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Research Articles

Photodynamic sensitizers assay: rapid and sensitive iodometric measurement

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Abstract. A rapid, sensitive and simple spectrophotometric method for the detection of $^1O_2$ produced by photodynamic photosensitizers in slightly acid and air-saturated aqueous solutions has been developed. The method is based on the reaction of $^1O_2$ (produced by photodynamic processes) with I^- in the presence of ammonium molybdate as a catalyst. The reaction product I_3^-, proportional to $^1O_2$, is followed spectrophotometrically at 355 nm. Several ways of avoiding interference with other oxidizing compounds, either present before or produced during the irradiation, are described.
The method could be used to measure the efficiency of water-soluble photodynamic photosensitizers.
Key words. Photosensitizers; singlet oxygen; iodometric measurement.

This study was prompted by the growing interest in the photodynamic therapy of major human diseases like cancer and obstructive atherosclerosis[1–5]. This technique relies largely on the preferential uptake of a photosensitizer (dye) by pathologically altered cells. Upon irradiation, the dyes and oxygen initiate the photooxidative damage of the tissue.
The mechanism of light–induced cell injury involves the production of singlet oxygen ($^1O_2$), a short-lived and highly reactive cytotoxic species. It is generally assumed that excited singlet oxygen is formed after illumination via an energy transfer process between the excited triplet state of the photosensitizer and molecular oxygen in its ground triplet state[6]. Alternative pathways for oxygen activated by photoexcited photosensitizers, e.g., the generation of the superoxide anion by electron transfer, are less common[7,8].
Since the $^1O_2$ possesses a rather short lifetime, particularly in aqueous solutions[8], the detection of $^1O_2$ is difficult. The generation of singlet oxygen has been observed directly using time-resolved spectroscopy, following the luminescence of $^1O_2$ at 1270 nm by the $^1\Delta_g \rightarrow {}^3\Sigma_g$ transition. This luminescence is very weak and occurs in a wavelength region where very sensitive instruments must be used for its detection[9,10].
Indirect evidence of $^1O_2$ involvement in the photosensitized photooxidation of several biological substrates has been provided by photokinetic studies in deuterated solvents, in which the lifetime of $^1O_2$ is significantly enhanced, or physically quenched in the presence of NaN_3 (see ref. 11).
Recently, the bleaching of 1,3-diphenylisobenzofuran (DPBF), a well-known singlet oxygen acceptor, has been used to measure the absolute quantum yield of the formation of $^1O_2$ generated by photosensitizers in solutions containing high percentages of methanol or formamide[7]. Unfortunately, this method cannot be used for aqueous solutions.
In neutral air–saturated aqueous solution another spectrophotometric bleaching method can be used. This is based on the bleaching of p-nitrosodimethylaniline induced by the reaction of $^1O_2$ with imidazole or histidine. The intermediate product of the reaction of $^1O_2$ with the imidazole derivative, a trans-annular peroxide, causes the bleaching of p-nitrosodimethylaniline that can be measured at 440 nm wavelength[12].
Recently, several authors indicated that some peroxocompounds, mainly lipid peroxides, could be determined by a simple iodometric procedure[13–15].
We found that a similar technique could also be used as a rapid and simple method for the indirect chemical detection of singlet oxygen in aqueous solutions, and is therefore very useful in comparative studies of the efficiency of some water-soluble photodynamic photosensitizers in tumor or atherosclerotic plaque phototherapy.

Materials and methods

The iodometric reagent was prepared from components as listed in the table and stored in a light–protected bottle. The composition is similar to the commercially

Table. The composition of iodometric reagent

Potassium phosphate, pH 6.2	0.2 M
Potassium iodide	0.12 M
Ammonium molybdate	10 μM

available kit for enzymatic determination of cholesterol and/or hydrogen peroxide (CHOD-iodide, Merck, Germany), but excludes the detergents and NaN_3.

Irradiation of this solution in the presence of micromolar concentrations of photosensitizer results in the production of I_3^- (absorbency maximum around 355 nm, molar extinction coefficient $2.47 \times 10^4 \times M^{-1} \times cm^{-1}$), proportional to the production of $^1O_2$.

