Singlet Oxygen Sensor Green is not a Suitable Probe for $^1\text{O}_2$ in the Presence of Ionizing Radiation

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A great number of fluorescent probes have been developed for detecting singlet oxygen ($^1\text{O}_2$), which is considered to be one of the most effective reactive oxygen species (ROS), especially in clinical applications. The commercially available fluorescent probe Singlet Oxygen Sensor Green (SOSG) is widely used due to its reported high selectivity to $^1\text{O}_2$. In this study, we carried out systemic experiments to determine the activation of SOSG in the presence of ionizing radiation. The results show that the SOSG probe exhibits a pronounced fluorescence increase as a function of radiation dose delivered by gamma-rays as well as X-rays, in conditions where the formation of singlet oxygen is not expected. Furthermore, scavenger tests indicate that hydroxyl radicals may be involved directly or indirectly in the activation process of SOSG although the exact mechanism remains unknown.

Reactive oxygen species (ROS) occur naturally in human cells and are known to play a role in various cancerous processes$^1$. At the same time, a number of cancer therapies rely on the generation of ROS to induce cell death$^2$. Among all ROS, singlet oxygen ($^1\text{O}_2$) is considered to be the most effective in killing tumour cells and has been widely used in photodynamic therapy (PDT)$^3$. To better understand the behaviour of photosensitizers generating ROS in PDT and consequently their biological effect, the detection of ROS, especially $^1\text{O}_2$, is of great importance. However, the short lifetime and the low concentration of $^1\text{O}_2$ tremendously complicate proper detection. Various approaches have been developed for the detection of $^1\text{O}_2$, including direct measurement of the $^1\text{O}_2$ luminescence at 1280 nm, electron spin resonance spectroscopy (ESR), fluorescent probes and others$^4$–$^6$. Fluorescent probes are widely employed due to their simplicity in utilization and high detection efficiency$^7$. The commercially available probe Singlet Oxygen Sensor Green (SOSG) is currently the preferred choice for $^1\text{O}_2$ detection due to its claimed specific sensitivity to singlet oxygen$^8$.

As shown in Fig. 1, the SOSG molecule has two parts: a trapping moiety and a fluorophore$^9$. The anthracene-derived trapping moiety, being an electron donor, quenches the luminescence of the fluorophore by photo-induced electron transfer. In an environment containing $^1\text{O}_2$, the trapping moiety will react with $^1\text{O}_2$ and form an endoperoxide anthracene moiety, which has a lower energy for the highest occupied molecular orbital (HOMO) than that of the fluorophore. Removing the quenching ability of the anthracene leads to fluorescence (FL) emission of the fluorophore under light excitation with a peak at around 530 nm. Despite the many advantages of SOSG, it was previously shown that this probe is not entirely reliable under certain conditions. The main drawback of SOSG is that, for instance when irradiated with UV light, its endoperoxide derivative acts as a photosensitizer itself, generating singlet oxygen which then induces even more fluorescence emission of SOSG$^9$–$^{11}$. Moreover, photo-bleaching of SOSG is observed even for short light exposure times$^9$.

In photodynamic therapy, singlet oxygen is typically generated by light irradiation of a photosensitizer. Due to absorption and scattering effects of tissue, the penetration depth of light in living tissue can be very shallow, which limits further clinical application$^{12}$. To overcome this challenge, one of the promising options is to combine PDT with radiotherapy. For example, nano scintillators conjugated to photosensitizers were developed which can convert ionizing radiation to light, producing ROS$^{13}$–$^{15}$. Very recently it was also suggested that radioactive isotopes (e.g. $^{18}\text{F}$, $^{64}\text{Cu}$) producing so-called Cerenkov light could function as internal light sources, which in combination with photosensitizers can be applied to induce tumour cell death$^{13,16}$. Moreover, in radiation-involved therapies,
the generation of ROS is also an indirect strategy to reduce the growth of tumour cells. In these therapies, probes are also used to prove the generation of $^{1}\text{O}_2$.17–19.

As far as we know, the influence of ionizing radiation on the photochemical behaviour of such probes has not been comprehensively evaluated. In this paper, the photochemical performance of SOSG was assessed when exposed to ionizing radiation under conditions similar to other studies13,19. In this study, we used gamma-rays (γ-rays) and X-rays, which are common types of ionizing radiation in external radiotherapy. After irradiation, the UV-vis absorption and fluorescence emission of SOSG were determined. We found that, under conditions that are unlikely to generate $^{1}\text{O}_2$, the SOSG probe shows an increasing fluorescence emission as the radiation dose is increased, without any detectable changes in UV-vis spectrum. Furthermore, scavenger tests suggest that the generation of hydroxyl radicals may be related to the increase in fluorescence emission of SOSG.

