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Felip-León, C.; Puche Panadero, M.; Miravet, JF.; Galindo, F.; Feliz Rodriguez, M. (2019). A spectroscopic study to assess the photogeneration of singlet oxygen by graphene oxide. *Materials Letters*. 251:45-51. <https://doi.org/10.1016/j.matlet.2019.05.001>



The final publication is available at

<https://doi.org/10.1016/j.matlet.2019.05.001>

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Additional Information

A spectroscopic study to assess the photogeneration of singlet oxygen by graphene oxide

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KEYWORDS: singlet oxygen, reactive oxygen species, graphene oxide, fluorescent probe

Abstract

The photogeneration of singlet oxygen ($^1\text{O}_2$) during short irradiation times of graphene oxide (GO) is assessed under visible light with soft irradiation conditions either *directly* monitoring the phosphorescence emission of $^1\text{O}_2$ at *ca.* 1275 nm, or *indirectly* by means of the fluorescent probe 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA). Results obtained using both methodologies lead to the conclusion that $^1\text{O}_2$ generation is negligible under our experimental conditions. In the case of using ABDA very small emission changes were recorded, which could be attributed to other side reactions. Special care should be taken when using this spectroscopic probe to assess the generation of $^1\text{O}_2$, since ABDA and related probes based on the reactivity of the anthracene fluorophore can also detect electron transfer processes. This kind of approaches have been less explored in the field of Materials Science at the nanoscale, and we believe that the knowledge on the lack of generation of $^1\text{O}_2$ by irradiated GO is informative and useful, especially for the assessment of the environmental and biological toxicity of nanomaterials based on GO.

1. Introduction

Carbon based nanomaterials are gaining increasing attention taking into account their outstanding physical properties. Numerous applications have been found in the fields of electronics, energy storage and biomedical areas, to mention only a few. The interest in this family of materials is expected to grow exponentially in the coming years [1-15]. Despite the efforts devoted to understand the chemistry of graphene and its derivatives still important questions remain to be answered. Some of those questions are concerns about their potential environmental toxicity [16-25], and to the *in vivo* toxicity of graphene, GO and derived hybrid nanocomposites used for biological imaging and therapy [15, 26, 27, 28, 29]. In this last realm, the use of short exposure times to light (minutes) is frequent, in contrast to environmental studies (hours). Reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$), superoxide radical anion ($\text{O}_2^{\cdot-}$) and hydroxyl radical ($\cdot\text{OH}$) have been detected upon irradiation of fullerenes [30], carbon nanotubes [31, 32], and graphene quantum dots [33]. However, the investigation of ROS production by GO and reduced graphene oxide (rGO) is much recent. Krishnamoorthy *et al.* reported on the behaviour of GO as a photoreductant but not informed about the generation of ROS [34]. More recently the group of Jafvert detected $\text{O}_2^{\cdot-}$ but not $^1\text{O}_2$ or $\cdot\text{OH}$ after irradiation of GO in water [35]. Later, the group of Sarkar reported that rGO was responsible for the production of ROS in aged samples of GO (suggesting the involvement of $^1\text{O}_2$, although not directly detected) [36]. More recently, Li, Keller *et al.* have studied the photochemistry GO and rGO, finding that $\text{O}_2^{\cdot-}$ was the most abundant ROS generated upon irradiation during long irradiation times (>24h) and using high-power light sources (800 W Xe lamp) [37, 38]. In this case, the concentration of $^1\text{O}_2$ was found $<3 \cdot 10^{-14}$ M.

The most frequently used probe for determining de $^1\text{O}_2$ production is FFA [39]. But the use of this molecule requires a centrifugation step and HPLC analysis for monitoring the reaction. In the last years, very sensitive spectroscopic probes based on fluorescence are gaining acceptance since they allow an *in situ* measurement of the reaction progress, especially for short irradiation times. In the course of our research on graphene composites [40], we were interested in the analysis of ROS produced by GO, and we hypothesized that highly-sensitive spectroscopic methods to detect $^1\text{O}_2$ could be used as a complement to chromatographic ones. Hence we turned our attention to fluorescent probes based on anthracene as $^1\text{O}_2$ indicators. Additionally, we combined this approach with the direct measurement of $^1\text{O}_2$ phosphorescence at *ca.* 1275 nm. Our investigation confirms that $^1\text{O}_2$ production upon irradiation of GO is minimal, as reported with the traditional FFA method [35, 37]. This finding is applicable specifically to the sample of GO prepared by us; samples with a different degree of oxidation or aging after preparation could have a different behaviour. This fact highlights the utility of having a variety of detection methods to assess the production of ROS in graphene derived materials. The need of a toolbox of probes for ROS is strongly recommended in research on GO, especially to evaluate the interaction of GO based nanomaterials with biological media [41, 42].

