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Intracellular singlet oxygen photosensitizers: On the road to solving the problems of sensitizer degradation, bleaching and relocalization.

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Insight, innovation, integration

There are many molecules designed to be incorporated into a cell and perform some function requiring UV/Vis irradiation. Fluorescent probes for structure/activity and sensitizers for photodynamic action are examples. Upon irradiation, and exploiting the desired function, the molecule can bleach, degrade and move to a different intracellular location. This problem can be exacerbated with sensitizers specifically designed to produce singlet oxygen, which is known to oxidize/oxygenate many molecules. With the advent of more accurate methods to monitor (a) intracellular singlet oxygen, and (b) a cell's response to singlet oxygen, the development of stable photosensitizers becomes correspondingly more critical. We address this issue as a means of developing better tools to elucidate singlet-oxygen-mediated mechanisms of cell signaling.

Abstract

Selected singlet oxygen photosensitizers have been examined from the perspective of obtaining a molecule that is sufficiently stable under conditions currently employed to study singlet oxygen behavior in single mammalian cells. Reasonable predictions about intracellular sensitizer stability can be made based on solution phase experiments that approximate the intracellular environment (*e.g.*, solutions containing proteins). Nevertheless, attempts to construct a stable sensitizer based solely on the expected reactivity of a given functional group with singlet oxygen are generally not sufficient for experiments in cells; it is difficult to construct a suitable chromophore that is impervious to all of the secondary and/or competing degradative processes that are present in the intracellular environment. On the other hand, prospects are reasonably positive when one considers the use of a sensitizer encapsulated in a specific protein; the local environment of the chromophore is controlled, degradation as a consequence of bimolecular reactions can be mitigated, and genetic engineering can be used to localize the encapsulated sensitizer in a given cellular domain. Also, the option of directly exciting oxygen in sensitizer-free experiments provides a useful complementary tool. These latter systems bode well with respect to obtaining more accurate control of the "dose" of singlet oxygen used to perturb a cell; a parameter that currently limits mechanistic studies of singlet-oxygen-mediated cell signaling.

Introduction

The oxygen-dependent photoinduced bleaching or degradation of dyes, fluorescent probes, and sensitizers is a topic that has long drawn the attention of the scientific and technological communities. In materials science, the issue of producing stable electroluminescent polymers, for example, has been an important limiting factor in the evolution of organic display screens.[1, 2] In biology, common photo-functional molecules that are likewise susceptible to bleaching include (a) fluorescent probes used to assess cell structure and/or activity,[3] and (b) sensitizers used to generate reactive oxygen species, ROS.[4-7] ROS play key roles in cell signaling,[8-11] among other processes, with ramifications that range from cell proliferation to cell death.[12, 13] For example, the sensitized production of ROS forms the basis for the medical procedure of photodynamic therapy, PDT, wherein undesired tissue (*e.g.*, cancerous tumors) can be removed by the action of light.[14-16]

Although the words "bleaching" and "degradation" are often used interchangeably, and this has been done in sections of the present text, it is desirable at the outset to provide definitions that distinguish between the two words in our context of photo-induced processes. Degradation is the more general phenomenon that refers to a chemical change in a given molecule (*e.g.*, the oxidation of a given functional group). Bleaching refers to a change in the absorbance or emission intensity of a given compound (generally a loss at a specified wavelength). As such, bleaching is an optical manifestation of a degradation reaction. It is important to note, however, that a degradation reaction may not involve the chromophore directly, but may only perturb a functional group elsewhere in the molecule which, in turn, may change the solubility properties and location of the molecule in a cell. A change in the local

environment of the chromophore may, in turn, change the probability of light absorption and emission giving the impression of bleaching.

The appropriate reference for biologically-pertinent photoinduced bleaching would be a citation to a large fraction of the photochemical literature published over the last ~ 100 years. Light-induced molecular rearrangements, bond making/breaking reactions, and functional group modifications all contribute to the phenomenon called bleaching. We can limit our perspective somewhat by focusing on oxygen-dependent events, but this is still a large field given the plethora of molecules that absorb light under conditions in which they are exposed to our ambient atmosphere.

