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ORIGINAL PAPER

Validation of Photodynamic Action via Photobleaching of a New Curcumin-Based Composite with Enhanced Water Solubility

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Abstract Motivated by the photochemical and photophysical properties of curcumin-based composites, the characteristics of a new curcumin-based water-soluble salt were investigated via absorption and fluorescence spectroscopy. Photobleaching was investigated using a set of LEDs in three different wavelengths (405 nm, 450 nm and 470 nm) to illuminate an aqueous solution of curcumin, evaluating its degradation for five different exposure times (0, 5, 15, 45 and 105 minutes). The results were compared with equivalent measurements of dark degradation and illumination in the presence of a singlet-oxygen quencher. Three solution concentrations (50, 100 and 150 μg/ml) were studied. To measure the fluorescence, it was used low power 405 nm excitation laser source. Time dependent photodegradation of curcumin was observed, as compared to the natural degradation of samples maintained on a dark environment. Two main absorption peaks were detected and their relation responded to both concentration and wavelength of the illumination source. A spectral correlation between absorption of curcumin and the emission bands of the sources showed an optimal spectral overlap for the 450 nm LED. For this source, photobleaching showed a less intense degradation on the presence of singlet oxygen quencher. This last result confirmed singlet oxygen production in vitro, indicating a strong potential of this composite to be used as a blue-light-activated photosensitizer.

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Introduction

Curcumin is a natural yellow-orange colored compound, with origins on the Zingiberaceae turmeric family. It is a diferuloylmethane ($HOC_6H_3(OCH_3)CH{=}HCO\big|_2CH_2$) which can assume two main chemical configurations: keto and enol. The enol form is the most energetically stable, and its most common commercial form have a molecular weight of 368.38 $g.mol^{-1}$. The term curcumin may refer to a mixture of three curcuminoids: curcumin, desmethoxycurcumin and bis-desmethoxycurcumin, with different number of hydrogen atoms substituting the $–OCH_3$ radical [1, 2].

Several reports have confirmed that curcumin-based compounds have natural anti-inflammatory, antimicrobial and anti-cancer properties [3–5]. Recent investigations on cell lines also indicated their potential application on Photodynamic Therapy (PDT) in vitro [6–10]. A potential application of curcuminoids on PDT protocols is to destroy superficial tumor lesions or localized and superficial infections. Because of its optical absorption located in the blue-green region of the electromagnetic spectrum [11], the irradiated photons do not affect interior structures. The curcumin-mediated PDT and its induced apoptosis were investigated in the treatment of carcinoma cells from human skin [6]. The curcumin prevented JNK activation induced by PDT, mitochondrial release of cytochrome c, caspase-3 and cleavage of PAK2. The synergetic effect of UVB and curcumin on cell death by apoptosis in HaCaT cells and the molecular mechanisms related to it were also investigated [7]. HaCaT cells undergo apoptosis evidenced by DNA defragmentation by the combination of curcumin with UVB when compared with UVB alone or curcumin. It was observed the activation of caspase-3, 8 and

9, along with the activation and following release of cytochrome c.

The preferential uptake of a curcumin composite in two different cell lines in vitro in comparison to healthy cell lines has also been demonstrated [12]. Besides the preferential uptake, the authors have shown an enhanced quantum yield occurring for the same curcumin concentration inside tumor cells. This last result has great impact on PDT applications, as they depend on the selective uptake of photosensibilizing agents.

The superficial penetration of the light necessary to photo activate curcumin composites makes them an excellent candidate for antimicrobial Photodynamic Therapy (aPDT), targeting bacteria [13, 14] and other microorganisms [15]. Microbial entities can cause extreme complications and infections during cavity preparation and other dentistry procedures. Recently, Dovigo *et.al*. demonstrated the antibacterial photodynamic effect against *Candida Albicans* [16–18], reinforcing the curcumin photodynamic properties. The effect of curcumin on gram-positive (GP) and gram-negative (GN) bacteria was also investigated, in which *Enterococcus faecalis* (GP) and *Escherichia coli* (NG) were used as targets. The dependence of the phototoxic effect on the curcumin concentration and dose of blue light radiated was evaluated [19, 20].