2. Materials and Methods

2.1. Chemicals

Graphite (powder <20 μm , synthetic), potassium permanganate, sulfuric acid, phosphoric acid, ABDA, 9,10-dimethylanthracene (DMA), Rose Bengal (RB), sodium azide, and fullerene- C_{60} have been obtained from commercial resources, and used as received.

2.2. Instrumentation

Combustion chemical analysis of the samples were carried out using a Fisons EA 1108-CHNS-O analyzer. Fourier-transform infrared spectroscopy (FT-IR) spectra were measured on KBr pellets with a Nicolet 8700 Thermo spectrometer. The Raman spectra were obtained from solid samples previously deposited onto aluminium or quartz wafers, indistinctively, using a “Reflex” Renishaw spectrometer, equipped with an Olympus microscope. The exciting wavelength was 514 nm of an Ar⁺ ion laser. The laser power on the sample was ~10-25 mW and a total of 20 acquisitions were taken for each spectra. UV-Vis spectra were recorded in solution on JASCO V-630 spectrophotometer. Fluorescence measurements were recorded using a JASCO FP-8300 apparatus. Solid-state ¹³C magic angle spinning nuclear magnetic resonance (MAS-NMR) spectra were recorded at RT by using a Bruker AV400WB spectrometer. The samples were spinning at the magic angle at 10 kHz, choosing $\mu/2$ pulses of 5 μ s and a recycle delay of 5 s. Powder X-ray diffraction (XRD) patterns were obtained by using a Philips X’Pert diffractometer and copper radiation ($\text{CuK}\alpha = 1.541178 \text{ \AA}$). X-ray photoelectron (XPS) spectra were collected using a SPECS spectrometer with a 150MCD-9 detector and using a non monochromatic AlK α (1486.6 eV) X-Ray source. Spectra were recorded using analyzer pass energy of 30 eV, an X-ray power of 50W and under an operating pressure of 10^{-9} mbar. During data processing of the XPS spectra, BE values were referenced to C1s peak (284.5 eV). Spectra treatment has been performed using the CASA software. Atomic force microscopy (AFM) images were recorded by using a Multimode Nanoscope 3A instrument operating in tapping mode and with a Si wafer as the substrate. Samples for high resolution electron microscopy (HR-TEM) were ultrasonically dispersed in Milli-Q water and transferred into carbon coated copper grids. HR-TEM images were recorded by using a JEOL JEM2100F microscope operating at 200 kV.

2.3. Synthesis and characterization of GO

GO has been prepared by following the improved Hummer's synthetic method, and by optimization of a previous reported procedure [43, 44]. A mixture of concentrated H₂SO₄/H₃PO₄ (360:40 mL) was added to a mixture of graphite (3 g) and KMnO₄ (18 g) to produce an exothermic reaction to 35-40 °C. The reaction was then heated to 50 °C and stirred for 13 h, then cooled to room temperature and poured onto 400 mL of ice with 30% H₂O₂ (3 mL). After air cooling, the suspension was filtered and washed first with an aqueous HCl (1:10, 37%) solution, and finally with water until pH 7. The resulting solid was suspended in water (400 mL) and sonicated for 30 min; then the suspension was centrifuged at 4000 rpm for 4 h and the solid was removed. The liquid suspension was newly centrifuged at 15000 rpm for 1 h, and the resulting solid was dried at 60 °C to afford *ca.* 1 g of a dark brown material identified as GO. This material has been characterized by FT-IR, Raman, XRD, XPS, solid-state ¹³C MAS-NMR, AFM, HR-TEM, and combustion chemical analyses (C 40.07%, H 2.19%, N 0.0%).

