JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Antioxidant β-Carotene Does Not Quench Singlet Oxygen in Mammalian Cells

Gabriela N. Bosio,[†,‡] Thomas Breitenbach,[‡] Julieta Parisi,[§] Miguel Reigosa,[§] Frances H. Blaikie,[‡] Brian W. Pedersen,[‡] Elsa F. F. Silva,[‡,∥] Daniel O. Mártire,[†] and Peter R. Ogilby*[,‡]

[†]Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), CCT-La Plata-CONICET, Universidad Nacional de La Plata, Casilla de Correo 16, Sucursal 4 (1900), La Plata, Argentina

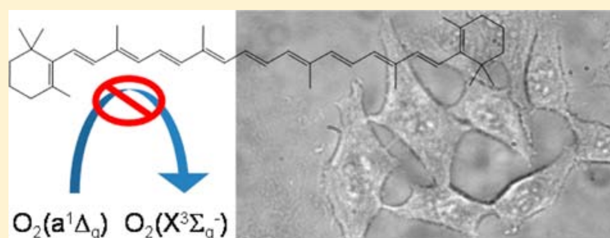
[‡]Center for Oxygen Microscopy and Imaging, Department of Chemistry, Aarhus University, Langelandsgade 140, Århus 8000, Denmark

[§]Instituto Multidisciplinario de Biologia Celular (IMBICE), CCT-La Plata-CONICET, Camino General Belgrano y 526, B1906APO, La Plata, Argentina

[∥]Department of Chemistry, University of Coimbra, 3004 Coimbra, Portugal

Supporting Information

ABSTRACT: Carotenoids, and β-carotene in particular, are important natural antioxidants. Singlet oxygen, the lowest excited state of molecular oxygen, is an intermediate often involved in natural oxidation reactions. The fact that β-carotene efficiently quenches singlet oxygen in solution-phase systems is invariably invoked when explaining the biological antioxidative properties of β-carotene. We recently developed unique microscope-based time-resolved spectroscopic methods that allow us to directly examine singlet oxygen in mammalian cells. We now demonstrate that intracellular singlet oxygen, produced in a photosensitized process, is in fact not efficiently deactivated by β-carotene. This observation requires a re-evaluation of β-carotene's role as an antioxidant in mammalian systems and now underscores the importance of mechanisms by which β-carotene inhibits radical reactions.



1. INTRODUCTION

Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest excited electronic state of molecular oxygen.[1] It can be generated in a variety of ways, many of which are pertinent to mammalian biology. These include inherent enzymatic and stress-response processes[2,3] as well as photosensitized processes wherein a photoexcited electronic state of a given molecule (the sensitizer) transfers its energy of excitation to the triplet ground state of oxygen, $O_2(X^3\Sigma_g^-)$.[4,5] The latter occurs routinely in nature given the ubiquity of good sensitizers, oxygen, and sunlight.

$O_2(a^1\Delta_g)$ has a unique chemistry that results in the oxygenation of many organic and bio-organic molecules.[6] In this way, $O_2(a^1\Delta_g)$ plays a role in mechanisms of oxidation-based protection and conversely in events that perturb cell homeostasis and ultimately result in cell death.[3,7]

One of the axioms upon which the chemistry of singlet oxygen is based is that carotenoids, in particular β-carotene, are the molecules that most efficiently deactivate $O_2(a^1\Delta_g)$ to $O_2(X^3\Sigma_g^-)$.[4,8−10] The mechanism for this process involves the collision-dependent spin-allowed energy transfer from $O_2(a^1\Delta_g)$ to produce the triplet excited state of the carotene. Indeed, of all organic molecules known, the carotenoids are unique in that they have a triplet energy that is degenerate with, or slightly lower than, the 94 kJ/mol excitation energy of $O_2(a^1\Delta_g)$.[9,11] This aspect of carotenoid behavior has long been used as an argument when explaining the antioxidative properties of the carotenes in mammalian biology, establishing a key tenet in the way diet can influence human health.[9,12−15]

We recently developed laser-based time-resolved photosensitized methods by which intracellular $O_2(a^1\Delta_g)$ can be selectively produced and spectroscopically monitored in single cells.[5,16,17] The latter relies on detecting $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence at ∼1275 nm. Moreover, we have been able to monitor cell response to the $O_2(a^1\Delta_g)$ thus produced in imaging-based experiments.[17,18] To facilitate the acquisition of the $O_2(a^1\Delta_g)$ phosphorescence signal and subsequent data analysis, it is beneficial to use cells in which the intracellular H_2O has been replaced by D_2O and as such exploit the fact that the lifetime of $O_2(a^1\Delta_g)$ in D_2O-based systems is much longer than that in H_2O-based systems.[5,16,17] In this way, we also make the system more sensitive to the effects of added quenchers.[17,19] Importantly, the cytotoxic effects of D_2O do not adversely influence our experiments over a period of ∼3−5 h.[20]

We have established that the well-known hydrophilic quencher of $O_2(a^1\Delta_g)$ sodium azide (NaN_3) can manifest its intracellular presence (1) through changes in the time-resolved