Curcumin is a hydrophobic polyphenol, which is mostly insoluble in water, even in moderately acid pH [21]. By changing the properties of the solvent, such as polarity, the spectroscopic properties may vary dramatically [22, 23]. In addition, curcumin can be synthetized with metallic ions in its structure, also changing its spectroscopic properties [24]. These results point to the possibility of producing synthetic curcumin based-compounds with specific spectroscopic properties. Despite its unique optical properties, curcumin's low solubility in water prevents it from surviving inside plasma and tissues for long periods, decreasing its biological availability [25]. Efforts are being conducted to produce new formulations with enhanced stability and solubility in aqueous solution [26]. This is a necessary step to attain full applicability of curcumin clinically.

Our goal is to validate the photodynamic properties of a new curcumin salt with enhanced water solubility. Recently published studies using the same formulation showed its photodynamic potential directly on bacteria and planktonic cultures [27, 28]. These facts reinforce the need of a fundamental investigation on the spectroscopic and photochemical properties of this new compound. The degradation induced by illuminating solutions with different light sources, irradiation times and concentrations, compared to dark environment degradation, was studied via absorption and fluorescence spectroscopy. It will be shown a relation between its degradation and the singlet oxygen production in vitro, a clear signature of its photodynamic action.

Materials and Methods

In this work, it was used a concentrated mother-solution (30 % in weight) of curcumin salt, containing the three main curcuminoids (curcumin, desmethoxycurcumin and bisdesmethoxycurcumin). It is prepared in water and N-Methyl-D-Glucamine and has a final average molecular weight of 730.32 $g.mol^{-1}$. (PDTPharma®, Cravinhos, Brazil). The addition of N-Methyl-D-Glucamine ($C_7H_{17}NO_5$) gives the composite a considerable higher stability in water solutions. The mother-solution was diluted again in distilled water, generating solutions in three different concentrations (50, 100 and 150 $\mu g.ml^{-1}$).

Absorption spectroscopy was carried out on a spectrophotometer (VARIAN - CARY 50 BIO UV-Visible Spectrometer), using quartz cuvettes with 1 cm of optical path. Fluorescence spectra were obtained by a probe-type fiber optics coupled to a spectrometer (SF2000, Ocean Optics Inc.) and a laser source (Eagle VIO 50 mW laser, 405 nm, Ocean Optics Inc.). Both absorption and fluorescence records were obtained immediately after dilution (at t=0 min) and subsequently at times of 5, 15, 45 and for 105 min in a dark environment. This procedure is important to evaluate the stability on the solution concentration.

Absorption spectra were obtained to evaluate the photobleaching caused by light illumination. To validate photobleaching, it is performed an in vitro illumination of the solutions containing the light absorbing molecule. The decay on the absorption or fluorescence bands of the studied molecule as a function of the illumination time is a strong indication of photobleaching. LEDs with different emission bands, centered at 405, 450 and 470 nm, were used as illumination sources. The illumination conditions appear on Fig. 1. The photon energies are inversely proportional to the central wavelength λ of the emission band and can be calculated by the expression $E_\lambda=(1.9914\times10^{-16}/\lambda)$J, where λ is already expressed in nanometers. The incident power on each side of the cuvette was of 10 mW and uniform, confirmed by the use of a power meter. The cuvette was filled with solutions until a height 3 cm of liquid column was attained, resulting on a 3 cm^2 of illuminated area on each side of the cuvette. The total effective energy and dose irradiated were calculated from the expressions $E_{total}=(1.2\Delta t)$J and $D_{total}=(0.4\Delta t)$ J/cm^2, where Δt is the illumination time expressed in minutes. Absorption spectra were obtained during the illumination procedure, at the same moments of dark experiments (t = 0, 5, 15, 45 and 105 min). Absorption and fluorescence spectra of the cuvettes containing water and N-Methyl-D-Glucamine were also obtained as control, in the same proportions of previous solutions.

The obtained spectra at t=0 min were correlated and compared to the emission spectra of the illumination LEDs. This is made by overlapping the normalized absorption spectrum and

