

Ultraweak chemiluminescence from γ -irradiated humic acids

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Abstract Dried humic acid and its aqueous solution in 0.1 M Na_2CO_3 were γ -irradiated with 1–90 kGy from ^{60}Co . Thereafter, a secondary ultraweak radiation in the spectral range of $\lambda = 340\text{--}650$ nm from aqueous solutions was recorded as long-lived chemiluminescence (CL). Only for absorbed doses lower than 10 kGy low intensity signals of the CL decay were measurable. For absorbed doses higher than 40 kGy residual flat and weak signals are observed. Humic acid irradiated in the dry form did not reveal statistically significant CL. Absorption spectra ($\lambda = 240\text{--}800$ nm) of irradiated solutions indicated the occurrence of a dominant degradation process of the humic acid macromolecular components. The effect of H_2O_2 and CL enhancers (luminol and lucigenin) on the intensity and kinetics of CL proved the participation of reactive oxygen species and the free radical mechanism in the CL and degradation processes. The dose-effect relationship (i.e. γ -radiation absorbed dose vs. intensity of γ -radiation-induced CL) shows a non-linear shape, especially in the range of 1–10 kGy, which suggests complex radical mechanisms. A possible ecological significance of the observed phenomena is briefly discussed.

Key words chemiluminescence • γ -irradiation • humic acid

Introduction

Humic substances, i.e. dark paramagnetic polymeric anionic polyelectrolytes, belong to the most widespread macromolecular substances of biogenic origin in soil and aquatic environments. The exact structure of humic substances is unknown because of their complexity resulting from unrecurring precursors, from their composition and their process of synthesis. Degradation processes of these substances can be a source of energy, carbon and nitrogen for soil and aquatic microorganisms. CO_2 released in the degradation processes contributes significantly to the greenhouse effect [3].

Humic substances effectively absorb non-ionizing radiation, such as ultraviolet (UV), visible or infrared radiation. Much less is known about their sensitivity to ionizing radiation, such as γ - or X-rays. They exhibit a permanent “dark” paramagnetism ($10^{16}\text{--}10^{18}$ spin/g), photoinduced transient electron paramagnetic resonance (EPR) signals, probably originating from redox semiquinone moieties [3]. Therefore, humic substances are an efficient target for solar radiation and most probably for ionizing radiation. Up to now, only few papers are available on the possible interaction of humic substances with ionizing radiation [4, 8], as compared to those dealing with the effect of UV [5, 8, 11]. The only substances, which have the elemental composition, molecular weight and physicochemical properties similar to humic substances, are melanins. Although their interaction with ionizing radiation is of high medical relevance [6], the possible secondary chemiluminescence induced by γ - or X-rays has not yet been investigated. Earlier studies were

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focussed only on proteins, nucleic acids and several polymers [9, 10].

In this paper, we present some preliminary results of our first study series on γ -induced secondary radiation (radiochemiluminescence) of an alkali-soluble fraction of humic acids. Our investigation is restricted to the kinetics of chemiluminescence (CL), dose-effect relationship and enhancement of ultraweak CL signals by highly efficient chemiluminogenic probes. Because of the lack of published data on this topic, we applied a broad range of absorbed doses 1–90 kGy.

Methods

In our experiments, commercial humic acid (Serva, Busch, CH) soluble in alkaline solution has been examined. Its elemental composition was: 38.54% C, 6.33% H, 0.67% N, 39.36% O, 16.10% ash (minerals). The colour coefficient was $Q_{4/6} = 8.60$ ($OD_{400\text{nm}}/OD_{600\text{nm}}$).

An aerated solution of HA (200 mg of HA in 1000 cm³ of 0.1 M Na₂CO₃) was irradiated in a glass vessel, in a RChM-gamma-20 (Co-60) equipment (Techsnabexport, Moscow, Russia). The system was calibrated with Fricke dosimeter. The absorbed doses were: 1, 2, 5, 10, 40, 50, 60 and 90 kGy. These doses required an irradiation time of 40 min to 4 h. Aliquots of irradiated solutions (15 ml) transferred from a stock solution were placed into liquid scintillation vials (Beckman) and γ -induced ultraweak CL was measured in a scintillation counter Beckman LS-100 C after every 12–15 min cycle and several days after irradiation.