We have long been interested in the photosensitized production and subsequent deactivation of one particular ROS, singlet oxygen, $O_2(a^1\Delta_g)$.[17] Singlet oxygen is the lowest excited electronic state of molecular oxygen,[18] and has a characteristic chemistry that results in the oxygenation/oxidation of many organic and bio-organic molecules.[19, 20] As such, singlet oxygen plays a key role in the oxygen-dependent photoinduced degradation and bleaching of many dyes. It follows that the effects of degradation and bleaching can be mitigated by (a) using dyes that have a low quantum yield of photosensitized singlet oxygen production, and/or (b) adding a molecule to the system that can deactivate any singlet oxygen that has been produced before it can react with the dye. A wonderful example of the latter is the addition of the amine DABCO (*i.e.*, 1,4-diazabicyclo[2.2.2]octane) to the dye solution used in dye lasers.[21]

Our interests include (1) monitoring and controlling the production of singlet oxygen in biologically-pertinent systems, and (2) assessing the effects of singlet oxygen on mammalian cells. As such, we work under conditions where organic molecules, be it a singlet oxygen

sensitizer or a fluorescent probe used to assess cell response, are exposed to an environment where photobleaching can be a problem. For one interested in elucidating the mechanisms of singlet-oxygen-dependent cell signaling, such photobleaching/photodegradation provides an appreciable hindrance; it limits the control over which the singlet oxygen dose can be delivered, and decreases the accuracy with which cell response can be assessed.

The present report on photobleaching has its origins in our work on individual cells where a focused laser is used to initiate the photosensitized production of singlet oxygen in a spatially-confined subcellular domain.[22-26] We have been able to monitor the singlet oxygen thus produced in time-resolved experiments using the weak 1275 nm $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence as a probe. Given the laser irradiance (*i.e.*, W/m^2) currently required in this phosphorescence experiment, bleaching of the photosensitizer generally occurs over the elapsed irradiation time used for signal averaging. Thus, the amount of singlet oxygen produced can vary with each successive laser pulse delivered over this time period, and this is then reflected in the intensity of the final singlet oxygen phosphorescence signal retained. Moreover, products of photobleaching reactions can also influence the decay kinetics, and hence diffusion distance, of singlet oxygen.[17, 26] Although the latter is only now beginning to be demonstrated for experiments at the level of an individual cell,[26, 27] it has long been known as a potential problem for *in vitro* solution phase singlet oxygen experiments.[28] These problems associated with sensitizer bleaching are pertinent not just for 1275 nm singlet oxygen phosphorescence experiments, but are equally relevant for experiments in which a fluorescent probe is used to indirectly monitor singlet oxygen.[29-31]

Photo-initiated reactions can also initiate processes that lead to the relocalization of a sensitizer into another subcellular domain and/or into the extracellular medium.[26, 32-36] This

relocalization may be a consequence of the degradation of the light-absorbing sensitizer itself and/or the degradation of molecules in the environment that immediately surrounds the sensitizer. In any event, the net adverse result with respect to an experiment on singlet-oxygen-mediated cell signaling is exacerbated: one loses control over the dose of singlet oxygen initially created. Deconvoluting the effects that sensitizer relocalization have on the intensities and decay kinetics of singlet oxygen phosphorescence signals can certainly complicate the process of assessing other, and arguably more meaningful, responses to singlet oxygen.[26, 37]

A corollary of this discussion on singlet oxygen sensitizers is that bleaching and relocalization can also occur with fluorescent probes used to assess cell structure and activity. Derivatives of fluorescein, for example, are a commonly used fluorophore in many assays.[3] A limitation of many of these derivatives, however, is that they can bleach under the conditions in which the cell is being examined.[3, 38-42] This characteristic of fluorescein and other fluorescent probes partly reflects the fact that, under many biologically-pertinent conditions, an excited state of the probe itself can sensitize the production of singlet oxygen,[4, 43, 44] or another ROS,[45] in appreciable yield. Because such self-sensitized ROS-dependent bleaching invariably involves the longer-lived triplet state,[40, 46, 47] not the shorter-lived fluorescent singlet state, the addition of a covalently-linked triplet state trap to the probe chromophore can be beneficial.[48] However, this methodology clearly cannot be applied to impart stability to a singlet oxygen sensitizer, where energy transfer to oxygen from the sensitizer triplet state is the desired feature.