Results and Discussion

The effect of ionizing radiation on the photochemical properties of SOSG was studied systematically using two different external radiation sources, i.e., a cobalt-60 ($^{60}\text{Co}$) γ-ray source and an X-ray source. The fluorescence spectra of SOSG after exposure to gamma rays are presented in Fig. 2a, showing that the FL intensity of the SOSG solution increased with increasing radiation doses. Figure 2b shows the UV-vis absorption spectra of SOSG at the same irradiation conditions, where the maximum band at 307 nm belongs to the fluorescein moiety, while the large peak at 257 nm and the two smaller peaks at 374 and 394 nm correspond to the methylanthracene moiety9. No noticeable changes were observed in the UV-vis spectra as the radiation dose increases, suggesting that the structure of SOSG is not affected or the concentration of activated SOSG remains low. To check whether SOSG also reacts with ionizing photons of lower energy, we exposed the probe solutions to X-rays with a maximum energy of 320 keV and monitored the fluorescence spectra and UV-vis absorption at different doses. As shown in Fig. 2c, the X-ray exposure also induces fluorescence emission of the SOSG probe, and the FL intensity exhibits an upward trend with increasing radiation doses. In fact, the FL intensity increase is comparable to the rise observed when using the $^{60}\text{Co}$ source for the same radiation dose, showing that this process is most likely not dependent on the photon energy. The UV spectra of SOSG appear unchanged for all X-ray radiation doses (Fig. 2d).

As a check, we also exposed the SOSG samples to UV light sources using two different wavelengths (365 nm and 400 nm). These experiments show that the SOSG fluorescence intensity increases significantly as a function of irradiation time (Fig. S1), which is likely caused by the generation of $^{1}\text{O}_2$ in the presence of UV light. Meanwhile, in UV-vis the intensity of the peaks at 257, 307 and 394 nm decreases upon UV irradiation, caused by the formation of SOSG-EP10.

Ionizing radiation can have direct and indirect effects on the behavior of SOSG. In direct interaction, ionizing radiation can result in breaking of chemical bonds, changing the SOSG structure. Indirect effects result from the generation of reactive oxygen species in the aqueous solution which can react with SOSG, or influence the intermolecular electron transfer processes, resulting in the fluorescence emission of SOSG. According to the unchanged UV-vis spectra of the irradiated SOSG samples, chemical bonds are most likely not broken which is expected considering the nature of radiation (i.e., photons) and the low radiation dose (max 25 Gy). Therefore, we focused mostly on any possible indirect effects.

Figure 3 shows various photochemical processes that may take place in the aqueous SOSG solution induced by ionizing radiation, which may influence the photochemical performance of the SOSG probe. The $^{60}\text{Co}$ is a radioisotope that decays by beta minus emission, producing in the process two energetic γ-rays of 1.17 MeV and 1.33 MeV18. These photons can interact with water via the Compton effect, giving part of their energy to electrons. The energy of these photons is high enough to provide electrons with energies above the threshold for the production of Cerenkov radiation (261 keV in water)21. It should be mentioned that Cerenkov light has a wide spectrum ranging from 250 to 800 nm with a maximum around 360 nm22,23, which overlaps with the absorption range of SOSG resulting in some probability of $^{1}\text{O}_2$ generation. Ionizing radiation also leads to the radiolysis of water which produces various reactive oxygen species, including OH•, $\text{O}_2$•− and $\text{H}_2\text{O}_2$24. This means that both
Figure 2. (a) Fluorescence spectra of SOSG solutions (5 µM) for varied radiation doses delivered by a $^{60}$Co source ($\lambda_{ex} = 504$ nm); (b) UV-vis absorption spectra of SOSG solutions (5 µM) for different radiation doses delivered by a $^{60}$Co source; (c) Fluorescence spectra of SOSG solutions (5 µM) for different X-ray doses; (d) UV-vis absorption spectra of the SOSG solution (5 µM) for different X-ray doses.

Figure 3. Possible reactive oxygen species generated under (a) gamma ray radiation and (b) X-ray (320 keV) radiation of aqueous SOSG solutions.
the increasing FL intensity of SOSG probe under exposed a simple SOSG solution and a SOSG solution mixed with Ce6 to gamma rays originating from the 60Co is able to generate Cerenkov light in the UV-Vis range, this light is likely not enough to activate the SOSG fluorescence of SOSG in these experiments is not induced by singlet oxygen. Although with N2 or normal air atmosphere (Fig. S8) shows no significant difference, which strongly suggests that the increasing signal as function of radiation dose. Furthermore, comparing the FL intensity of SOSG solutions saturated doses, although no firm conclusion can be drawn due to the large uncertainty of the measured FL intensity. Still, it is smaller than the signal from the solution containing the photosensitizer. These results also support that the SOSG probe becomes fluorescent when exposed to either γ-rays or X-rays. SOSG shows increased fluorescence intensity as function of radiation dose, which appears not to be related to singlet oxygen formation. Scavenger tests reveal that the suppression of hydroxyl radicals lead to a decrease in induced fluorescence intensity, which suggests that 