The dead time between the end of irradiation and the first measurement was about 5 min. The LS-100C was equipped with an S-11-type photocatode PMT sensitive to the spectral range of 320–600 nm. The counting (sampling) time was 10 s. Non-irradiated empty scintillation vials, Na₂CO₃ + HA solution and Na₂CO₃ solution alone were measured as a control.

Absorption spectra of the control and irradiated HA solutions were measured in the range 240–800 nm using a Beckman DV-7000 spectrophotometer. In order to enhance a weak CL signal and to acquire information about the intermediates and mechanisms involved in the measured secondary emission, hydrogen peroxide, luminol and lucigenin in μM concentrations were added to the solutions following their irradiation, or to the non irradiated solutions for the control, respectively.

Results and discussion

Radiolysis of components

High energy γ -radiation from ⁶⁰Co (1.17 and 1.33 MeV), interacting with the complex system of humic acid macromolecules in air-saturated aqueous solutions of Na₂CO₃, initiates a wide spectrum of radical and ion-radical processes involving electronic-excited states and reactive oxygen species.

The γ -irradiated Na₂CO₃ solution (pH = 10.4) alone produces a long living CL by a the signal-to-noise ratio (S/N) of ≈ 3 –4. This emission is probably due to the reactions of OH \cdot and O₂ \cdot^- with HCO₃ \cdot^- ions (see Eq. (1)):

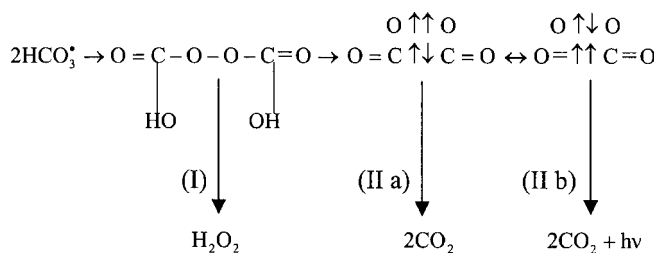
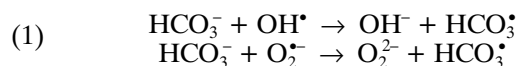


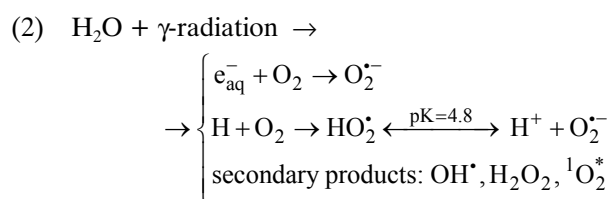
Fig. 1. A possible mechanism of chemiluminescence arising from carbonate radicals and dioxethanone (arrows indicate spins of electrons).



Recombination of O₂ \cdot^- or COO \cdot^- ion-radicals may generate energy-rich precursors (II a, II b) of electronically excited molecules or directly electronic excitation (Fig. 1). For this mechanism, the spin reversion can be achieved by emission of radiation $h\nu$ (II b) in the near UV-blue light as predicted from calculations [13], and as observed in the auto-oxidation system: L-dopa + O₂ + COO \cdot^- [12].

However, clear two-phase chemiluminescence kinetics with relatively “flat” chemiluminescence kinetic curves, slightly higher than 28 ± 8 cps, were observed hundreds of minutes. Therefore, we can assume that a stable molecular product of the radiolysis of the reaction system Na₂CO₃–O₂–H₂O has to be formed. Then, it is gradually consumed in the reactions with HCO₃ \cdot^- + CO₃ \cdot^{2-} . The most probable product is H₂O₂ which decomposes to OH \cdot and/or O₂ \cdot^- .

During irradiation of dilute water solutions of humic substances, the radiation energy is predominantly absorbed by H₂O₂ molecules, which undergo radiolysis. The action of radiation is thus mainly based on the reactions of water radiolysis products with humic substance molecules. Under the irradiation conditions employed (air atmosphere), H atoms and e_{aq} \cdot^- are scavenged by O₂ at the diffusion – limited rate (see Eq. (2)):



Intermediates and stable products of water radiolysis include OH \cdot , O₂ \cdot^- , HO₂ \cdot , H₂, ¹O₂ \cdot and H₂O₂. The effect of ionizing radiation on solutions of biopolymers leads to diverse macromolecular modification, which may result in the state of aggregation or degradation. The mechanisms of damage to complex sub- and supramolecular structures of humic substances due to ionizing radiation and products of radiolysis are completely unknown. In contrast, the radiosensitivity of melanins, as a similar group of natural polymers, has been widely studied because there was a need due to melanoma therapy with ionizing radiation. However, detailed mechanisms of melanin degradation are also unknown.