Received: September 8, 2012
Published: December 11, 2012

 © 2012 American Chemical Society

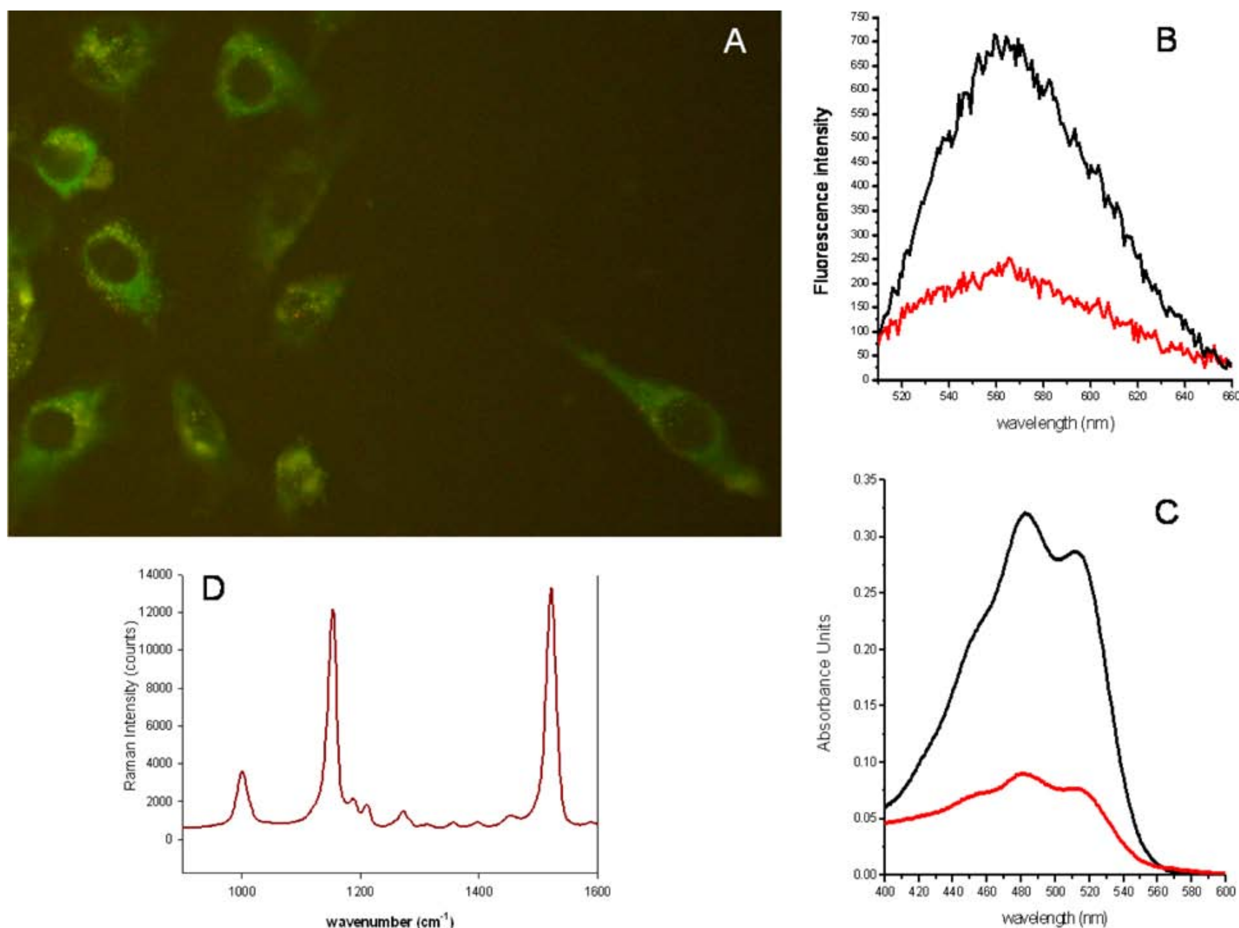


Figure 1. (A) Image of HeLa cells based on the fluorescence of incorporated β-carotene; λ_{exc} = 480 nm; λ_{detect} > 515 nm. (B) Fluorescence spectrum obtained from extracts of HeLa cells that had been incubated with β-carotene (red). To obtain the control spectrum (black), β-carotene-free HeLa cells were extracted, and β-carotene was then added to yield a 7 μM solution. In both cases, cell extracts were redissolved in CS_2. (C) Absorption spectra of the solutions described above. (D) Raman spectrum directly obtained from HeLa cells that had been incubated with 10 μM β-carotene for 48 h.

$O_2(a^1\Delta_g)$ phosphorescence signal and (2) by imparting protection against the cytotoxic effects of $O_2(a^1\Delta_g)$.[17,19,21] To complement our NaN_3 studies, we set out to use our single-cell methods to examine the behavior of a hydrophobic quencher of $O_2(a^1\Delta_g)$. The logical choice of a molecule to this end was β-carotene.

We now demonstrate using hydrophilic as well as hydrophobic sensitizers and two cell lines (adherent HeLa and HL-60 suspensions) that intracellular β-carotene does not quench $O_2(a^1\Delta_g)$ as manifested in the time-resolved $O_2(a^1\Delta_g)$ phosphorescence signal. This unexpected result requires a re-evaluation of the role(s) played by β-carotene as an antioxidant in mammalian systems.

2. RESULTS

2.1. Incorporation of β-Carotene in Cells. A key aspect of this study is to ensure that an appreciable amount of β-carotene can be incorporated into the cells examined. The $O_2(a^1\Delta_g)$ lifetime in an unperturbed D_2O-incubated cell is ~15 μs.[17] The rate constant for $O_2(a^1\Delta_g)$ quenching by β-carotene obtained in liquid solutions, $\sim 1 \times 10^{10}$ s^{-1} M^{-1},[22] is characteristic for a process that occurs at the diffusion-controlled limit. In the more viscous intracellular environment, however, this rate constant will be smaller. If we assume a value of $\sim 5 \times 10^8$ s^{-1} M^{-1} for the quenching of $O_2(a^1\Delta_g)$ by β-carotene in a cell,[19] then we would only expect to see a noticeable change in the $O_2(a^1\Delta_g)$ lifetime at effective intracellular concentrations of β-carotene that exceed ~10 μM.

Following literature precedence,[23] we could readily incorporate an appreciable amount of β-carotene into the cells over a period of 48 h when the incubating medium contained 0.1% (by volume) of a 1:1 mixture of THF and DMSO. The evidence for β-carotene incorporation comes in four parts:

(a) After incubating HeLa cells with 10 μM β-carotene, we washed the cells with medium lacking β-carotene. The cells were then trypsinated, suspended, and lysed, and the β-carotene was extracted into hexane and dried. High-performance liquid chromatography (HPLC) was then performed using independent solutions of β-carotene as the standard (see the Supporting Information). We found that β-carotene is incorporated in the amount of $\sim 2 \times 10^{-15}$ mol/cell, a value that is consistent with what has been published using the same methods.[23,24] If the diameter of a suspended cell is ~20 μm, the corresponding cellular volume of ~4 pL then yields an intracellular β-carotene concentration of ~500 μM. Of course, the pertinent hydrophobic intracellular volume in which β-carotene is localized will be smaller than ~4 pL, and hence, the effective concentration will be greater than 500 μM.

(b) Extracts of β-carotene-incubated HeLa cells, prepared as described above and redissolved in CS_2, were examined using the absorbance and fluorescence of β-carotene as a probe (Figure 1). Independent solutions of β-carotene were again used as a standard to correlate the observed data to a concentration of β-carotene. The results yield an intracellular β-carotene amount of $\sim 1 \times 10^{-15}$ mol/cell, which is consistent with the HPLC data above.