**Conclusions**

In this paper, we studied the effect of ionizing radiation on the photochemical behavior of SOSG, showing that this probe becomes fluorescent when exposed to either γ-rays or X-rays. SOSG shows increased fluorescence intensity as function of radiation dose, which appears not to be related to singlet oxygen formation. Scavenger tests reveal that the suppression of hydroxyl radicals lead to a decrease in induced fluorescence intensity, which suggests that
these species play some role in the activation of the SOSG probe in the presence of ionizing radiation. In contrast, when exposed to UV-light sources, SOSG is efficiently activated through the formation of the SOSG endoperoxide, caused by reaction with singlet oxygen. In addition, another two commercially available probes used for the

**Figure 4.** Fluorescence spectra ($\lambda_{ex} = 504$ nm) of SOSG solutions (a) with varied SOSG concentrations, (b) reacted with $\text{H}_2\text{O}_2$ solutions with varied concentrations, (c) reacted with $\text{NaN}_3$ solutions with different concentrations and (d) in the addition of varied amount of ethanol under 20 Gy radiation dose from a $^{60}\text{Co}$ source; Fluorescence spectra of SOSG solutions (5 $\mu\text{M}$) (e) saturated with $\text{N}_2$ for varied radiation doses delivered by a $^{60}\text{Co}$ source; (f) The FL intensity determined at 529 nm of air-saturated and $\text{N}_2$-saturated SOSG solutions as function of radiation dose.
detection of singlet oxygen, i.e. 1,3-diphenylisobenzofuran and 9,10-anthracenediyl-bis(methylene) dimalic acid, were also evaluated under ionizing radiation conditions. These two probes appeared also to be activated by ionizing radiation. The exact mechanism leading to the activation of SOSG as well as the two other probes when exposed to ionizing radiation remains unclear but this study does clearly demonstrate that such probes should be cautiously used under such conditions.

**Experimental Section**

**Materials.** Singlet Oxygen Sensor Green was purchased from Thermo Fisher; Chlorin-e6 (C34H36N4O6) was purchased from Bio-connect life sciences (Huissen, the Netherlands); Sodium azide, sodium acetate, ammonium molybdate ((NH4)6Mo7O24•4H2O), potassium iodide (KI), sodium iodide (NaI) were obtained from Merck (Darmstadt, Germany); Methanol, hydrogen peroxide (H2O2, 30% (W/W)), acetic acid, 1,3-diphenylisobenzofuran (DPBF) and 9,10-anthracenediyl-bis(methylene) dimalic acid (ABDA) were bought from Sigma Aldrich (Zwijndrecht, the Netherlands). Ethanol was bought from Brenntag (Zwijndrecht, The Netherlands). Dimethyl Sulfoxide (DMSO) was bought from Biosolve B. V. (Valkenswaard, The Netherlands). All chemicals were used without further treatment. Water used in these experiments was prepared with the in-house Milli-Q system from Merck Millipore.

**Sample preparation.** All samples were prepared in dim environment.

SOSG stock solution (10 µM): Typically, 100 µg of SOSG was dissolved in 33 µL of methanol, and then this yellow solution was added to 16.467 mL of MQ water. For preparing SOSG solutions with different concentrations, the volume of MQ water was changed as required. ABDA stock solution (2 mM): 1.64 mg of 9,10-anthracenediyl bis(methylene) dimalic acid was dissolved in 2 mL of DMSO, then ultrasonicated for 10 min. DPBF stock solution (2 mM): 2.7 mg of 1,3-diphenylisobenzofuran was dissolved in 5 mL of DMSO and ultrasonicated for 10 min. Ce6 stock solution (10 µM): 1.79 mg of Ce6 was dissolved in 300 mL MQ water and ultrasonicated for 20 min to obtain a Ce6 solution. NaN3 stock solution (21 mM): 6.825 mg of NaN3 was dissolved in 5 mL of MQ water and ultrasonicated it for 10 min to obtain a transparent solution. NaN3 solutions with the concentration of 4.2, 2.1, 1.05 and 0.21 mM were obtained by dilution of the NaN3 stock. KI stock solutions (1 M): 2.656 g of KI was added to 16 mL of MQ water and ultrasonicated for 10 min. Ammonium molybdate ((NH4)6Mo7O24•4H2O) stock solution (5 mM): 98.9 mg of (NH4)6Mo7O24•4H2O was dissolved in 16 mL of CH3COONa/CH3COOH buffer solution (1M) then ultrasonicated for 10 min.