Radiative relaxation of excited molecules leads to the observed secondary radiation. This ultraweak radiation has to be chemiluminescence, because due to the delay between

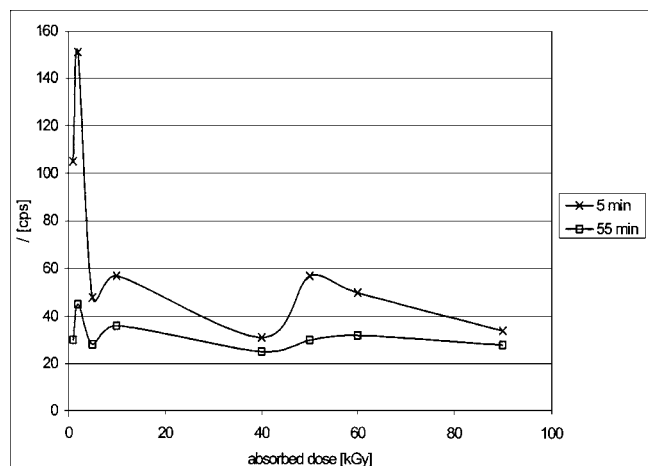


Fig. 2. Chemiluminescence intensity I of HA after dissolving the irradiated dry HA in 0.1 M non irradiated Na_2CO_3 aqueous solution as a function of the absorbed dose (the parameter of the curves – start of the CL measurement after the end of irradiation process). For the sake of clarity, curves $I = f(D)$ for 15, 25, 35 and 45 min are not presented.

the end of γ -irradiation and the start of measurement of this secondary radiation it is so long. Three groups of experimental data characterizing the observed CL and the underlying processes are analyzed as follows.

Chemiluminescence intensity and kinetics – absorbed dose dependence

Humic acid in the dry form that has been γ -irradiated with absorbed doses of 10–90 kGy does not reveal statistically significant CL. Only after dissolving, the irradiated dry humic acid in 0.1 M non irradiated CO_3^{2-} aqueous solution a trace of CL was observed. This suggests (Fig. 2) that the deposition of high doses of γ -radiation in humic acids does not generate long-lived radicals or trapped e^- s at concentrations high enough to produce CL measurable by means of the used detection system and experimental conditions. For all doses of ionizing radiation, only the first 20–50 min of kinetic processes are statistically important (Fig. 3). The average noise N-value is 28 ± 8 cps, γ -irradiated solutions of HA in 0.1 M Na_2CO_3 give weak distinct decaying CL

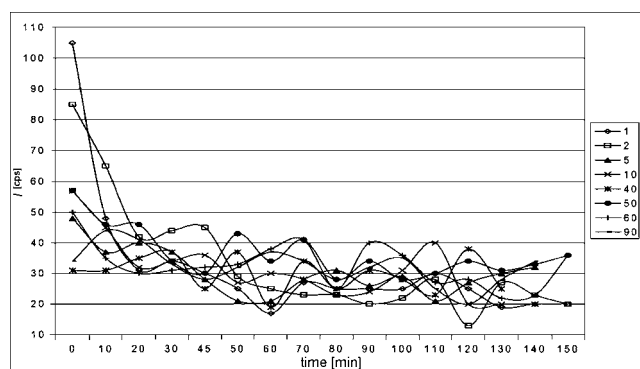


Fig. 3. Chemiluminescence intensity I of HA- Na_2CO_3 solution as a function of the time (the parameter of the curves – absorbed dose in kGy). For times higher than 20 min all values are fluctuated around the background.