(c) Using β-carotene fluorescence, we recorded images of HeLa cells that had been incubated with 10 μM β-carotene (Figure 1). Because the fluorescence quantum yield of β-carotene is small (6×10^{-5}),[25] the data are consistent with an appreciable amount of incorporated β-carotene. The latter point was confirmed by comparing the integrated fluorescence intensity of β-carotene from one cell with that from a cell containing rhodamine 123 as a fluorescent standard (see the Supporting Information). These data yield an intracellular amount of β-carotene that is consistent with the data obtained from the cell extracts (vide supra). Moreover, the fluorescence spectrum of β-carotene recorded from a single HeLa cell (see the Supporting Information) matches that obtained from both the cell extracts (Figure 1B) and from hydrophobic solvents (e.g., in CS_2[25]). Thus, the intracellular β-carotene does not appear to aggregate, or rather, there is an appreciable nonaggregated concentration of intracellular β-carotene.

(d) Suspensions of HL-60 cells and, independently, adherent HeLa cells were incubated with 10 μM β-carotene, washed with medium lacking β-carotene, and placed on a microscope coverslip (cell density $\sim$9 cells/1.6×10^3 μm^2). Raman spectra obtained from these samples conform to what is expected for β-carotene (Figure 1).[24] This observation complements the fluorescence spectrum recorded from a single cell (vide supra) indicating that there is an appreciable nonaggregated concentration of intracellular β-carotene.

The amount of β-carotene incorporated into a cell correlates with the concentration of β-carotene in the incubating medium, a point that is likewise consistent with published data.[23] Although the intracellular β-carotene will be inhomogeneously distributed, we nevertheless conclude that the effective intracellular concentration of β-carotene in our experiments indeed exceeds the minimum threshold of $\sim$10 μM required to observe the effect of $O_2(a^1\Delta_g)$ quenching.

2.2. Time-Resolved $O_2(a^1\Delta_g)$ Phosphorescence Studies.

The most direct and accurate method to assess the effect of a $O_2(a^1\Delta_g)$ quencher is to monitor the way it changes the kinetics of $O_2(a^1\Delta_g)$ removal in a time-resolved photosensitized $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence experiment.[4,5,22] Although such experiments are routinely performed in cuvette-based studies, we have recently shown that they can also be used in unique microscope-based experiments performed at the level of a single adherent mammalian cell.[5,16,19]

Monitoring the kinetics of $O_2(a^1\Delta_g)$ removal in photosensitized $O_2(a^1\Delta_g)$ phosphorescence experiments from cells is a nontrivial endeavor that in part is hampered by the fact that $O_2(a^1\Delta_g)$ is cytotoxic.[5,17] Thus, over the time course in which kinetic data are recorded, changes in cell morphology and sensitizer localization, among other things, cause a corresponding change in the $O_2(a^1\Delta_g)$ phosphorescence signal.[5,17,26] Nevertheless, under controlled conditions and with the judicious use of control experiments, accurate measurements to assess the effect of β-carotene incorporation can be made.

Although time-resolved $O_2(a^1\Delta_g)$ phosphorescence experiments performed using cell suspensions have limitations,[17] they can be used to complement single-cell experiments and still provide useful information. A key characteristic of using cell suspensions is that the data can be influenced by $O_2(a^1\Delta_g)$ produced in the extracellular medium (i.e., by sensitizer that diffuses out of the cell and that is irradiated by the exciting laser beam that propagates through the suspension).[17] In contrast, sensitizer irradiation in single-cell experiments is achieved using a focused laser. Although scattered light will excite sensitizers throughout the cell under these latter conditions (i.e., when using irradiation wavelengths that correspond to a resonant one-photon transition in the sensitizer, scattered light is readily absorbed), excitation is nevertheless effectively confined to the cell itself, and the $O_2(a^1\Delta_g)$ phosphorescence data obtained reflect intracellular phenomena.[18]

The results of time-resolved $O_2(a^1\Delta_g)$ phosphorescence experiments performed using hydrophilic and hydrophobic sensitizers are shown in Table 1. The single-cell experiments were performed using adherent HeLa cells, whereas the cell-suspension experiments were performed using undifferentiated HL-60 cells. The data shown reflect averages from independent experiments ($n > 5$ in each case). Given the caveats regarding the difficulties of recording such kinetic data,[5,16,17] we report relative values of the $O_2(a^1\Delta_g)$ lifetime, τ_Δ^{rel}. Specifically, to

Table 1. Relative Values of the $O_2(a^1\Delta_g)$ Lifetime, τ_Δ^{rel}, Obtained under a Variety of Conditions from Experiments Using Cell Suspensions and Single Cells

sensitizer	condition	τ_Δ^{rel} /μs cell suspension	τ_Δ^{rel} /μs single cell
hydrophobic			
PPa	only sensitizer	18.8 ± 1.2	15.2 ± 1.7
	+ BSA	19.5 ± 1.1[a]	16.9 ± 2.1
	+ NaN_3	4.0 ± 2[b]	7.0 ± 0.9
	+ BSA + NaN_3	9.1 ± 0.3	
	+ β-carotene	32 ± 4	16 ± 2
	+ β-carotene + BSA	21 ± 2	17.2 ± 1.6
	+ β-carotene + NaN_3	10 ± 5	6.3 ± 0.7
FCh	only sensitizer	20 ± 1	19.1 ± 0.4
	+ BSA	19 ± 2	
	+ NaN_3		14.0 ± 0.7
	+ β-carotene	24 ± 5	21.6 ± 0.7
	+ β-carotene + BSA	17 ± 1	
	+ β-carotene + NaN_3	9 ± 4	15.5 ± 0.6
	+ β-carotene + BSA + NaN_3	3.3 ± 0.5	
hydrophilic			
$AlPcS_4$	only sensitizer	29.4 ± 2.8	33.6 ± 2.4
	+ BSA	13.3 ± 0.9	36 ± 6
	+ NaN_3	4 ± 1[c]	
	+ BSA + NaN_3		23 ± 2
	+ β-carotene	36.8 ± 1.3	30.5 ± 1.7
	+ β-carotene + BSA	14 ± 1	32.7 ± 2.5
	+ β-carotene + BSA + NaN_3	12.9 ± 0.8	26.8 ± 2.5

[a]In all cases, [BSA] = 2 mM; BSA = bovine serum albumin. [b]In all cases, [NaN_3] = 2 mM except where indicated. [c][NaN_3] = 0.1 M.

dx.doi.org/10.1021/ja308930a | J. Am. Chem. Soc. 2013, 135, 272−279

avoid complicating details about the effects of elapsed irradiation, among other things, we opted to reduce the pertinent information to the parameter τ_Δ^{rel} obtained from a single-exponential fit to the decay of the time-resolved $O_2(a^1\Delta_g)$ phosphorescence signal from D_2O-incubated cells (e.g., Figure 2). Thus, data recorded using a given sensitizer at a given incident laser power for a given period of irradiation, and shown as a group in a given column of Table 1, can be compared.