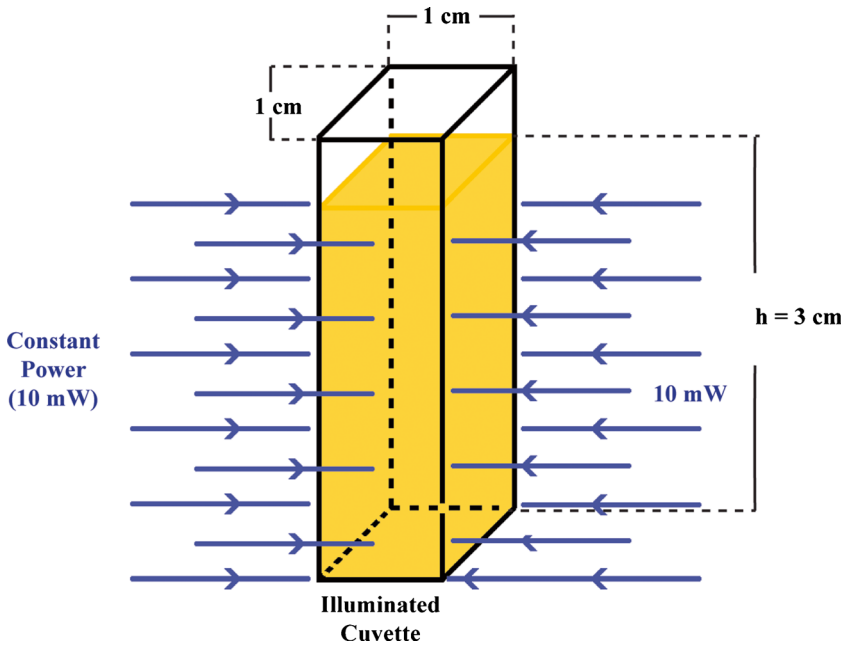

Fig. 1 Photobleaching illumination of the solutions in a quartz cuvette

the emission spectra from the sources. This analysis reveals the optimal LED source for PDT by computing a numerical quantity defined as the Spectrum Utilization Rate [29]:

$$\eta_\lambda = \frac{\sum_i A_i \cdot E_i}{\sum_i A_i \cdot A_i} \quad (1)$$

where the numerator corresponds to the sum of the product, point by point, of the absorption curve (A_i) by the emission curve (E_i) for the three illumination wavelengths. As the equipment did not generate readings exactly at the same spectral points, mathematical interpolation was performed in order to correctly execute the operation of equation (1).

After confirming the LED source which most effectively caused photodegradation, the photobleaching experiments were repeated with the addition of sodium azide (NaN_3) to the solution. Sodium azide is a known singlet oxygen quencher and tends to decrease the time of permanence of singlet oxygen in the solution [30]. Singlet oxygen is responsible for the Type-II photodynamic effect, although it is believed that such effect may also be caused by the presence of oxidative free radicals (Type-I mechanism) [31, 32]. The decrease of the photobleaching effect by adding sodium azide would indicate the production of singlet oxygen. This composite was added in a proportion of 3:1 of molar concentration.

Results

In Fig. 2-a, the collected absorption spectra, in dark environment, are shown for the recorded times and one extra record at 24 hours. In the scale of Fig. 2, none of the solutions containing N-Methyl-D-Glucamine and water only showed expressive absorption and fluorescence intensities, when compared to the curcumin salt. Two main absorption peaks (~345 and ~422 nm) presented considerable variations for longer record times, showing a time dependent degradation in the presence of water. Figure 2-b shows the fluorescence spectra, which exhibits a large emission band in the green region (peak at 528 nm), for all three curcumin concentrations. It is worth mentioning that the fluorescence showed a decay pattern for dark degradation similar to the absorption measurements (data not shown).

At Fig. 3-a and b the degradation patterns of curcumin during violet (405 nm) and blue (470 nm) illumination are respectively shown. The relation between the two main absorption peaks presents significant changes when the sample is illuminated by different wavelengths. Violet light at 405 nm produced a dramatic degradation of both absorption peaks, while light at a longer wavelength (470 nm) produced a milder degradation for only one of t absorption peak. We stress that the slight differences in the absorption spectra at t=0 min are mainly related to small variations in the environment conditions generated during the sample preparation and manipulation prior to the measurement. However, these differences do not influence the analysis of the photobleaching process, as detailed below.