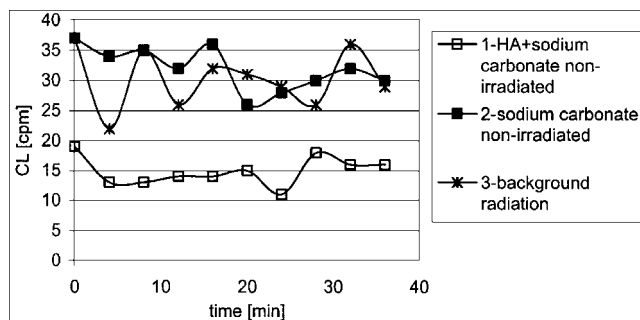


Fig. 4. Chemiluminescence of the background and non-irradiated solutions.

signals lasting ca. 50 min. These signals are measurable only for low doses and short time interval after γ -irradiation.

An aqueous solution of Na_2CO_3 (a solvent) γ -irradiated with 1–5 kGy emits measurable quasi-stationary CL in the (S/N) ratio equal to ≈ 5 . When non-irradiated humic acid solution was added to the irradiated Na_2CO_3 solution, then a distinct quenching of CL was observed. Humic substances have antioxidizing capacity and are able to quench, e.g., electro-CL of radical reactions [2].

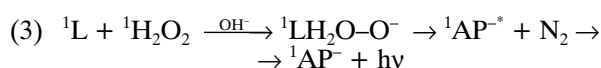
However, after irradiation of HA + Na_2CO_3 , the resulting CL is about 6 times stronger than that of Na_2CO_3 irradiated alone. This means that both compounds undergo radiolytic reactions and contribute to the generation of excited states and a secondary radiation (Fig. 4). For non-irradiated solutions of reactants, the single photon counting (SPC) measurements give the following results: CL intensity of 0.1 M Na_2CO_3 varies from a minimum 26 cpm to 37 cpm (average 35 ± 9), while that of 0.01% HA + 0.1 M Na_2CO_3 from 11–19 cpm (average 15 ± 7). These data were obtained from 10 measurements taken during initial 36 min (Fig. 4). The intensity of CL of HA + Na_2CO_3 (non-irradiated) is statistically lower than that of the background and Na_2CO_3 alone. This can be easily explained by the well-known strong absorption of 0.01% humic acids and scavenging of reactive oxygen species present in trace amounts in solutions. Also, a residual phosphorescence of an empty vial is quenched by the HA solutions.

For high absorbed doses of γ -radiation (i.e. 10–90 kGy), only flat, residual low-intensity CL kinetics of the irradiated system can be recorded (see Figs. 2 and 3). A complex shape of $I_{\text{CL}} = f(D)$ functions indicates that different doses cause variable kinetics and generate a complex mechanism of chemiluminescence.

Effect of CL enhancers after different times and doses of γ -irradiation

Luminol

5-amino-2,3-dihydrophthalazine-1,4-dione (luminol, L, SIGMA) is a widely used CL compound for sensitive detection of reactive oxygen species (ROS). Luminol can be oxidized by $1-e^-$ and $2-e^-$ oxidants (see Eq. (3)):



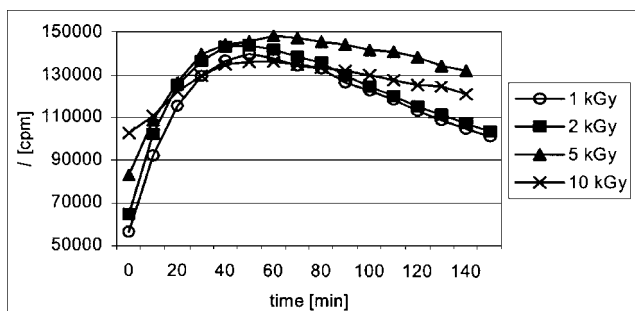


Fig. 5a. Chemiluminescence intensity I of γ -irradiated humic acid and Na_2CO_3 solution as a function of the time (the parameter of the curves – absorbed dose, only 1–10 kGy) after adding luminol.

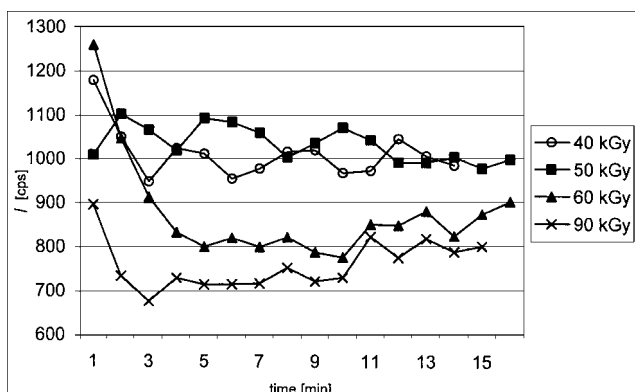


Fig. 5b. Chemiluminescence intensity I of γ -irradiated humic acid and Na_2CO_3 solution as a function of the time (the parameter of the curves – absorbed dose, only 40–90 kGy) after adding luminol.