In light of the bleaching-dependent problems associated with the use of singlet oxygen sensitizers, we set out to (a) identify sensitizers that are sufficiently stable under the conditions of our single cell experiments, (b) determine if one could have readily identified such sensitizers on the basis solely of the functional groups present in the molecule, and (c) ascertain if *in vitro*

solution phase experiments can be used to accurately model the behavior of a given sensitizer when located inside a cell.

Our initial perspective in this regard was partly influenced by a number of points:

(1) Despite the fact that many of the porphyrin-based molecules commonly desired for use as an intracellular sensitizer readily photo-bleach,[14, 49] conjugated dimers of metallo-porphyrins have been shown to be reasonably stable when incorporated into a cell and irradiated.[50] Moreover, these molecules sensitize the production of singlet oxygen in appreciable yield,[50, 51] and give rise to good intracellular singlet oxygen phosphorescence signals.[37] Thus, it is reasonable to look for porphyrin-based sensitizers that are sufficiently stable under our experimental conditions. One feature of these particular porphyrin dimers is that the conjugating bridge consists of a butadiyne moiety.[50] This leads to our second point.

(2) We have shown that molecules containing alkyne-conjugated phenyl moieties are more stable than the corresponding molecule with alkene-based units of conjugation upon exposure to singlet oxygen.[52] Likewise, we have shown that the judicious placement of electron-withdrawing substituents on an alkene-based conjugation unit markedly decreases the rate of singlet-oxygen-mediated photobleaching (singlet oxygen is an electrophile).[53] The use of electron-withdrawing substituents to impart stability against singlet oxygen has also been achieved by replacing the C-H bonds in the given molecule with C-F bonds.[54-57] Thus, it appears reasonable to use some basic chemical principles to methodically design and construct a sensitizer that would be more stable under selected conditions of irradiation. This leads to our third point.

(3) Electron-withdrawing substituents in the *ortho* positions of *meso*-phenyl-substituted porphyrin derivatives increase the oxidation potential and concomitantly decrease the photodecomposition quantum yield of that porphyrin.[56] Moreover, bulky substituents at these same *ortho* positions can provide steric hindrance against attack by molecular oxygen and may further stabilize the sensitizer.[58] This leads to our fourth point.

(4) Evidence has been presented to indicate that encasing/encapsulating a sensitizer or fluorophore can increase its stability towards oxygen-dependent degradation.[27, 59-62] Although this observation may reflect a number of phenomena, the relative importance of which depends on the nature and composition of the encasing material, a key factor is that the chromophore can be shielded from reactive species that only diffuse over a finite distance in a given period of time (*e.g.*, singlet oxygen, hydroxyl radical, etc.).[31]

(5) Krieg and Whitten[63] reported in 1984 that the *in vitro* oxygen-dependent photobleaching of protoporphyrin IX is significantly enhanced in the presence of selected amino acids and, independently, in the presence of erythrocyte ghosts which contain lipids as well as membrane proteins. A mechanism was proposed in which singlet oxygen creates, for example, a protein-based oxygenated intermediate (*e.g.*, a long-lived peroxide) which, in turn, can then oxidize the porphyrin. This interpretation is consistent with independent work on protein oxidation,[64] and has since been substantiated in studies where the photobleaching of selected chlorins was enhanced upon the addition of bovine serum albumin to the solution.[65, 66] On this basis, one could infer that the rate and extent of sensitizer bleaching in a cell will depend on the immediate environment and, hence, intracellular location of that sensitizer. It has been shown that rates of fluorophore bleaching depend on the intracellular location of the dye.[67]

For the present study, we examined the behavior of seven singlet oxygen sensitizers, both in solution phase experiments and in cultured cells. We conclude that predictions about intracellular stability based solely on the chemical structure of the sensitizer can be erroneous. Indeed, we go further to say that attempts to design a stable sensitizer based solely on principles of functional group reactivity will continue to be a challenge. On the other hand, attempts to impart stability through encapsulation of the chromophore appear promising. The data obtained thus contribute to the ultimate goal of designing singlet oxygen sensitizers whose intracellular behavior can be controlled under a variety of oxidizing conditions.