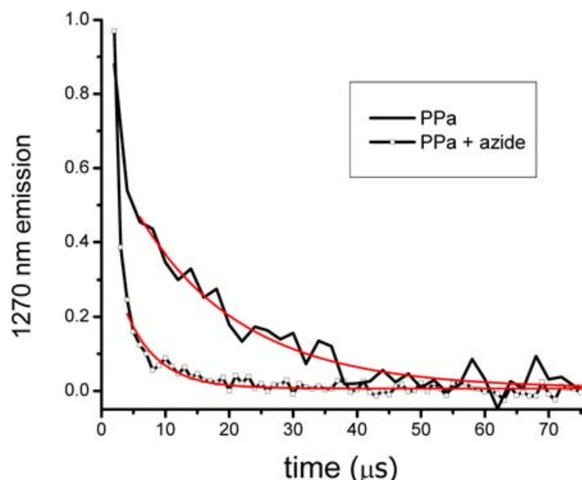


Figure 2. Examples of PPa-sensitized time-resolved $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence traces observed in microscope-based single-cell experiments. The trace yielding the longer value of τ_Δ^{rel} (~ 15 μs) was obtained from a D_2O-incubated control cell lacking added quenchers, whereas the trace yielding the shorter value of τ_Δ^{rel} (~ 7 μs) was obtained from a D_2O-incubated cell with added NaN_3. Also shown are the single-exponential fits that yield τ_Δ^{rel}. Data recorded upon the addition of β-carotene were identical to those obtained from the control cells.

Experiments with β-carotene were first performed using cells incubated with low levels of β-carotene (i.e., < 15 μM in the medium). With the absence of any observed effect of added β-carotene on our $O_2(a^1\Delta_g)$ kinetic traces under these conditions, we repeated experiments using cells incubated with a higher (and potentially toxic, vide infra) concentration of 25 μM β-carotene. Under these latter conditions, we nevertheless always recorded data from cells whose bright field images revealed no obvious signs of death. In any event, kinetic data recorded from dying cells should not appreciably influence our conclusions regarding the ability of β-carotene to quench $O_2(a^1\Delta_g)$.[17,19]

Other tests performed to establish confidence in the accuracy of our conclusions regarding the effect of β-carotene include the addition of bovine serum albumin (BSA) and NaN_3 to the extracellular medium. BSA is a protein that efficiently quenches $O_2(a^1\Delta_g)$.[27] However, with the cells and conditions in our experiments, BSA remains in the extracellular medium.[16,17,27] Thus, a change in the $O_2(a^1\Delta_g)$ lifetime upon the addition of BSA indicates the presence of $O_2(a^1\Delta_g)$ in the extracellular medium. The latter could occur as a consequence of intracellularly produced $O_2(a^1\Delta_g)$ and/or the sensitizer itself diffusing across the cell membrane. NaN_3 is likewise an efficient quencher of $O_2(a^1\Delta_g)$. It readily crosses the cell membrane and quenches intracellular $O_2(a^1\Delta_g)$.[17,19,27] Moreover, although NaN_3 can be cytotoxic over long periods of exposure, it appears to be benign over the time course of our experiments. Indeed, it imparts protection against the short-term cytotoxic effects of $O_2(a^1\Delta_g)$.[21] Although NaN_3 is hydrophilic, its effects can nevertheless also be seen when using hydrophobic sensitizers; it will interact with $O_2(a^1\Delta_g)$ that diffuses from hydrophobic into hydrophilic domains, and it also likely penetrates a modest distance into the interfacial region delimiting hydrophobic domains.[17,19]

Two different hydrophobic photosensitizers were used: pyropheophorbide *a* (PPa) and a fluorinated chlorin (FCh). The expectation here was that, since $O_2(a^1\Delta_g)$ would be produced in lipophilic domains and since β-carotene is likewise lipophilic, we had the greatest chance of observing an effect of added β-carotene on the lifetime of $O_2(a^1\Delta_g)$. The NaN_3 data indicate that, with these sensitizers, there is indeed a $O_2(a^1\Delta_g)$ population that can be deactivated by an added quencher. The BSA data indicate that the sensitizer remains in the cell over the course of the experiment, an observation consistent with earlier results using hydrophobic sensitizers.[17] The BSA data also suggest that added β-carotene may promote relocalization of the sensitizer such that a greater population of extracellular $O_2(a^1\Delta_g)$ can be produced. Specifically, upon the addition of β-carotene in the cell-suspension study, we observe a longer $O_2(a^1\Delta_g)$ lifetime that, in turn, is susceptible to the addition of BSA. However, the most important observation is the clear absence of a shorter $O_2(a^1\Delta_g)$ lifetime upon the addition of β-carotene. This is best underscored in the single-cell experiments where the $O_2(a^1\Delta_g)$ phosphorescence signal is not influenced by extracellular $O_2(a^1\Delta_g)$.

Data recorded using hydrophilic aluminum phthalocyanine ($AlPcS_4$) as sensitizer yield complementary results. Specifically, although the addition of BSA and NaN_3 clearly cause a decrease in the $O_2(a^1\Delta_g)$ lifetime, the presence of β-carotene does not change the $O_2(a^1\Delta_g)$ lifetime. The BSA effect observed in the cell-suspension study is consistent with independent results that show that hydrophilic sensitizers can readily diffuse out of a cell into the extracellular medium.[17]

It is also important to note that the τ_Δ^{rel} value recorded using this hydrophilic sensitizer in the D_2O-based single-cell experiment (~35 μs) is appreciably longer than that recorded using the hydrophobic sensitizers (~15−20 μs). As we have already established, single-cell experiments are less sensitive to the effects of extracellular $O_2(a^1\Delta_g)$. As such, and as examined in independent reports,[17,19,26] the present single-cell data instead likely reflect differences in the extent to which irradiation of a given sensitizer perturbs a cell and that, with this hydrophilic sensitizer, we are recording data from cells that are "more dead". Specifically, longer $O_2(a^1\Delta_g)$ lifetimes tend to be observed from cells that are dying or dead.[17]