The photobleaching process can be analyzed in terms of the spectral correlation. Figure 4 shows the overlapping of curcumin normalized absorption at t=0 min and the emission bands of the applied illumination sources. By means of Eq. 1, the data analysis showed that the 450 nm LED is the most energetically available illumination source for the present

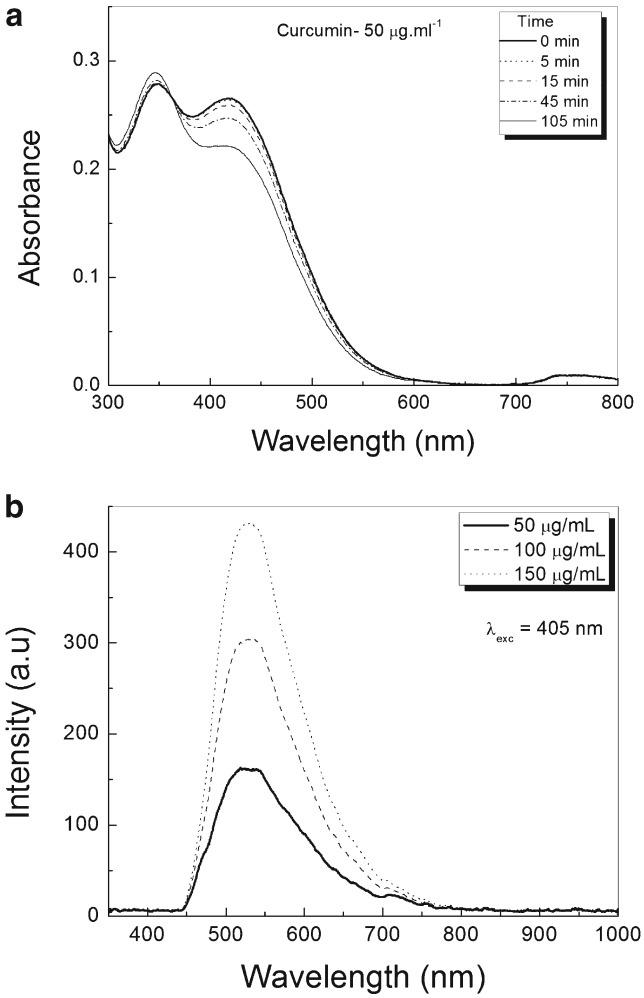

Fig. 2 (**a**) Absorption spectra in the dark for the most diluted solution (50 μg/.mL) and distinct times (lower curve corresponds to the longer time). (**b**) Fluorescence spectra for distinct concentrations at t=0 min (lower curve corresponds to the smallest concentration). The measured dimensionless absorbance corresponds to the negative logarithm of the transmittance with respect to the reference of a quartz cuvette containing only the solvent

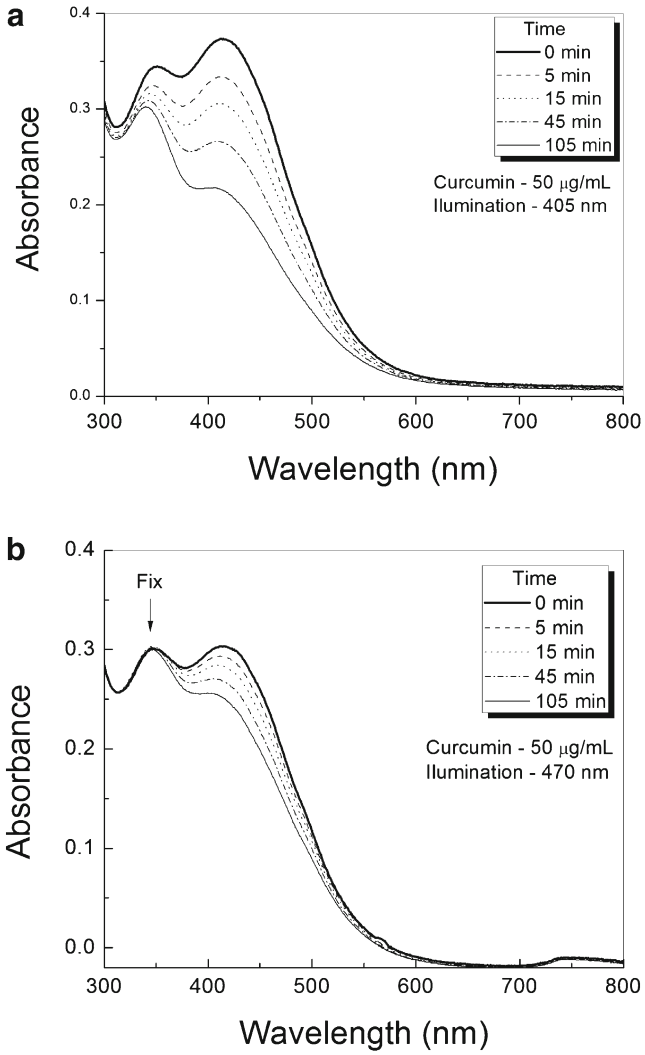

Fig. 3 Absorption spectra for (**a**) 405 nm and (**b**) 470 nm LED illumination. Lower curves correspond to longer exposition times. Photodegradation is stronger for violet illumination (405 nm)

compound. The spectrum utilization rate η_λ is shown in Fig. 5. It is worth mentioning that this parameter does not takes into account the concentration of the composite or the emitted power of the illumination source.