The procedure is based on the reaction of singlet oxygen (as a product of photodynamic reaction) with I^- in a neutral aqueous solution by the following mechanism[16]:

$F_0 + h\nu \rightarrow {}^1F$ excitation by light

${}^1F \rightarrow {}^3F$ intersystem crossing

${}^3F + {}^3O_2 \rightarrow F_0 + {}^1O_2({}^1\Delta_g)$ energy transfer

F_0, ^1F and ^3F denotes photosensitizer in ground, singlet and triplet excited state respectively.

$$^1O_2(^1\Delta_g) + I^- \rightarrow IOO^- \xrightarrow{H_2O} IOOH$$

$$HOOI + I^- \rightarrow HOOI_2^- \rightarrow I_2 + HO_2^- \xrightarrow{I^-,H_2O} I_3^- + H_2O_2 + OH^-$$

If the reaction is carried out in slightly acid buffered solution (pH = 6.2) in the presence of ammonium molybdate as a catalyst, another step takes place:

$$H_2O_2 + 2I^- + 2H^+ \rightarrow I_2 + 2H_2O$$

$$I_2 + I^- \rightarrow I_3^-$$

This technique of evaluation of photodynamically produced singlet oxygen was tested using four well-known water soluble photodynamic photosensitizers: tetrasodium salt of tetra(4-sulfonatophenyl)porphine ($TPPS_4$), Porphyrin c (Pc), Rhodamine B (RB) and Bengal Rose (BR). RB and BR were obtained from Aldrich (Heidenheim, Germany), Pc and $TPPS_4$ were synthesized according to ref. 17 and 18, respectively. The purity of all photosensitizers was tested by TLC, UV-VIS and IR spectroscopy. H_2O_2 and KO_2 were obtained from Aldrich (Heidenheim, Germany); catalase from beef liver (2000 units per mg) was obtained from Reanal (Budapest, Hungary).

Results

In a typical experiment the solution described earlier was irradiated in 1 × 1 cm closed quartz cuvette in a thermostated steel chamber fixed to an optical bench at 25 °C. The radiation was obtained from a monochromatic SHC 400 W high-pressure sodium arc at 589 nm or from a polychromatic 250 W stabilized halogen lamp with normalized interference filters. The wavelength of excitation light was chosen according to the absorbance maxima of photosensitizers. After irradiation UV-VIS spectra were recorded by SPECORD M-42 (Germany), and data obtained were processed with the aid of in-built and personal computers.

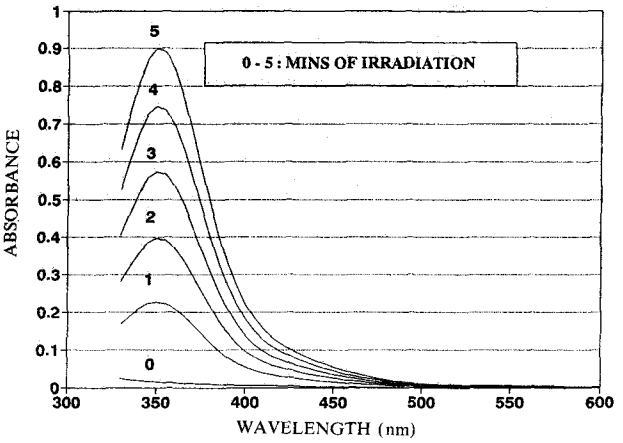

Figure 1. Absorbance spectrum after irradiation of 4 ml iodometric solution in the presence of Porphyrin c (16 μM). Absorbance spectrum of Porphyrin c was subtracted.

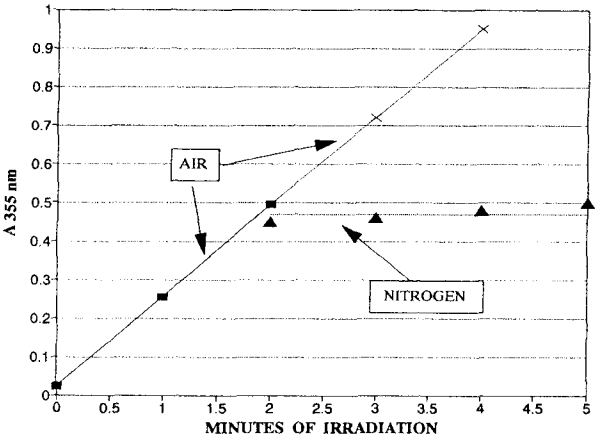

Figure 2. Peak absorbance at 355 nm was measured at one minute intervals, and after 2 minutes of irradiation one half of the sample was irradiated under nitrogen. Otherwise see legend for figure 1.

The relation between the change of absorbance spectrum of the solution (absorbance spectrum of photosensitizer is subtracted) and the time of irradiation is depicted in figure 1. Under the conditions described a measurable change in peak absorbance could easily be detected after less than 60 s irradiation.

The absorbance maximum increases linearly for up to 4–5 min of irradiation (fig. 2). The time course remains linear even in a more acid environment. The diminished slope in acid pH is related to the photodynamic process and not to iodide oxidation, as indicated by similar results using p-nitroso-N, N'-dimethylaniline with imidazole for the detection of singlet oxygen[12].