2.3. Monitoring Cytotoxic Effects. When assessing the effects of potentially cytotoxic agents, it is desirable to use a variety of assays to test for different cell responses. In this regard, it is important to recognize that a given perturbation (e.g., adding β-carotene or the presence of $O_2(a^1\Delta_g)$) may influence some processes more than others and thus may yield apparently contradictory assay results. Here, cell viability was assessed using four tools: (1) Bright field microscope images for morphology changes (e.g., membrane located vacuole formation, chromatin condensation). This is an established approach that combines accuracy with ease of implementation.[17,18,21,28−30] (2) The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for enzymatic activity.[20,31,32] In this test, a metabolically active cell will reduce MTT to a blue derivative, formazan, the amount of which can be quantified using an absorption spectrometer. (3) The

 dx.doi.org/10.1021/ja308930a | J. Am. Chem. Soc. 2013, 135, 272−279

Trypan Blue assay for membrane permeability.[33] (4) The Giemsa stain for morphology changes.[33]

Experiments to monitor cytotoxic effects were performed using cells cultured and incubated in H_2O-based media. We elaborate on the ramifications of choosing H_2O, as opposed to D_2O, in the Discussion section .

2.3.1. Inherent Toxicity of β-Carotene. When present in a cell, β-carotene can be inherently toxic.[34,35] However, this is an overly simplistic statement in that the adverse/beneficial effects of β-carotene have long been a topic of investigation and debate.[15,34,36] In the least, a great deal depends on the conditions under which toxicity is assessed, the cell line studied, whether differentiated or undifferentiated cells are used, the conditions of cell handling, and whether the oxidation products of β-carotene could play a role in the study. In some cases, an apparent threshold for β-carotene toxicity has been observed.[34,35] In this regard, and using the Trypan Blue assay, we determined that incubation of HeLa cells for 48 h with media containing less than ∼15 μM β-carotene does not apparently have an adverse effect, whereas incubation for 48 h with media containing greater than ∼15 μM β-carotene has an adverse effect (see the Supporting Information). On the other hand, undifferentiated HL-60 cells appeared to be more sensitive to the effects of β-carotene; using the Trypan Blue assay, we noted a change in cell viability upon incubation with only 5 μM β-carotene (see the Supporting Information). Interestingly, and most importantly for the aspect of our present study on photosensitized effects shown in Figure 3, the MTT assay gave no clear evidence that, under our experimental conditions, β-carotene has an inherent adverse effect on HeLa cells incubated in media containing less than ∼40 μM β-carotene (see the Supporting Information).

2.3.2. Irradiation of Hydrophobic Sensitizers. In one set of experiments, an ester derivative of tetraphenylporphine (TPPCOOMe) was used as the $O_2(a^1\Delta_g)$ sensitizer, and the MTT assay was used to assess photoinitiated toxicity (Figure 3A). The first point of note is that the THF/DMSO solution used to facilitate β-carotene incorporation does not have an adverse effect; within our errors, the formazan level is the same as in the control population. Upon irradiation of the intracellular TPPCOOMe and the production of $O_2(a^1\Delta_g)$, we adversely affect the cell's ability to reduce MTT to formazan, as expected. Subsequent experiments were performed in the same way, only with increasing amounts of added β-carotene. As the concentration of β-carotene in the incubating medium was increased to 10 μM, the data indicate that β-carotene indeed imparts a significant protective effect. The data also indicate that increasing the concentration of β-carotene in the medium above ∼10 μM has no additional effect on the cell's ability to reduce MTT to formazan (i.e., the maximum protective effect appears to have been reached).

Experiments using TPPCOOMe, where bright field images were used to assess cell response, yield data that are consistent with what is shown in Figure 3A. However, when PPa was the $O_2(a^1\Delta_g)$ sensitizer, HeLa cell death was observed in all cases (i.e., there was no indication of a β-carotene-dependent protective effect at low β-carotene concentrations) (see the Supporting Information).

2.3.3. Irradiation of Hydrophilic Sensitizers. In other experiments, a cationic porphyrin, TMPyP, was the $O_2(a^1\Delta_g)$ sensitizer, and the MTT assay was likewise used to assess cytotoxicity (Figure 3B). The data thus obtained nicely complement the data obtained in the TPPCOOMe-sensitized experiments, showing that β-carotene indeed has a protective effect against the cytotoxic effects of TMPyP-sensitized $O_2(a^1\Delta_g)$ production.

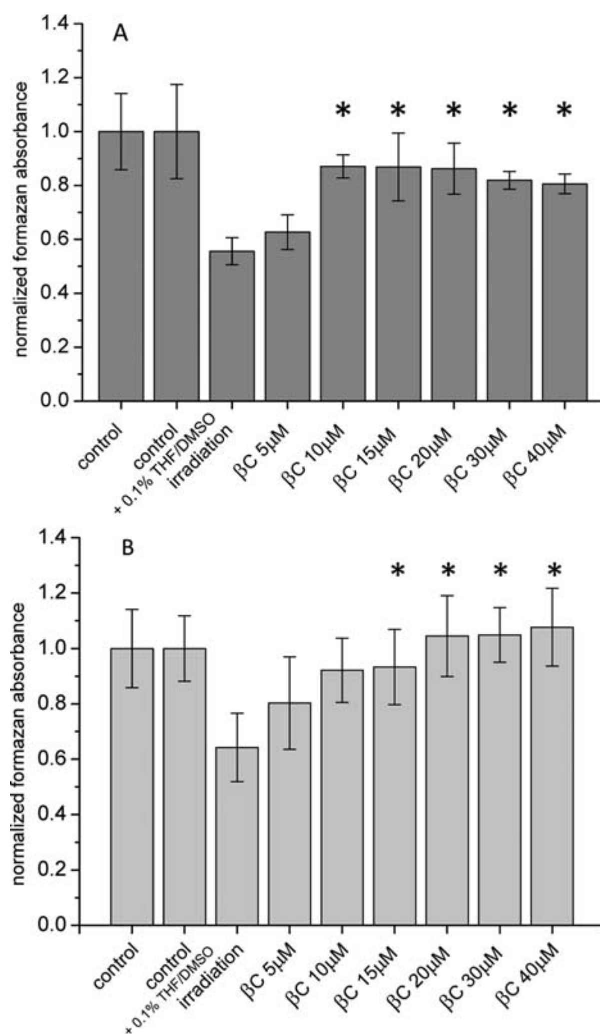


Figure 3. Histograms showing the results of the MTT assay on HeLa cells. Values of formazan absorbance were normalized against average values obtained from cells to which a sensitizer and β-carotene had not been added. The data shown were obtained from experiments in which (A) the hydrophobic sensitizer TPPCOOMe and (B) the hydrophilic sensitizer TMPyP were irradiated. Error bars refer to one standard deviation; in each case, the number of samples examined was at least 8 and as large as 16. The asterisk indicates that there is a statistically significant difference between this particular number and the number obtained from the irradiated sample without β-carotene and with only 5 μM β-carotene in the medium (one-way ANOVA with Tukey's posthoc test; $p < 0.05$).