The previous results show that the 450 nm LED is the most spectrally efficient light source to produce photodegradation in this curcumin formulation. This source was selected to evaluate the production of oxygen reactive species. The previous photobleaching experiment was repeated for this illumination source only, while adding sodium azide (NaN_3) to the solutions. A prior absorption spectrum was collected before this step in order to guarantee that the sodium azide would not react with curcumin and degrade it. The absorbance of the 422 nm absorption peak was monitored through the previously studied times, normalized to unity at t=0 min. The graph in Fig. 6 shows the results for the 422 nm peak photobleaching,

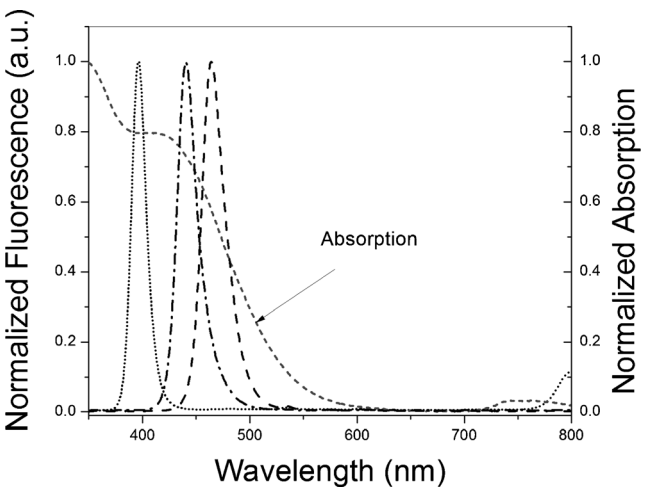

Fig. 4 Spectral overlap of the normalized emission bands of the illumination sources and the Curcumin absorption

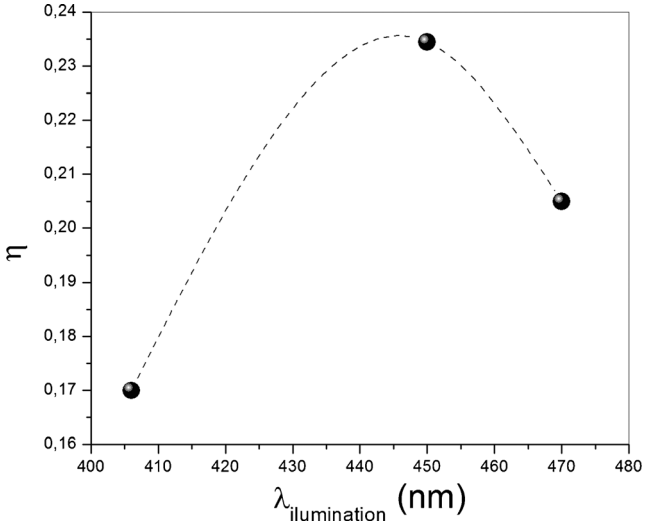

Fig. 5 Spectral utilization rate (η_λ). Optimal photobleaching efficiency is obtained for the 450 nm LED illumination

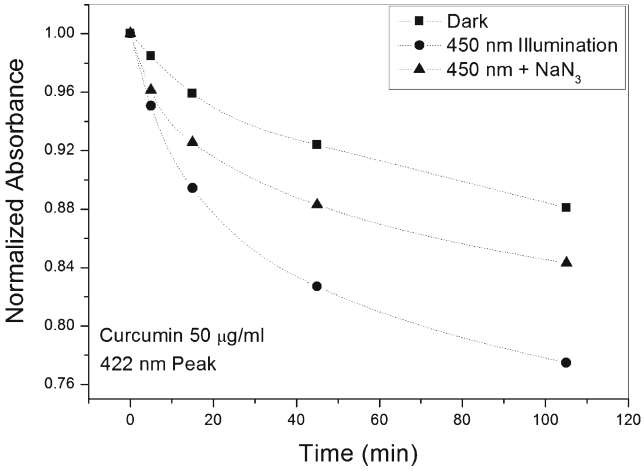

Fig. 6 The 422 nm absorption peak normalized absorbance, in three different conditions: dark, 450 illumination, and 450 nm illumination + NaN_3

in the presence of sodium azide, plotted alongside with the previously performed dark measurement and illumination without NaN_3.