2.4. Chemical trapping of ¹O₂

Photo-oxidation reactions were performed under air inside 3 mL fluorescence quartz cuvettes (1 cm light path) containing aerated aqueous solutions of the singlet oxygen trap ABDA (3 mL, 50 μM) and RB (4 μM) or heterogeneous GO (0.05 mg/mL) photosensitizers. Prior to irradiation, GO was dispersed in distilled water (1mg/mL), sonicated for 60 min, and 150 μL of the resulting dispersion was diluted to 3 mL and sonicated again for 1 min. Irradiations were carried out, with continuous stirring, using a cylindrical reactor (equipped with LED lamps, 400-700 nm emission output, 15.6 mW/m²) placed 12 cm away from the cuvette. The evolution of the photoreactions was

monitored over time (maximum 16 min.) by means of fluorescence spectroscopy (decrease of fluorescence emission at $\lambda_{em} = 430$ nm, $\lambda_{exc} = 375$ nm). The initial points of the kinetic traces were fitted to a pseudo-first order model ($\ln C/C_0 = -k_{obs} \cdot t$, where C is the concentration of ABDA at a certain time t and C_0 is the initial concentration of ABDA (for low concentrations it can be assumed that fluorescence intensity is proportional to concentration)). The same methodology was followed by using fullerene- C_{60} (0.05 mg/mL) as photosensitizer and DMA (3 mL, 50 μ M) as a probe. Control experiments were performed by using ABDA in the absence of the photosensitizer, in the dark or under N_2 atmosphere, using GO as photosensitizer. Quenching experiments were done in the presence of NaN_3 (10 mM).

2.5. Direct singlet oxygen detection

The singlet oxygen phosphorescence decay traces after the laser pulse were registered at *ca.* 1275 nm employing a Peltier-cooled (-62.8 °C) A pulsed Nd:YAG L52137 V LOTIS TII was used at the excitation wavelength of 355 nm. The single pulses were *ca.* 10 ns duration, and the energy was lower than 5 mJ per pulse. The system consisted of a pulsed laser, a 77250 Oriel monochromator coupled to a Hamamatsu NIR detector and an oscilloscope connected to the computer. The output signal was transferred from the oscilloscope to a personal computer. All measurements were made at room temperature, under air atmosphere, and using the chosen solvent (MilliQ water for previously ultrasonically dispersed GO, and toluene for fullerene- C_{60}) in 10×10 mm² quartz cells with a capacity of 4 mL. The absorbance of the freshly prepared samples was adjusted to 0.28 for the singlet oxygen measurements at the laser excitation wavelength.

3. Results and Discussion

GO was synthesized by an optimization of the improved Hummers method [43, 44]. The characterization of the material was done using spectroscopic, X-ray, morphological, and combustion chemical analyses. [13] The identification of hydroxyl, epoxy and carboxylic groups in the GO structure has been confirmed by infrared, solid-state ^{13}C MAS-NMR and XPS analyses (Fig. 1, A-C). [45, 46] The Raman spectrum of GO (Fig 1D) shows the characteristic D (1346 cm^{-1}) and G bands (1605 cm^{-1}), where the G band is associated to the carbon-carbon vibrations of the aromatic rings, and the D band to the presence of defects and to the grade of disorder introduced by the oxygen functionalities. [47-50] The XRD pattern of GO nanosheets (Fig. 1E) reveals the most prominent (001) diffraction peak centered at 2Θ 11.5° , which corresponds to an interlamellar spacing (7.64°) associated to the grade of oxidation of the graphene sheets, and a turbostratic stacking arrangement of the structure. [51, 52] The presence of single and 2-4 layers of GO have been confirmed by HR-TEM and AFM techniques (Fig. 2). [53, 54]