Experiments performed in which $AlPcS_4$ was irradiated and in which bright field images were used to assess cell response yield data that are consistent with what is shown in Figure 3B. Specifically, and as illustrated in Figure 4, in the absence of added β-carotene, irradiation of $AlPcS_4$ clearly results in morphological features associated with cell death. However, the presence of β-carotene imparts a protective effect under the same conditions.

3. DISCUSSION

Despite pre-experiment expectations, the absence of a β-carotene-mediated change in the lifetime of intracellular

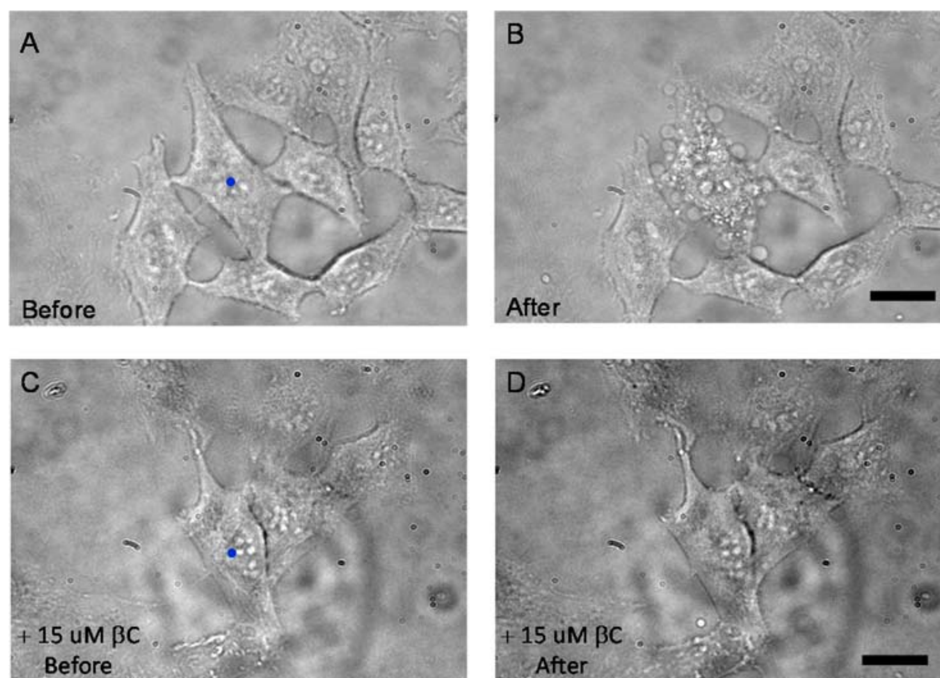


Figure 4. Bright field images of HeLa cells containing the hydrophilic sensitizer $AlPcS_4$. (A) Before irradiation of $AlPcS_4$. (B) After irradiation of $AlPcS_4$, showing vacuoles characteristic of cell death. (C) Before irradiation of $AlPcS_4$ in a cell that had also been incubated with 15 μM β-carotene. (D) After irradiation of $AlPcS_4$, showing the protective effect of β-carotene. Scale bar = 20 μm. The blue dots show the approximate position at which the irradiating laser was focused.

$O_2(a^1\Delta_g)$ can in fact be reasonably explained. First, the $O_2(a^1\Delta_g)$ lifetime in a D_2O-incubated cell is ~15−35 μs, depending on the conditions of measurement.[5,17] In turn, this means that the distance over which $O_2(a^1\Delta_g)$ will diffuse from its point of production at the sensitizer is limited (~230−350 nm over 3 lifetimes for root-mean-square radial diffusion with a coefficient of 2×10^{-6} cm^2 s^{-1}).[5,37] This distance will be appreciably shorter in H_2O-incubated cells where the $O_2(a^1\Delta_g)$ lifetime is less than ~3 μs.[5,17] Given the concentrations and distributions of the sensitizer and β-carotene in the cell and given that these larger molecules will be comparatively immobile in the viscous intracellular environment,[26,37] the probability of the required collision between a sufficient number of $O_2(a^1\Delta_g)$ and β-carotene molecules is indeed likely to be small. With respect to the hydrophobic sensitizers and given that β-carotene is likewise hydrophobic, it is possible that the displacement of one molecule by another in selected membranes may increase the distance between β-carotene and the sensitizer and thereby further decrease the chance for the required collision between $O_2(a^1\Delta_g)$ and β-carotene. In any event, it is significant to note that, with this model in mind, our data are independent of the sensitizer used. Specifically, using a variety of hydrophilic and hydrophobic sensitizers, each of which will localize in different intracellular domains allowing for different distances between the sensitizer and β-carotene, we find that added β-carotene (1) has no effect on the lifetime of intracellular $O_2(a^1\Delta_g)$ but (2) still imparts a significant protective effect against photoinduced cell death.

Carrying these points further, it is important to recognize that our $O_2(a^1\Delta_g)$ lifetime experiments were performed using D_2O-based media where, because of the comparatively long $O_2(a^1\Delta_g)$ lifetime, $O_2(a^1\Delta_g)$ will be more sensitive to the effects of added β-carotene. However, experiments to assess the protective effects of β-carotene were performed using H_2O-based media where the $O_2(a^1\Delta_g)$ lifetime is much shorter and as such $O_2(a^1\Delta_g)$ will arguably be less cytotoxic and thus less sensitive to the effects of β-carotene (e.g., in the kinetic competition between reaction of $O_2(a^1\Delta_g)$ with a given protein that can lead to cell death and the solvent-mediated deactivation of $O_2(a^1\Delta_g)$, the latter is more important in H_2O-based media than in D_2O-based media).