It is clear that photobleaching efficiency is decreased in the presence of sodium azide, as a sign of singlet oxygen production. This result is sufficient to prove the occurrence of at least one of the PDT mechanisms (type II mechanism).

Discussions

The curcumin formulation used in this work has an average molecular weight of 730.32 g.mol^{-1}, which means that more than one type of curcuminoid are present. We believe that this collection of curcuminoids contributes to the form of the absorption spectra at t=0 min and the relation between the two main absorption peaks during the photobleaching.

The photobleaching is a phenomenon that occurs when, by the strongly efficient optical excitation of a photosensitizing molecule, oxidative chemical species are formed, causing the destruction of the closest neighboring molecules, which may be of the same kind of the absorbing molecule. This fact occurs due to the short lifetime of the formed reactive species. This photobleaching reduces the PDT efficiency and its outcome [33]. The formation of oxidative chemical species is also the principle of the photodynamic action. Then, the recognition of a new composite as a photosensitizer to use in PDT can rely on the photobleaching evidence. Absorption and fluorescence are important and simple measurements in this context because they are able to show the survival of curcuminoids in water solutions.

Looking closely to the graph in Fig. 2-a, it can be observed that the 422 nm peak decreases as the 345 nm slightly increases. This is an indicative of the natural instability of curcumin-based compounds in the presence of water due to solvation [34]. The absorption spectrum shown is a collective contribution of every curcuminoid type, with one type contributing more to an absorption peak than to another. If one type of curcuminoid is more instable in the presence of water, its contribution to a peak will become less evident in the collective spectrum. The result of this process is the relative change over time in the absorption peaks.

In addition, the concept of photon energy can be explored in order to better understand the results when illumination is present. The individual photon energies involved in this work were calculated as being $E_{405} = 5.03 \times 10^{-19}$ J, $E_{450} = 4.53 \times 10^{-19}$ J and $E_{470} = 4.29 \times 10^{-19}$ J , based on the emission peaks of Fig. 4. The absorption curve in the visible region of the spectrum is due to electronic transitions occurring in the molecules. The observed changes in the absorption spectra in Fig. 3-a and 3-b reveals that the 405 nm LED degrade both peaks, while the 470 nm LED only degrades the larger wavelength peak. The incoming photons can only affect the processes that involve an energy comparable to or lower than its own energy. As a result, the 405 nm photons have energy that can affect the electronic processes associated to both absorption peaks, making it possible to degrade different types of curcuminoids. The 470 nm photons have lower energy, comparable only with the 422 nm absorption band, which may be associated to one type of curcuminoid only, confirmed by the spectral overlap of Fig. 4.

The photodynamic action of the present curcumin formulation was confirmed for one Type-II photodynamic mechanism, namely by singlet oxygen formation. This is the main reason why the photodegradation was not completely canceled with sodium azide addition to the solution. Other types of reactive molecules and radicals may have been formed,

thus leading to the additional degradation observed on Fig. 6 (Type-I mechanism).

It is worth mentioning the fact that, even at t=105 min of light exposure in water solution, there is still an expressive number of curcumin surviving molecules, responsible for the absorption at this time. This large number of surviving molecules is responsible for the continued photodegradation seen in Fig. 6. This characteristic makes possible the application of the present compound to clinical applications, where the treatment times should not be long.

Conclusion

Full confirmation of photodynamic action was performed for a new curcumin-based formulation with enhanced water solubility. The present results open the possibility of curcumin-based photodynamic therapy applications with higher biocompatible solutions. As the present compound is better optically excited in the blue-green region of the spectrum, it would be a good candidate for treating superficial infections caused by microbiological biofilms, as well as superficial cancer lesions, because of the low tissue penetration of the possible excitation wavelengths in biological media. It would be interesting to have future in vitro as well as *in vivo* experiments aiming to explore these potential applications. We hope the present results will stimulate further contributions along these lines.

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