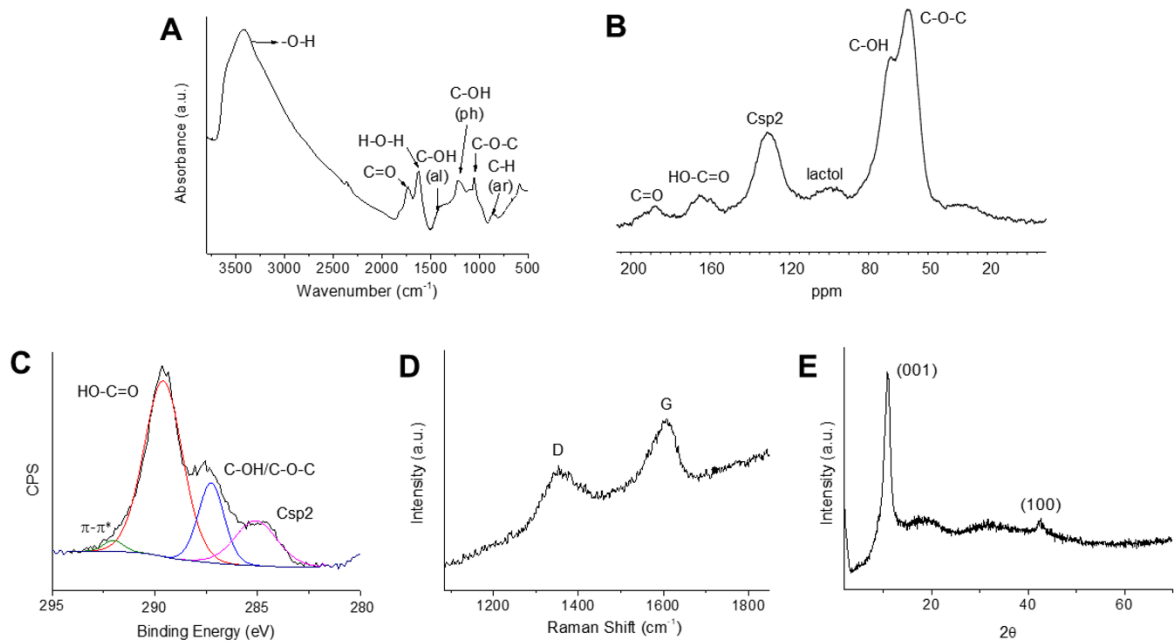


Fig. 1. (A) FT-IR absorption spectrum of GO (ar = aromatic; ph = phenolic; al = alcoholic); (B) solid-state ^{13}C MAS-NMR spectrum of GO; (C) C1s XPS spectrum; (D) Raman spectrum of GO measured at 514 nm; (E) XRD pattern of GO.

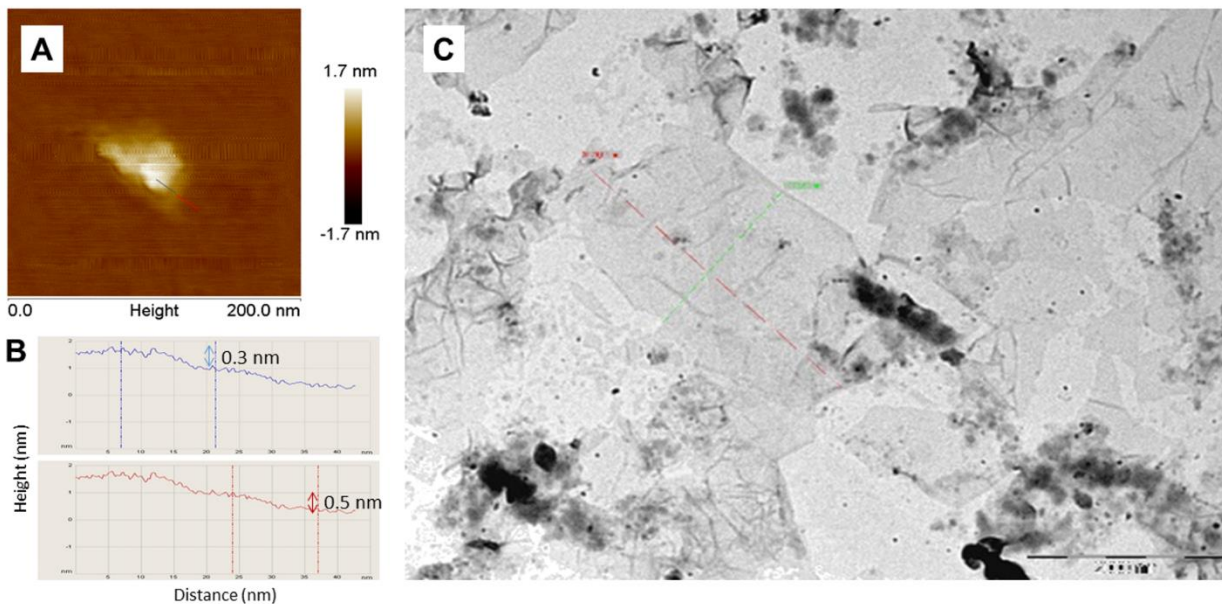


Fig. 2. (A) AFM image of GO; (B) the height profile of the AFM image; (C) HR-TEM image of GO.