Because we see protective effects of β-carotene, but we do not see an effect of β-carotene on the $O_2(a^1\Delta_g)$ lifetime, we infer that the principal mechanism of β-carotene action under our conditions likely involves trapping of radicals that are also produced as a consequence of sensitizer irradiation and the subsequent reactions of $O_2(a^1\Delta_g)$. It is known that β-carotene indeed exhibits such chemistry.[9,13] Moreover, a given radical precursor formed as a consequence of a reaction with $O_2(a^1\Delta_g)$ (e.g., a hydroperoxide, ROOH) may diffuse over a greater distance than $O_2(a^1\Delta_g)$ and thus have a greater probability of encounter with β-carotene.

Our observation that β-carotene does not alter the $O_2(a^1\Delta_g)$ lifetime in HeLa and HL-60 cells is an apparent contradiction to two reports published in 1993−1994.[38,39] In the latter studies, performed using $O_2(a^1\Delta_g)$ phosphorescence measurements with human lymphocytes, β-carotene-dependent changes in the $O_2(a^1\Delta_g)$ lifetime were observed. However, these earlier studies are characterized by three features that will appreciably influence the data obtained: (1) a water-soluble derivative of β-carotene was used, (2) only water-soluble $O_2(a^1\Delta_g)$ sensitizers were used, and (3) data were recorded from cell suspensions. On the basis of what is now known about recording $O_2(a^1\Delta_g)$ phosphorescence data from such systems, certainly in contrast to single-cell $O_2(a^1\Delta_g)$ measurements,[17] it is reasonable to suggest that the observations reported in these earlier studies reflect the behavior of extracellular $O_2(a^1\Delta_g)$. Moreover, we have established that extracellular $O_2(a^1\Delta_g)$ can be cyto-

dx.doi.org/10.1021/ja308930a | J. Am. Chem. Soc. 2013, 135, 272−279

toxic.[21,40] Thus, it is reasonable to observe that water-soluble extracellular β-carotene will impart a protective effect against extracellular $O_2(a^1\Delta_g)$ and that this effect could correlate with the ability of β-carotene to deactivate $O_2(a^1\Delta_g)$.

4. CONCLUSIONS

In conclusion, although experiments with liquid solutions have long established that β-carotene is one of the most efficient quenchers of $O_2(a^1\Delta_g)$ known, we find that β-carotene does not alter the lifetime of intracellular $O_2(a^1\Delta_g)$. Thus, intracellular β-carotene cannot exert its antioxidative protective effect through a direct reaction with $O_2(a^1\Delta_g)$. Although this conclusion goes against conventional expectation, it is reasonable given the requirement of a collision between $O_2(a^1\Delta_g)$ and β-carotene for the quenching interaction to occur. Indeed, in natural photosynthetic systems, the protective carotenoids are judiciously placed immediately adjacent to the light absorbing, and $O_2(a^1\Delta_g)$-producing, chromophores thus avoiding this diffusion-dependent limitation.[41,42]

5. EXPERIMENTAL SECTION

5.1. Chemicals. All *trans*-β-carotene (>97%, Sigma-Aldrich), pyropheophorbide *a*, PPa (Frontier Scientific), aluminum(III) phthalocyanine chloride tetrasulfonic acid, $AlPcS_4$ (Frontier Scientific), 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine, TMPyP (Sigma-Aldrich), bovine serum albumin, BSA (~65 kDa, Sigma-Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Sigma-Aldrich), and Trypan Blue (Sigma-Aldrich) were used as received. 5,10,15,20-Tetrakis(2,6-difluoro-5-*N*-methylsulfamoylphenyl)chlorin, FCh, was a gift from Luzitin SA (Coimbra, Portugal), and 5-(4-Methoxycarboxyphenyl)-10,15,20-triphenyl-21*H*,23*H*-porphyrin, TTPCOOMe, was synthesized according to a published procedure. Details are provided in the Supporting Information.

5.2. Cells. The methods used to culture and prepare HeLa and HL-60 cells for our single-cell and cell-suspension experiments have been described.[17,18] In some cases, the exchange of intracellular H_2O with D_2O was achieved by changing the tonicity of the medium, as previously described,[20] with the exception that, in the current case, cells were only exposed to the D_2O-based medium for a period that did not exceed ~15 min and the laser experiment was performed immediately thereafter. Under all conditions, the cells used showed no adverse effects of the H_2O/D_2O exchange. All manipulations with β-carotene were performed using ambient light at a wavelength not absorbed by β-carotene to avoid photooxidation of the carotene.[43]

5.3. Instrumentation. The instrumentation, methods, and conditions used to produce and spectroscopically detect $O_2(a^1\Delta_g)$ in microscope-based time-resolved single-cell experiments have been described.[16,17,26] Note that, in ref 17, an erroneous laser fluence of 7 kJ/cm^2 was published. The correct value of laser fluence used both in ref 17 and in the present study was 0.29 J/cm^2. With this fluence, $O_2(a^1\Delta_g)$ kinetic traces were typically accumulated over an irradiation period of 3 min. PPa and AlPcS4 were irradiated at 675 nm whereas FCh was irradiated at 650 nm. The approaches used to produce and monitor (1) $O_2(a^1\Delta_g)$ in cell suspensions and (2) fluorescence from single cells have likewise been described.[17,28] Control in these microscope-based experiments was achieved using μManager.[44] The Raman microscope used to document the presence of β-carotene in our cells is described in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Data pertinent to (1) incorporation of β-carotene into cells, (2) cytotoxic effects of β-carotene, and (3) sensitizer synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

progilby@chem.au.dk

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from the Danish National Research Foundation and ANPCyT (Argentina). G.N.B. thanks CONICET (Argentina) for a scholarship. D.O.M. is a research member from CIC (Province of Buenos Aries, Argentina). E.F.F.S. thanks the Portuguese Foundation for Science and Technology for a fellowship.

■ REFERENCES

(1) Paterson, M. J.; Christiansen, O.; Jensen, F.; Ogilby, P. R. *Photochem. Photobiol.* **2006**, *82*, 1136−1160.

(2) Kanofsky, J. R. *Chem.−Biol. Interact.* **1989**, *70*, 1−28.

(3) Klotz, L.-O.; Kröncke, K.-D.; Sies, H. *Photochem. Photobiol. Sci.* **2003**, *2*, 88−94.

(4) Schweitzer, C.; Schmidt, R. *Chem. Rev.* **2003**, *103*, 1685−1757.

(5) Ogilby, P. R. *Chem. Soc. Rev.* **2010**, *39*, 3181−3209.

(6) Clennan, E. L.; Pace, A. *Tetrahedron* **2005**, *61*, 6665−6691.