The emitter is 3-aminophthalate ($\lambda_{\text{max}} = 425$ nm and the quantum yield ≈ 0.02) [7].

Addition of μ -molar concentrations of luminol to irradiated solution of Na_2CO_3 or $\text{HA} + \text{Na}_2\text{CO}_3$ brings about very strong chemiluminescence (Fig. 5a, b) with the (S/N) ratio > 5000 . $I = f(t)$ curves at 1–10 kGy clearly show a consecutive character of CL kinetics (Fig. 5a). This proves the formation of reactive oxygen species containing higher amounts of H_2O_2 , OH^\bullet , OOH^\bullet , $^1\text{O}_2$ and ROO^\bullet than superoxide anion $\text{O}_2^{\bullet-}$ (compare results for lucigenin).

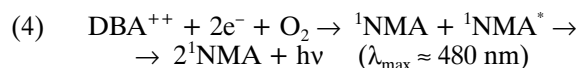
Analogous kinetic curves for 40–90 kGy absorbed doses reveal only a flat, residual CL with (S/N) ≈ 40 and a reciprocal relationship between D and I (Fig. 5b). At higher doses, requiring longer irradiation time, only a residual CL is observed. The rate of radioluminescent processes is probably high enough to consume HA , Na_2CO_3 , and $\text{H}_2\text{O}/\text{O}_2^-$ intermediates can be derived already during the irradiation. A fast flow system would be required to measure these fast processes in the initial phase of irradiation. There is no effect of added H_2O_2 that means H_2O_2 is formed during the radiolysis.

From the ascending part of $I = f(t)$ curves, it follows that the chemiluminescent reactions are slowly developing, i.e. in the irradiated solutions there are still substrates for such reactions. The proportionality between the absorbed dose D and the initial CL intensity is also seen.

Lucigenin

The lucigenin (dimethylbiacridinum dinitrate DBA, SIGMA) luminescent reaction is a reductive dioxygenation [1] (NMA

denotes N-methylacridone) (see Eq. (4)):



Lucigenin may, therefore, serve as a chemiluminescent probe for high sensitivity assay of superoxide ion-radical $\text{O}_2^{\bullet-}$ or organic peroxides produced in the radiolysis of humic acids.

CL curves for 1–10 kGy are shown in Fig. 6a and analogous curves for 40–90 kGy in Fig. 6b. Again, high doses and long-lasting irradiation cause total consumption of the reactants and a flat residual CL response (except 40 kGy) is observed. Addition of μ -molar quantities of H_2O_2 after irradiation, increases only slightly the I_{CL} values that indicates close-to-saturation amounts of radiolytically formed H_2O_2 . The enhancing effect of luminol is several times higher than that of lucigenin. Luminol is known to be less selective than lucigenin towards ROS. Lucigenin gives preferentially strong CL with H_2O_2 and O_2^- .

These enhancers are very useful to increase the lower limit of detectability of CL and, thus, to study possible effects of still lower doses of IR.

Optical absorbance – absorbed dose of γ -irradiation

After irradiation, the humic acid solutions changed their colour from dark-brown to straw-like. After higher than 60 kGy absorbed doses, the change of colour was more

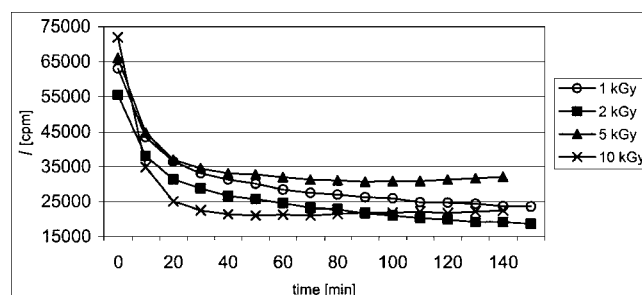


Fig. 6a. Chemiluminescence intensity I of γ -irradiated humic acid and Na_2CO_3 solution as a function of the time (the parameter of the curves – absorbed dose, only 1–10 kGy) after adding lucigenine.