In order to assess the production of $^1\text{O}_2$, the probe ABDA (Fig. 3) was used since it is widely employed as a trap for this species in the biomedical realm and can be monitored easily by UV-vis absorption or fluorescence spectroscopies [55, 56]. The underlying operational principle for ABDA involves the disappearance of its main absorption centred at 375 nm (and hence its fluorescence emission at $\lambda = 430$ nm) after reaction with $^1\text{O}_2$ (ABDA $\cdot\text{O}_2$ endoperoxide depicted in Fig. 3 is formed). In Fig. 3 it is shown a representative series of fluorescence spectra, specifically monitoring the reaction of ABDA with $^1\text{O}_2$ generated by the well-known photosensitizer Rose Bengal (RB).

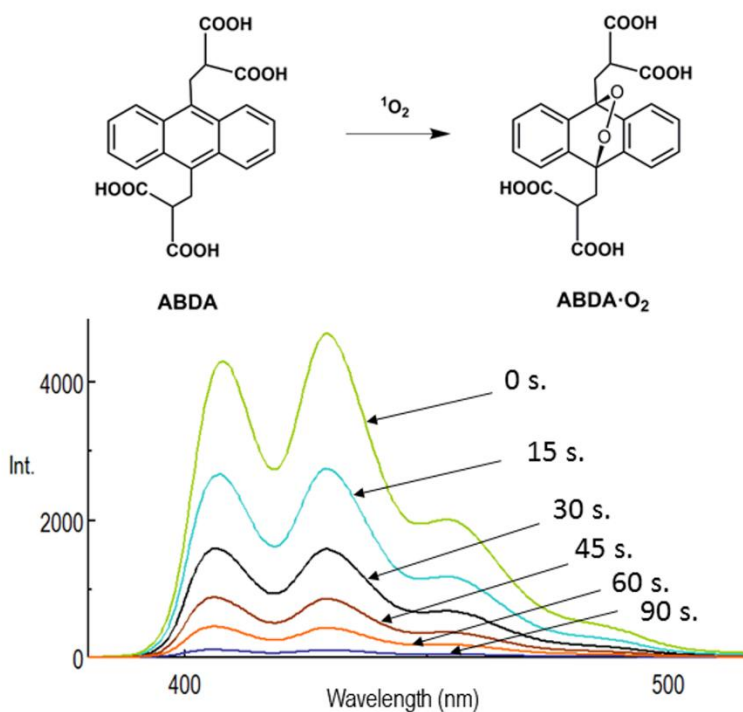


Fig. 3. Representative irradiation of the well-known photosensitizer RB in D₂O and monitoring of the generation of ¹O₂ by means of ABDA (see details in entry 14 in Table 1).

An aqueous suspension of GO (0.05 mg/mL) was prepared, in the presence of ABDA (50 μM), and irradiated with a photo-reactor containing white light emitting diodes (LED 400-700 nm). The fluorescence emission of ABDA was recorded and the intensity plotted against the irradiation time (Fig. 4). The data were fitted to a pseudo-first order model, and k_{obs} are compiled in Table 1. The reaction was repeated in deuterated water since it is reported that the rates of singlet oxygen mediated reactions are enhanced in this medium (about ten-fold) due to the longer lifetime of ¹O₂ as compared to water (67.9 μs vs 3.45 μs in D₂O and H₂O, respectively) [57]. The isotopic effect can be expressed as the ratio of the rate constants in water and D₂O ($k_{\text{D}}/k_{\text{H}}$) resulting a value of *ca.* 3. Finally the use of a specific quencher of singlet oxygen, like NaN₃, was assayed [58]. The azide quencher did not cause any relevant decrease in the rate, resulting a value for this effect of $k_{\text{H}}/k_{\text{H}}(\text{N}_3^-) = 1.1$. A well-known photosensitizer like RB was tested as a control. In this case the isotopic effect was found according to the expected value ($k_{\text{D}}/k_{\text{H}}$ is *ca.* 7) and more importantly the azide effect was as pronounced as described in the literature ($k_{\text{D}}/k_{\text{D}}(\text{N}_3^-) = 47.6$). See all the kinetic traces in Fig. 4 and a compilation of the values in Table 1. Overall, considering the weak isotopic effect and absence of quenching by azide, in the case of GO, it must be concluded that the drop in the emission of ABDA would not be attributable to ¹O₂ but to another side reaction.