The reaction was absolutely dependent on oxygen (fig. 2). The dependence of the photoeffect on the concentra-

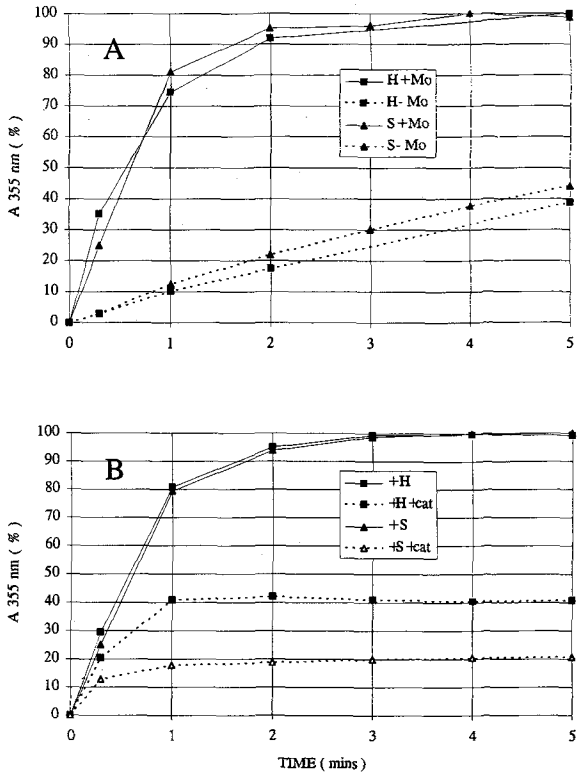

Figure 3. *A*) The formation of I_3^- in the 2.5 ml of iodide solution following the addition of H_2O_2 (H) or KO_2 (S) to a final concentration of 39.2 µM and in the presence or absence of ammonium molybdate (+Mo or −Mo) to a final concentration of 10 µM. Maximum absorbance was 1.46 units.
B) The formation of I_3^- in iodide solution following the addition of H_2O_2 (H) or KO_2 (S) to a final concentration of 39.2 µM and the inhibition by catalase (+cat) at a concentration of 400 units per ml. Maximum absorbance was 1.55 units.

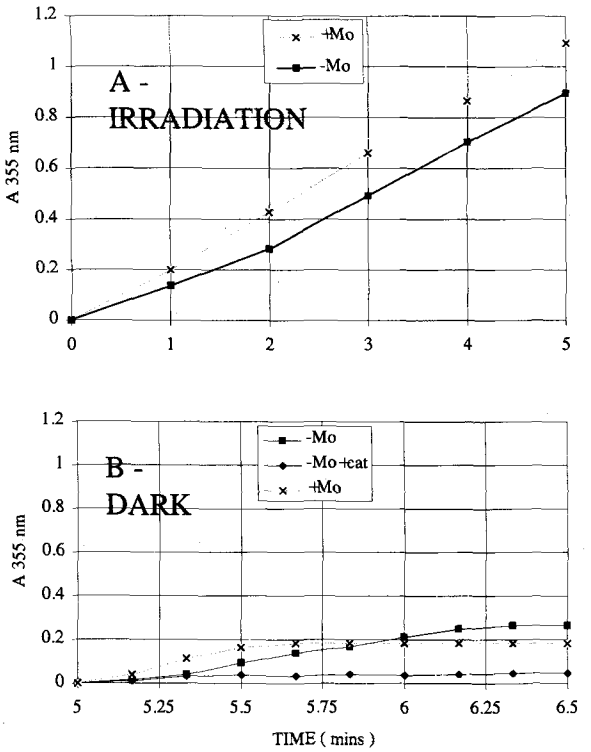

Figure 4. *A*) The formation of I_3^- in 2.5 ml of iodide solution plus Bengal Rose (final concentration 16 µM) during irradiation. Ammonium molybdate (10 µM) was either present (+Mo) or absent (−Mo).
B) The continuation of I_3^- formation after the irradiation has been stopped in the experiment illustrated in panel A. Note that the continuing oxidative reaction was faster and stopped sooner in the presence of molybdate (+Mo) than in its absence (−Mo), and that it could be inhibited by the addition of catalase at a concentration of 400 units per ml (+cat).

tion of Porphyrin c as a photodynamic photosensitizer was linear in the range from 0.25 to 2 µM.

All the above mentioned properties for each of the tested photosensitizers appeared to be in good agreement with the data obtained using bleaching of p-nitroso-N, N′-dimethylaniline for the detection of singlet oxygen.

The reaction specificity was not complete, since iodide solution could be oxidized not only by singlet oxygen but also by other active oxygen species like hydrogen peroxide or superoxide anion. Both of these are likely to arise during the irradiation under certain conditions[6]. To address this problem we tested the effectiveness of these compounds in separate experiments.