Experimental

Instrumentation. The details of the femtosecond (fs) and nanosecond (ns) laser systems as well as the lamps used for sample irradiation have been previously described.[26, 68-70] The instrumentation and methods used to monitor sample luminescence, create images of a cell, and record emission spectra from molecules in a cell have likewise been described.[24, 31, 71]

Cells. To maintain continuity with our previous work, HeLa cells on a polylysine substrate were used in this study. The preparation of samples has been described previously.[26, 72, 73] Cell death was assessed using a variety of morphological features as previously described.[24]

Chemicals. 5,10,15,20-Tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine, TMPyP, was obtained as the tetrasulfonate salt from Porphyrin Systems and used as received. Riboflavin 5'-monophosphate sodium salt dehydrate (*i.e.*, flavin mononucleotide, FMN), *N,N*-

dimethylfulleropyrrolidiniuim iodide, DMP-C_{60}, phosphate-buffered saline solution, PBS, and bovine serum albumin, BSA, were obtained from Sigma-Aldrich and used as received. Benzo[*cd*]pyren-5-one, BP,[74] and 1,4-bis[4-(*N,N*-diphenylamino)phenylethynyl]-2,5-dibromobenzene, BBB,[52] were prepared as outlined in the papers respectively cited. 5,10,15,20-tetrakis(2,6-difluoro-3-sulfophenyl)porphine, TDFPPS, (and the corresponding bacteriochlorin, TDFPBS)[56, 75] were gifts of Luzitin SA (Coimbra, Portugal). A plasmid for the protein-encased FMN, the so-called miniSOG,[76] was obtained as a gift from Prof. Roger Y. Tsien (University of California-San Diego), and was expressed as previously described.[77, 78]

Photobleaching quantum yields. A 3 mL sample of the photosensitizer dissolved in a given solvent was placed in front of the irradiation source (a pulsed laser) in a quartz cuvette. Absorption and fluorescence spectra were recorded at different times from the sample that was constantly stirred during irradiation. Photobleaching quantum yields, ϕ_{pb}, were obtained as the quotient (initial rate of photosensitizer disappearance)/(initial rate of photon absorption) by quantifying time-dependent changes in sensitizer absorbance and/or fluorescence intensity at a given wavelength.[79, 80] Further details are provided in the Supporting Information. A calibrated power meter was used to quantify the irradiance. Because mechanisms of photobleaching can be sensitizer dependent, and yield products that absorb/emit over a wide range of wavelengths, care must be exercised not to over-interpret values of ϕ_{pb} thus obtained; they should simply be viewed as a general qualitative guide to the bleaching process. To underscore this point, we have not applied error bars to the values of ϕ_{pb} reported.

Results and discussion

The seven singlet oxygen sensitizers studied can be classified into two groups: non-porphyrin-based and porphyrin-based molecules (Figures 1 and 2, respectively).

For well-founded reasons, porphyrin-based molecules have long been used as singlet oxygen sensitizers, particularly for PDT-related applications.[14] The properties of these molecules are readily tuned by changing substituents and/or the extent to which the macrocycle is reduced (*i.e.*, chlorins and bacteriochlorins). Most importantly, these compounds are generally not cytotoxic in the absence of light. We examined two porphyrins and one bacteriochlorin. The hydrophilic porphyrin TMPyP has been the basis for much of our single cell singlet oxygen phosphorescence work over the years.[23, 25, 36] The hydrophilic sulfonated porphyrin TDFPPS (and the corresponding bacteriochlorin, TDFPBS) likewise have good singlet oxygen yields and are readily incorporated into a cell.[26, 75, 81]

There is also a vast literature on non-porphyrin-based singlet oxygen sensitizers,[4] and we opted to examine three molecules that are reasonably photostable when dissolved in a liquid solvent: BP,[74] DMP-C_{60},[82] and BBB.[52] In light of recent work on protein-encased sensitizers,[7, 76, 77, 83] we also examined flavin mononucleotide, FMN, both as a free solvated chromophore and when encased in a LOV2-based protein.