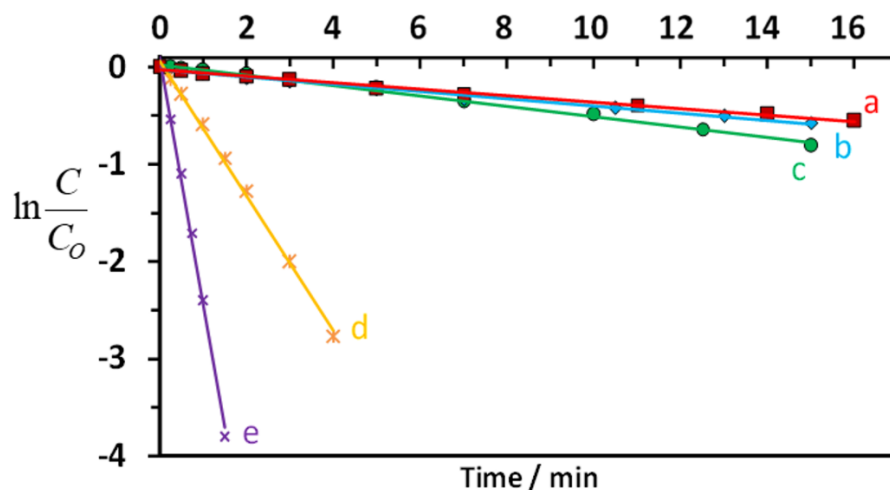


Fig. 4. Selected irradiations conducted in this study, using the quenching of ABDA fluorescence as spectroscopic test: a) GO in D₂O with N₃⁻ as quencher; b) GO in D₂O; c) RB in D₂O with N₃⁻ as quencher; d) Fullerene-C₆₀ in toluene; e) RB in D₂O. All heterogeneous photooxidations were performed at 0.05 mg GO/ml; [RB] = 4 μM; [ABDA] = 50 μM; [N₃⁻] = 10 mM.

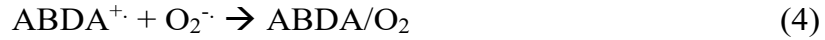
Table 1. Kinetic determinations using ABDA for different irradiations

| Entry | Material | Probe | Solvent | Atmosphere | Quencher | Light source | k _{obs} (min ⁻¹) |
|-------|------------|-------|------------------|------------|-----------------------------|--------------|---------------------------------------|
| 1 | GO (run 1) | ABDA | H ₂ O | air | - | LED | 0.0110 |
| 2 | GO (run 2) | ABDA | H ₂ O | air | - | LED | 0.0118 |
| 3 | GO (run 3) | ABDA | H ₂ O | air | - | LED | 0.0103 |
| 4 | GO (run 1) | ABDA | D ₂ O | air | - | LED | 0.0328 |
| 5 | GO (run 2) | ABDA | D ₂ O | air | - | LED | 0.0373 |
| 6 | GO (run 3) | ABDA | D ₂ O | air | - | LED | 0.0368 |
| 7 | GO (run 1) | ABDA | D ₂ O | air | N ₃ ⁻ | LED | 0.0335 |

| | | | | | | | |
|-----------|---------------------------|------|------------------|--------------------------|-----------------------------|-------------|--------|
| 8 | GO (run 2) | ABDA | D ₂ O | air | N ₃ ⁻ | LED | 0.0331 |
| 9 | GO (run 3) | ABDA | D ₂ O | air | N ₃ ⁻ | LED | 0.0342 |
| 10 | - (control) | ABDA | H ₂ O | air | - | LED | <0.001 |
| 11 | GO | ABDA | H ₂ O | N ₂ (control) | - | LED | <0.001 |
| 12 | GO | ABDA | H ₂ O | air | - | - (control) | <0.001 |
| 13 | RB | ABDA | H ₂ O | air | - | LED | 0.3834 |
| 14 | RB | ABDA | D ₂ O | air | - | LED | 2.576 |
| 15 | RB | ABDA | D ₂ O | air | N ₃ ⁻ | LED | 0.0533 |
| 16 | Fullerene-C ₆₀ | DMA | toluene | air | - | LED | 0.6920 |