(7) Redmond, R. W.; Kochevar, I. E. *Photochem. Photobiol.* **2006**, *82*, 1178−1186.

(8) Foote, C. S.; Denny, R. W. *J. Am. Chem. Soc.* **1968**, *90*, 6233−6235.

(9) Edge, R.; McGarvey, D. J.; Truscott, T. G. *J. Photochem. Photobiol., B* **1997**, *41*, 189−200.

(10) Farmilo, A.; Wilkinson, F. *Photochem. Photobiol.* **1973**, *18*, 447−450.

(11) Marston, G.; Truscott, T. G.; Wayne, R. P. *J. Chem. Soc., Faraday Trans.* **1995**, *91*, 4059−4061.

(12) Foote, C. S.; Chang, Y. C.; Denny, R. W. *J. Am. Chem. Soc.* **1970**, *92*, 5216−5218.

(13) Böhm, F.; Edge, R.; Truscott, T. G. *Acta Biochim. Pol.* **2012**, *59*, 27−30.

(14) Stahl, W.; Sies, H. *Biochim. Biophys. Acta* **2005**, *1740*, 101−107.

(15) Sen, S.; Chakraborty, R. *ACS Symp. Ser.* **2011**, *1083*, 1−37.

(16) Snyder, J. W.; Skovsen, E.; Lambert, J. D. C.; Poulsen, L.; Ogilby, P. R. *Phys. Chem. Chem. Phys.* **2006**, *8*, 4280−4293.

(17) Silva, E. F. F.; Pedersen, B. W.; Breitenbach, T.; Toftegaard, R.; Kuimova, M. K.; Arnaut, L. G.; Ogilby, P. R. *J. Phys. Chem. B* **2012**, *116*, 445−461.

(18) Pedersen, B. W.; Breitenbach, T.; Redmond, R. W.; Ogilby, P. R. *Free Radical Res.* **2010**, *44*, 1383−1397.

(19) Kuimova, M. K.; Yahioglu, G.; Ogilby, P. R. *J. Am. Chem. Soc.* **2009**, *131*, 332−340.

(20) Hatz, S.; Lambert, J. D. C.; Ogilby, P. R. *Photochem. Photobiol. Sci.* **2007**, *6*, 1106−1116.

(21) Pedersen, B. W.; Sinks, L. E.; Breitenbach, T.; Schack, N. B.; Vinogradov, S. A.; Ogilby, P. R. *Photochem. Photobiol.* **2011**, *87*, 1077−1091.

(22) Wilkinson, F.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1995**, *24*, 663−1021.

(23) Scita, G.; Aponte, G. W.; Wolf, G. *J. Nutr. Biochem.* **1992**, *3*, 118−123.

(24) Briviba, K.; Bornemann, R.; Lemmer, U. *Mol. Nutr. Food Res.* **2006**, *50*, 991−995.

(25) Gillbro, T.; Cogdell, R. J. *Chem. Phys. Lett.* **1989**, *158*, 312−316.

(26) Kuimova, M. K.; Botchway, S. W.; Parker, A. W.; Balaz, M.; Collins, H. A.; Anderson, H. L.; Suhling, K.; Ogilby, P. R. *Nat. Chem.* **2009**, *1*, 69−73.

(27) Skovsen, E.; Snyder, J. W.; Lambert, J. D. C.; Ogilby, P. R. *J. Phys. Chem. B* **2005**, *109*, 8570−8573.

(28) Breitenbach, T.; Kuimova, M. K.; Gbur, P.; Hatz, S.; Schack, N. B.; Pedersen, B. W.; Lambert, J. D. C.; Poulsen, L.; Ogilby, P. R. *Photochem. Photobiol. Sci.* **2009**, *8*, 442−452.
(29) Häcker, G. *Cell Tissue Res.* **2000**, *301*, 5−17.
(30) Rello, S.; Stockert, J. C.; Moreno, V.; Gámez, A.; Pacheco, M.; Juarranz, A.; Cañete, M.; Villanueva, A. *Apoptosis* **2005**, *10*, 201−208.
(31) Nikkhah, G.; Tonn, J. C.; Hoffmann, O.; Kraemer, H.-P.; Darling, J. L.; Schönmayr, R.; Schachenmayr, W. *J. Neuro-Oncol.* **1992**, *13*, 1−11.
(32) Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. *J. Neurochem.* **1997**, *69*, 581−593.
(33) Freshney, R. I. *Culture of Animal Cells: A Manual of Basic Technique*; Alan R. Liss, Inc.: New York, 1987.
(34) Palozza, P.; Serini, S.; DiNicuolo, F.; Piccioni, E.; Calviello, G. *Mol. Aspects Med.* **2003**, *24*, 353−362.
(35) Palozza, P.; Serini, S.; Torsello, A.; Boninsegna, A.; Covacci, V.; Maggiano, N.; Ranelletti, F. O.; Wolf, F. I.; Calviello, G. *Int. J. Cancer* **2002**, *97*, 593−600.
(36) Wang, X.-D.; Russell, R. M. *Nutr. Rev.* **1999**, *57*, 263−272.
(37) Hatz, S.; Poulsen, L.; Ogilby, P. R. *Photochem. Photobiol.* **2008**, *84*, 1284−1290.
(38) Böhm, F.; Haley, J.; Truscott, T. G.; Schalch, W. *J. Photochem. Photobiol., B* **1993**, *21*, 219−221.
(39) Tinkler, J. H.; Böhm, F.; Schalch, W.; Truscott, T. G. *J. Photochem. Photobiol., B* **1994**, *26*, 283−285.
(40) Pimenta, F. M.; Jensen, R. L.; Holmegaard, L.; Esipova, T. V.; Westberg, M.; Breitenbach, T.; Ogilby, P. R. *J. Phys. Chem. B* **2012**, *116*, 10234−10246.
(41) Polivka, T.; Sundström, V. *Chem. Rev.* **2004**, *104*, 2021−2071.
(42) McDermott, G.; Prince, S. M.; Freer, A. A.; Hawthornthwaite-Lawless, A. M.; Papiz, M. Z.; Cogdell, R. J.; Isaacs, N. W. *Nature* **1995**, *374*, 517−521.
(43) Scita, G. *J. Nutr. Biochem.* **1992**, *3*, 124−128.
(44) Edelstein, A.; Amodaj, N.; Hoover, K.; Vale, R.; Stuurman, N. *Curr. Protoc. Mol. Biol.* **2010**, *92*, 1−17.