6–8], which may trigger biological damage. This reaction cascade can lead to undesired adverse effects, such as drug-induced phototoxicity [9,10], but can also be exploited to produce beneficial effects as in photodynamic therapy [11,12].

Production of $^1O_2$ by a photosensitizer is a classical example of photoinduced energy transfer: after absorption of light, the photosensitizer reaches its singlet excited state and subsequently crosses to its triplet excited state. Then, the triplet ground state of molecular oxygen is promoted to the $^1\Delta_g$ state through triplet–triplet energy transfer [13].

For many biological and biomedical studies, it is essential to detect the production of $^1O_2$ and quantify its production yield. Among the available methods, detection of the characteristic 1270-nm phosphorescence of singlet oxygen by time-resolved near-infrared (TRNIR)[1] emission constitutes the most direct and unambiguous proof [14,15]. However, the required equipment is not always available in biochemical laboratories.

An alternative indirect method that has been widely applied is electron paramagnetic resonance (EPR) in combination with a $^1O_2$ probe. Upon reaction with $^1O_2$, the trapping molecule gives rise to a detectable spin-active species with a distinctive line pattern. Thus, oxidation of TEMP (2,2,6,6-tetramethylpiperidine) by singlet oxygen yields the TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxyl) free radical easily detected by EPR (Fig. 1) [16]. Although the TEMPO/EPR method has been widely employed [17–25], a systematic investigation of the scope and limitations of this technique has never been performed. For instance, amines are widely known for their ability to quench excited states, so a probable source of artifacts may be the interaction between the excited photosensitizer and TEMP [26–29]. The aim of the present study was to compare the results obtained for the detection and quantification of singlet oxygen by means of the direct method (TRNIR emission)

Abbreviations: ACN, acetonitrile; BAHA, tris(4-bromophenyl)aminium hexachloroantimonate; BP, benzophenone; CBZ, carbazole; EPR, electron paramagnetic resonance; LFP, laser flash photolysis; NP, naphthalene; PET, photoinduced electron transfer; PN, phenalenone; RB, rose Bengal; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxyl; TRNIR, time-resolved near infrared

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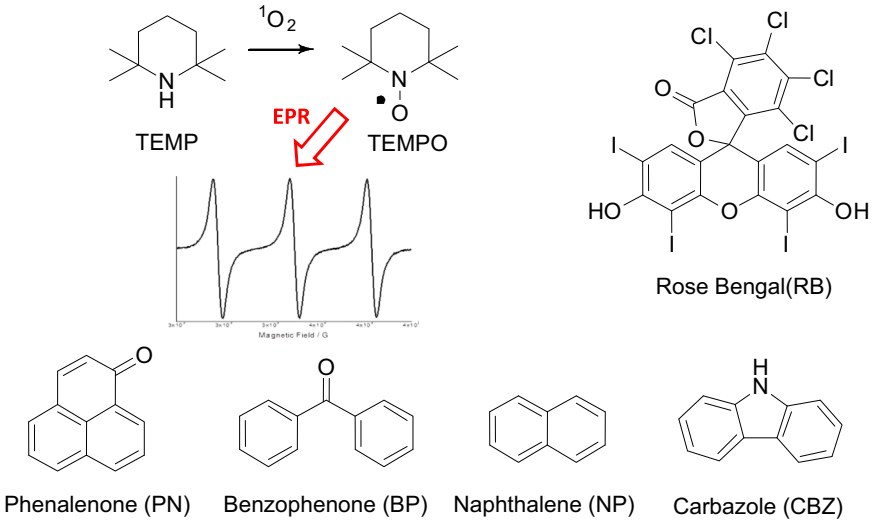

Fig. 1. Structure of the molecules involved in the study and EPR signal of the TEMPO radical.

and the indirect $^1O_2$ trapping mode (TEMPO/EPR method), using a set of well-known photosensitizers. The basis of the TEMPO method and the chemical structure of the selected photosensitizers are shown in Fig. 1. The results obtained reveal that the EPR method leads to significant overestimation of singlet oxygen production when the singlet or triplet excited state of the photosensitizers is efficiently quenched by TEMP, acting as electron donor.