Although it was not possible to set samples with comparable absorptions due to the dispersion of GO, it seems clear, although only qualitatively, that the kinetics of RB is notably faster than the rate measured for GO. As a matter of fact, RB depletes the emission of ABDA in seconds (Fig. 3), whereas the photoirradiation of GO leads to minor detectable changes in ABDA fluorescence only after minutes. This supports the view that the amount of generated ¹O₂ by GO must be minimal. Just for qualitative assessment another well-known photosensitizer, fullerene-C₆₀, was used to certify that GO generates very low amounts of ¹O₂. The purpose of this assay was to use an excellent photosensitizer (ϕ_{Δ} = 0.96) with a comparable absorption to GO [59]. Hence, a sample of 0.05 mg/mL of fullerene-C₆₀ was irradiated in toluene, since this photosensitizer generates highly efficiently ¹O₂ in organic apolar medium (but not in water). In this case another anthracene derivative was employed (dimethylantracene, DMA) since ABDA is not soluble in toluene. The measured reaction rate was 0.6920 min⁻¹. Considering that the lifetime of singlet oxygen in toluene (30.3 μ s) is about half of the value in D₂O [57], this result supports the idea of minimal amount of ¹O₂ generated by GO upon irradiation.

A question that can be raised is whether the slight decrease of the ABDA fluorescence could be attributed to other species different from $^1\text{O}_2$. It is described that anthracene and its derivatives (An) are very prone to oxidation leading to the corresponding radical cation (An^+). This species react with superoxide to give an adduct (AnO_2) identical to the one obtained by reaction with singlet oxygen. This reaction has been described many times for anthracene and electron transfer photosensitizers [60]. As a matter of fact, the specificity of anthracenic probes for the detection of $^1\text{O}_2$ has been studied in detail recently: the genetically encodable fluorescent tag miniSOG has been reported to produce $^1\text{O}_2$ with a notable quantum yield (0.47) using the probe ADPA [61]. However, Nonell and Flors have measured a significantly smaller value (0.03) [62]. This discrepancy was attributed to the ability of miniSOG to photooxidize the probe and hence lead to reaction with superoxide. Hence, ADPA would be actually measuring both ROS produced by type I (electron transfer) and type II (energy transfer) mechanisms. In another example, the endoperoxide of DMA was formed upon irradiation of alkyloxo(methoxo) tetraphenylporphyrinato-antimony *via* photoinduced electron transfer mechanism involving DMA^+ and O_2^- exclusively [63]. Accordingly, the mechanism involving O_2^- described in eqs.1-4 can be proposed to account for the minimal bleaching of ABDA upon irradiation of GO. In this case, apart from $^1\text{O}_2$, the superoxide anion would be involved in the reaction with ABDA. Unfortunately the slopes of the kinetics recorded in this study are too close to the control irradiations to allow any conclusion on this regard. What it can be affirmed, however, is that $^1\text{O}_2$ production by irradiated GO (under visible light with soft irradiation conditions and short times) is close to the detection capability of the technique, and hence disregard any reasonable toxicity associated to the production of this specific ROS by our material.



It is not ruled out that ageing of the GO samples would lead to the formation of byproducts similar to rGO and low molecular-weight species, as recently demonstrated by other groups[37, 38, 64]. If this is the case, the origin of $\text{O}_2^{\cdot-}$ would be this phototransformed fraction of the sample since it has been demonstrated the reducing capacity of rGO. However this possibility is very unlikely in our case since photoreduction of GO to rGO has been described using high energetic UV light during several hours, whereas we are using a source of visible light and very short irradiation periods (minutes), precisely to avoid photoageing of our GO. The ageing of GO upon irradiation is a matter of debate that falls out of the scope of this research, which is focused on the use of sensitive spectroscopic probes to study GO, as a complementary tool to FFA and chromatography.