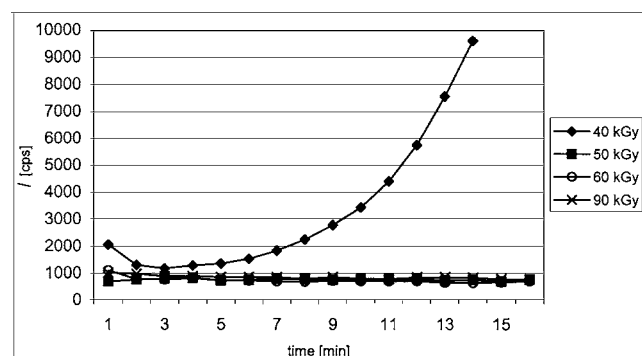


Fig. 6b. Chemiluminescence intensity I of γ -irradiated humic acid and Na_2CO_3 solution as a function of the time (the parameter of the curves – absorbed dose, only 40–90 kGy) after adding lucigenine.

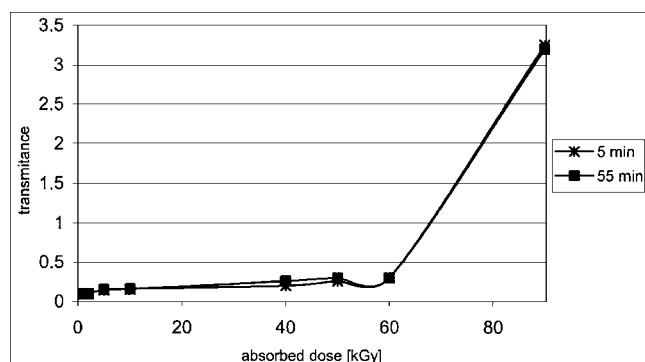


Fig. 7. Relation between transmittance and absorbed dose of humic acid and Na_2CO_3 solution after γ -irradiation for $\lambda = 254$ nm (5 min and 55 min after the end of irradiation process).

effective, which may be explained by the absorbance (or transmittance) at $\lambda = 254$, 400 and 600 nm (Fig. 7). Basic indicators of humic acid degradation are colour coefficients, Q_{ij} , where Q is the ratio of absorbance at selected wavelengths, and indices i/j indicate the absorption wavelength. Q_{ij} the value reflecting the condensation degree of the aromatic moieties of humic acid macromolecules and correlating with the chromophore and/or auxochrome concentration, molecular weight and intrinsic free radical concentration [3].

Calculated values of Q_{ij} for $\lambda = 270$, 400 and 600 nm indicate a degradation of long wavelength absorbing chromophores to simpler aliphatic compounds. The decrease of Q_{ij} observed for lower doses and shorter irradiation times is probably induced by competitive minor secondary reactions such as cross-linking of subunits and formation of higher polymerized products.

Conclusions

1. Irradiation of alkaline solutions of humic acids by high-energy γ -quanta initiates prolonged secondary emission, i.e. radiochemiluminescence in the spectral region of 320–600 nm. Humic acids in the dry form, irradiated with 10–90 kGy, do not reveal statistically significant chemiluminescence. Only absorbed doses lower than 10 kGy generate very weak short CL signals.
2. The data obtained reflect “residual” (final) kinetics $I = f(t)$ of the total $I' = f(t')$ as there is a various long delay between the end of irradiation and the start of measurement.
3. Combinations of substances, irradiated and non-irradiated, indicate that all components of the reaction system: H_2O , O_2 dissolved, Na_2CO_3 and humic acids contribute to the observed delayed radiochemiluminescence and its very complex mechanisms.
4. The use of chemiluminescent enhancers luminol and lucigenin proved the formation of reactive oxygen species, especially of H_2O_2 , OH^\bullet and $\text{O}_2^{\bullet-}$. The lack of the H_2O_2 effect indicates the presence of radiolytically formed H_2O_2 .

5. γ -Irradiation of humic acids induces oxidative degradation of their macromolecules to smaller and more acidic, hydrophilic fragments, which is shown by the increase in values of the optical indices $Q_{4/6}$ and $Q_{2,6/4}$.
6. Future studies require a stopped-flow technique or a deep-frozen sample irradiation to avoid long dead times between irradiation and measurements.

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