As shown in figure 3, the addition of both H_2O_2 and KO_2 to the iodide reagent results in the oxidation of I^-, and in both cases the oxidation was substantially inhibited by the omission of the catalyst $(NH_4)_2MoO_4$ (fig. 3A) or by the addition of catalase in a concentration of 400 units per ml (fig. 3B). The same amount of albumin added instead of catalase had no effect.

The generation of pure hydrogen peroxide or superoxide anion by irradiation is less likely than generation in combination with singlet oxygen. The pathway is already given by the equations describing the consecutive reactions during the irradiation of iodometric reagent (see 'Materials and methods'), where a partial conversion of singlet oxygen to hydrogen peroxide is indicated. In such cases and as demonstrated in figure 4, we find a slight but reproducible reduction of the oxidizing rate in the absence of ammonium molybdate (fig. 4A), and a slow oxidative reaction continuing after the end of illumination. The latter could be virtually stopped by the addition of catalase (fig. 4B). These findings suggest that the singlet oxygen as a primary product of irradiation was followed by the generation of a small amount of hydrogen peroxide and/or peroxide compounds.

The reaction principle can also be applied to the detection of and discrimination between photosensitive compounds after separation by thin layer chromatography. The thin layer containing a starch support could be sprayed with iodide solution and irradiated. When using paper or thin layer support not containing strach, about 4% of amylose has to be added to the iodide solution. The photosensitive compounds are revealed after about

5 s of irradiation using a 50 W halogen lamp at a distance of 20 cm as dark-blue or brownish spots.

Discussion

In this paper we describe a simple and sensitive spectrophotometric method for the detection of singlet oxygen produced by photosensitizing dyes in aqueous solution. The method can be used to compare the relative singlet oxygen-generating efficiencies of various potential photosensitizers.

The iodide solution described above is relatively stable in that no appreciable alteration was noted after several months. This stability to atmospheric oxygen and common laboratory light is of great practical advantage. Absorbance maxima of I^- and I_3^- at near UV wavelengths are convenient since there is minimum interference with the absorbance spectrum of photosensitizers having maxima mainly in the visible part of the spectrum.

The iodide method is simpler, cheaper and faster than the method based on the bleaching of p-nitroso-N, N'-dimethylaniline with imidazole. An increase rather than decrease of absorbance is measured. This makes it possible to use an excess of acceptor (I^-) for $^1O_2$. The method is surprisingly sensitive; one reason may be the influence of a heavy atom of iodine[19] which promotes intersystem crossing of excited states of the photosensitizers.

As can be seen from the mechanism of reaction described in 'Materials and methods', during the irradiation of the iodometric reagent a partial conversion of singlet oxygen to H_2O_2 takes place. This is consonant with the influence of $(NH_4)_2MoO_4$ (catalyzing the reaction of I^- with H_2O_2 in acid environment) on the production of I_3^- during the photodynamic reaction, as can be seen in figure 4. Thus using ammonium molybdate in the iodometric reagent is recommended because the time of reaction in the dark is shorter and thus the measurement variation smaller.

A slightly acid aqueous environment of the iodometric reagent is optimal for the rapid conversion of superoxide anion to hydrogen peroxide and oxygen. The dismutation rate constant will be approximately $k = 0.4 \times 10^4 \times M^{-1} \times s^{-1}$ (ref. 20). Consequently the absence of ammonium molybdate or the presence of catalase has a similar effect as hydrogen peroxide.

To prevent the iodide reagent interfering with oxidizing agents except singlet oxygen, several points should be noted:

1) The unknown or newly synthesized photosensitizer should not be contaminated by any oxidizing reagents. The presence of such compounds can be readily detected using the same experimental protocol but omitting the irradiation.

2) In rare cases, depending on the photosensitizer used and the presence of other compounds in the irradiated solution, other active oxygen species like superoxide anion or hydrogen peroxide could increase and oxidize I^- during irradiation. This is more likely to occur if change transfer from the singlet excited state of the photosensitizer to oxygen takes place, or if the reduction of the photosensitizer in triplet excited state (and consequently that of the oxygen by any reducing agents), by hydrogen or electron transfer occurs[6]. To detect the presence of the abovementioned oxygen species the assay solution without KI should be used. The accumulation of hydrogen peroxide as the final product of photoreaction may be detected after irradiation following the addition of KI and the increase in I_3^- absorbance. No such effect would be observed if only extremely short-living singlet oxygen has been formed.

Another indirect test to determine whether the generation of singlet oxygen was accompanied by hydrogen peroxide and/or superoxide anion formation is to repeat the assay with iodide solution but in the absence of $(NH_4)_2MoO_4$.

The addition of catalase before the irradiation cannot be used because, as with other proteins, it non-specifically quenches singlet oxygen in photodynamic reactions[6].

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