Finally, a direct measurement of the phosphorescence of $^1\text{O}_2$ at *ca.* 1275 nm was also attempted [57], since this is an irrefutable probe of the existence of this ROS. Hence, GO dispersed in water and irradiated at 355 nm yielded only a noisy signal (red line, Fig. 5), in contrast with the strong intensity showed by irradiated fullerene- C_{60} in toluene (black line, Fig. 5) used as a positive control. This experiment also confirms the negligible generation of $^1\text{O}_2$ when our GO is irradiated, at least up to the limit of detection of the employed methodologies.

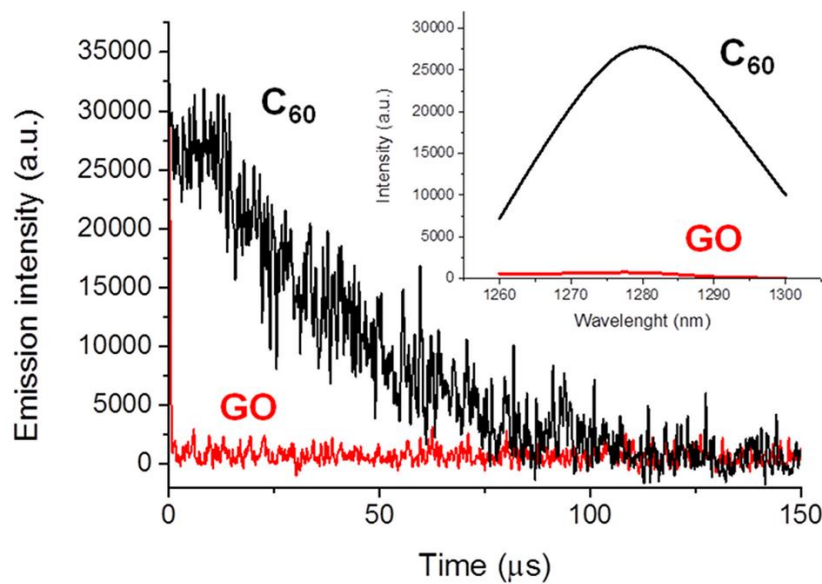


Fig. 5. Time profiles of the $^1\text{O}_2$ phosphorescence signal produced by fullerene- C_{60} (black line) and GO (red line) in water and toluene, respectively. Inset: singlet oxygen emission spectra for fullerene- C_{60} and GO acquired at *ca.* 10 μs after the laser pulse. The samples were adjusted to the same absorbance at the excitation wavelength (355 nm) and the phosphorescence of $^1\text{O}_2$ was recorded at *ca.* 1275 nm.

4. Conclusions

We have found that generation of singlet oxygen upon short periods of visible light irradiation of samples of freshly prepared GO is almost negligible, using a high sensitivity spectroscopic fluorescent probe like ABDA. Exact quantification of this species remained elusive due to the dispersion of GO in water (light scattering), however there is enough qualitative evidence to suggest that the irradiation of GO with visible light during short periods of time gives rise to negligible amounts of this ROS. These results confirm recent

measurements reported by other groups using FFA and chromatographic analyses as tools. We hope, firstly, that this study will contribute to answer some questions regarding the potential toxicity of nanomaterials derived from graphene and, secondly, that it will add ABDA, or other fluorescence probes, along with direct detection via phosphorescence emission, to the toolbox that environmental scientist use in their researches.

Acknowledgements

Ministerio de Economía y Competitividad of Spain (grant CTQ2015-71004-R) and Universitat Jaume I (grant P1.1B2015-76) are thanked for their financial support. C.F-L. thanks the Ministerio de Economía y Competitividad of Spain for a FPI fellowship. Technical support from SCIC of University Jaume I is acknowledged. The Instituto de Tecnología Química (ITQ) thanks the support of the Severo Ochoa Program (SEV-2016-0683), and Consejo Superior de Investigaciones Científicas (I-Link1063). We would like to thank Dr. Francisco Bosca for his technical assistance with the singlet oxygen emission measurements at the ITQ.

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