SOSG sample (5 µM): 1 mL of SOSG solution was added to a glass vial (4 mL) wrapped tightly with aluminium foil, followed by the addition of 1 mL MQ water. A SOSG (5 µM) + Ce6 (5 µM) solution: 1 mL of SOSG stock solution was added to a glass vial which was also tightly wrapped with aluminium foil, followed by the addition of 1 mL of Ce6 stock solution. DPBF sample: 0.1 mL of DPBF base was diluted by 2 mL of MQ water, achieving a final concentration of 0.1 mM. ABDA samples: 0.05 mL of ABDA base and 2 mL of MQ water was mixed well to form a mixture with the concentration of 0.05 mM.

Samples for the scavenger tests: NaN3 addition: 0.1 mL of NaN3 solution with different concentrations were added to 2 mL of SOSG sample in a closed covered glass vials; Ethanol addition: expected amounts of ethanol were added to the SOSG samples (5 µM). Before radiation, all samples were thoroughly mixed.

N2-saturated solutions were prepared by bubbling the samples with N2 for 20 min.

**Irradiation experiments.** Gamma irradiation: A Cobalt 60 (60Co) radioactive source (GC220, Nordion) was used for the gamma irradiations. The aluminium foil covered vials were placed in the centre of the source.

![Figure 5](https://example.com/image.png)

**Figure 5.** (a) Comparison of the fluorescence intensity at 529 nm for SOSG solutions (5 µM) at different time intervals after γ-ray irradiation of different radiation doses; (b) Comparison of the fluorescence intensity at 529 nm between pure SOSG solutions (5 µM) and SOSG solutions (5 µM) containing Ce6 (5 µM) (λex = 504 nm). (Error bars represent the standard deviation of at least 3 replicates).
The exposure periods were controlled to obtain irradiation doses of 1, 5, 10, 20 and 40 Gy; (The dose rates of $^{60}$Co was calculated using Fricke dosimetry corrected for the 2,778 day half-life of Cobalt-60).

X-ray irradiation: The X-ray irradiation was carried out using an X-ray source (Philips MCN 321 variable-energy X-ray tube) with a voltage of 320 kV and current of 3 mA, without filter. The samples were placed on a horizontal platform located 50 cm from the X-ray window (the dose rate was 1.56 Gy/min). The exposure doses were 5, 10, 15, 20 and 25 Gy.

UV (365 nm/254 nm) irradiation: A commercial UV source (UVGL-58 handheld UV Lamp) was employed as the light source. The aluminium foil was first removed from the vials, followed by placing these vials in a dim box. Then the light source was turned on to irradiate these samples for fixed periods of time.

UV (400 nm) irradiation: A LED light connected to the FL spectrometer was employed as the light source. Samples were firstly transferred from vials to cuvettes, and then these cuvettes were placed in the sample tank, the light source was turned on (0.3–0.5 A and 0.3 kV) to start the exposure.

Characterization. Mass spectra of SOSG solutions were recorded using an ESI mass spectrometer (LCMS-2010A, Shimadzu). A UV-vis-NIR spectrophotometer (UV-6300PC, VWR) was used to measure the optical absorption of the prepared samples. A Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies) was employed to characterize the fluorescence emission spectra of the samples. The emission peak for SOSG was located at 529 nm using a 504 nm excitation light. The slits of excitation and emission are 5 nm, if not be mentioned specially.

The generation of H$_2$O$_2$ was detected by Ghormley’s triiodide method\textsuperscript{32}; the standard samples were prepared by diluting the H$_2$O$_2$ to obtain different concentrations; then 0.1 mL of the KI solution and 0.1 mL of the ammonium molybdate solution was added, and leaving it to react for 10 min, after which the UV intensity at 350 nm was measured\textsuperscript{33}

Detection of H$_2$O$_2$ generation after ionizing radiation exposure: after the radiation treatment, 0.1 mL of the KI solution and 0.1 mL of the ammonium molybdate stock solution was immediately added to the samples, and the UV spectra was measured after reaction time of 10 min.

References

Acknowledgements
Authors gratefully acknowledge the financial support by the China Scholarship Council under Grant Number 201707040083. We also thank for B. Fan for the mass spectrum measurement.

Author Contributions
Huanhuan Liu performed the experiments, data analysis and drafted the manuscript. Philippe J.H. Carter conducted exploratory work on the SOSG probe. Adrie C. Laan provided input on spectroscopic analysis. Rienk Eelkema and Antonia G. Denkova guided the experimental process and data analysis, and revised the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44880-2.

Competing Interests: The authors declare no competing interests.

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