Materials and methods

Chemicals

TEMP, tris(4-bromophenyl)aminium hexachloroantimonate (BAHA), phenalenone (PN), benzophenone (BP), naphthalene (NP), carbazole (CBZ), rose Bengal (RB), and acetonitrile (ACN) were from Sigma–Aldrich. TEMP was freshly distilled at 152 °C before use.

Absorption and fluorescence spectra

UV–Vis absorption spectra were recorded on a commercial spectrophotometer (λ650; PerkinElmer). Fluorescence spectra were measured using 1-nm steps and 0.5-s dwell time, at right-angle detection (FLSP920; Edinburgh Instruments). Slits were kept narrow to 1 nm for excitation and 1 or 2 nm for emission; where necessary, a cutoff filter was used. All the measurements were carried out at 295 K in quartz cuvettes with a path length of 1 cm. The fluorescence spectra were obtained for air-equilibrated solutions with $A < 0.1$ over the whole absorption range to avoid inner filter effects and reabsorption of emission. Quenching of CBZ and NP fluorescence intensity by TEMP upon excitation at 331 and 278 nm, respectively, was performed by adding increasing amounts of TEMP to the solution. For NP measurements, the fluorescence intensities were corrected for the inner filter effect due to absorption of TEMP at 278 nm. The following equation was used to determine K_{sv}, the Stern–Volmer quenching constant:

$$F_0/F = 1 + K_{sv}[Q]. \quad (1)$$

In Eq. (1), F_0 and F are the fluorescence intensities, respectively, in the absence and presence of the quencher Q; [Q] is the quencher concentration (M); and K_{sv} is the Stern–Volmer constant. The bimolecular quenching rate constant k_q ($M^{-1}\,s^{-1}$) was obtained dividing K_{sv} by the fluorescence lifetime.

Fluorescence lifetimes

Fluorescence decay was measured in air-equilibrated solutions with a time-correlated single-photon counting apparatus (IBH 5000F) equipped with a TBX picosecond photon detection module. A nano-LED pulsed excitation source at 331 and 278 nm was used and the emission was collected at right angle at 341 or 320 nm using a long-pass cutoff filter at 305 nm. Fluorescence decay profiles were fitted using a monoexponential function of the decay analysis software DAS6 provided by the manufacturer with deconvolution of the instrumental response function.

Laser flash photolysis measurements

The beam of a pulsed Nd:YAG laser, operating at 532 or 355 nm (20 ns FWHM, 2 Hz, 2.7 mJ/pulse), was suitably shaped to pass through a 3-mm-high and 10-mm-wide rectangular window and provide a fairly uniform energy density of 9 mJ/cm^2 incident onto the sample cell. A front portion of 2-mm depth of the excited solution was probed at right angle, the useful optical path for analyzing light being 1 cm. All transient spectra were recorded with 3 ml of sample solutions in 1×1-cm^2 quartz cells; when specified ACN solutions were bubbled for 10 min with Ar before data acquisition. The absorbance of the samples was kept in the range 0.30–0.40 at the laser wavelength. Stock solutions of the quenchers were prepared, so that addition of microliter volumes to the sample cell allowed us to obtain the appropriate quencher concentration.

The bimolecular rate constant k_q ($M^{-1}\,s^{-1}$) for quenching of the triplet states was calculated from the slope of linear plots of the observed triplet decay rate constant k_{obs} (s^{-1}) versus the quencher concentration, applying Eq. (2),

$$k_{obs} = k_0 + k_q[Q], \quad (2)$$

where k_0 is the triplet decay rate constant in the absence of quencher and [Q] is the quencher molar concentration (M).

Singlet oxygen TRNIR emission measurements

The pulse of a Nd:YAG laser, operating at 355 or 266 nm (20-ns FWHM), was used for excitation of the samples dissolved in air-equilibrated acetonitrile. A preamplified (low impedance) Ge photodiode (Applied Detector Corp., Model 403HS, time resolution 300 ns), cooled at 77 K and equipped with a 5-mm-thick

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