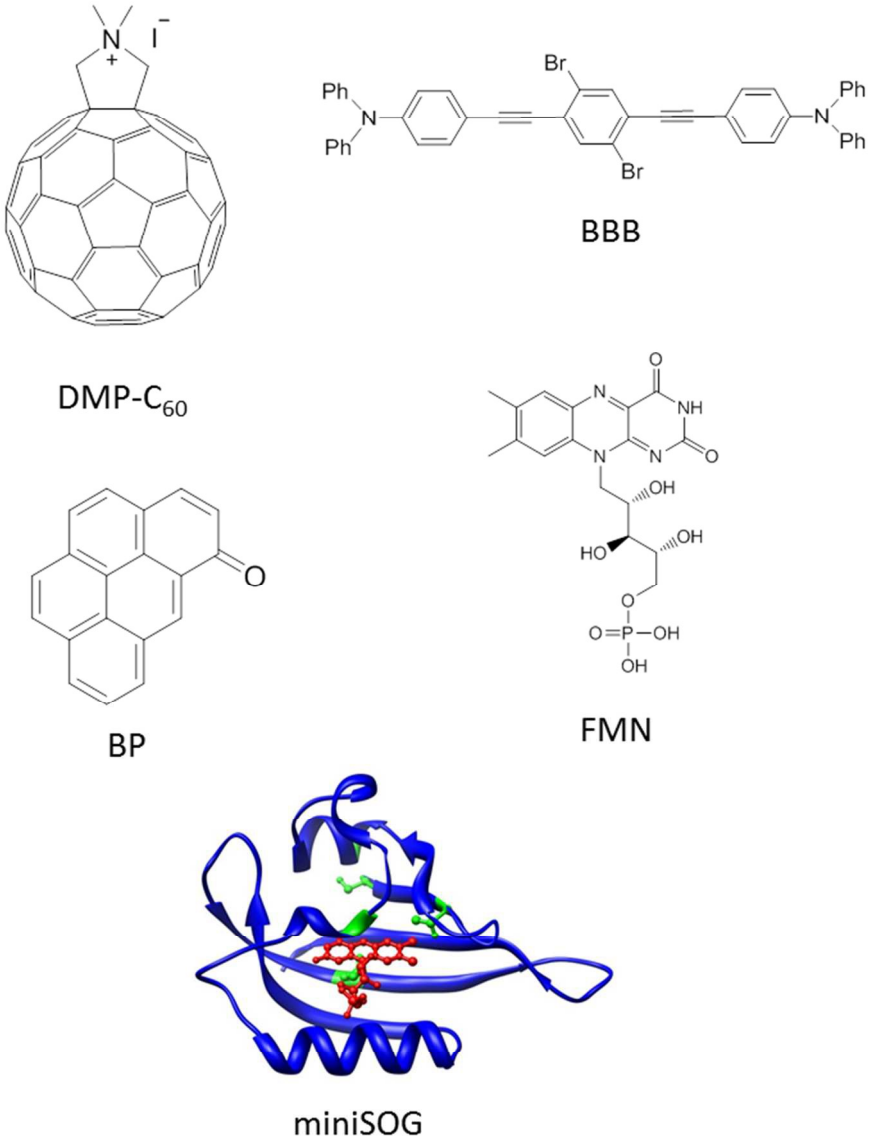

Fig. 1. Non-porphyrin-based singlet oxygen sensitizers examined in this study. The green residues shown in the miniSOG structure indicate the mutations made to the parent LOV2 protein (see Shu, *et al*.,[76] Pimenta, *et al*.,[78] and Westberg, *et al*.[77]).

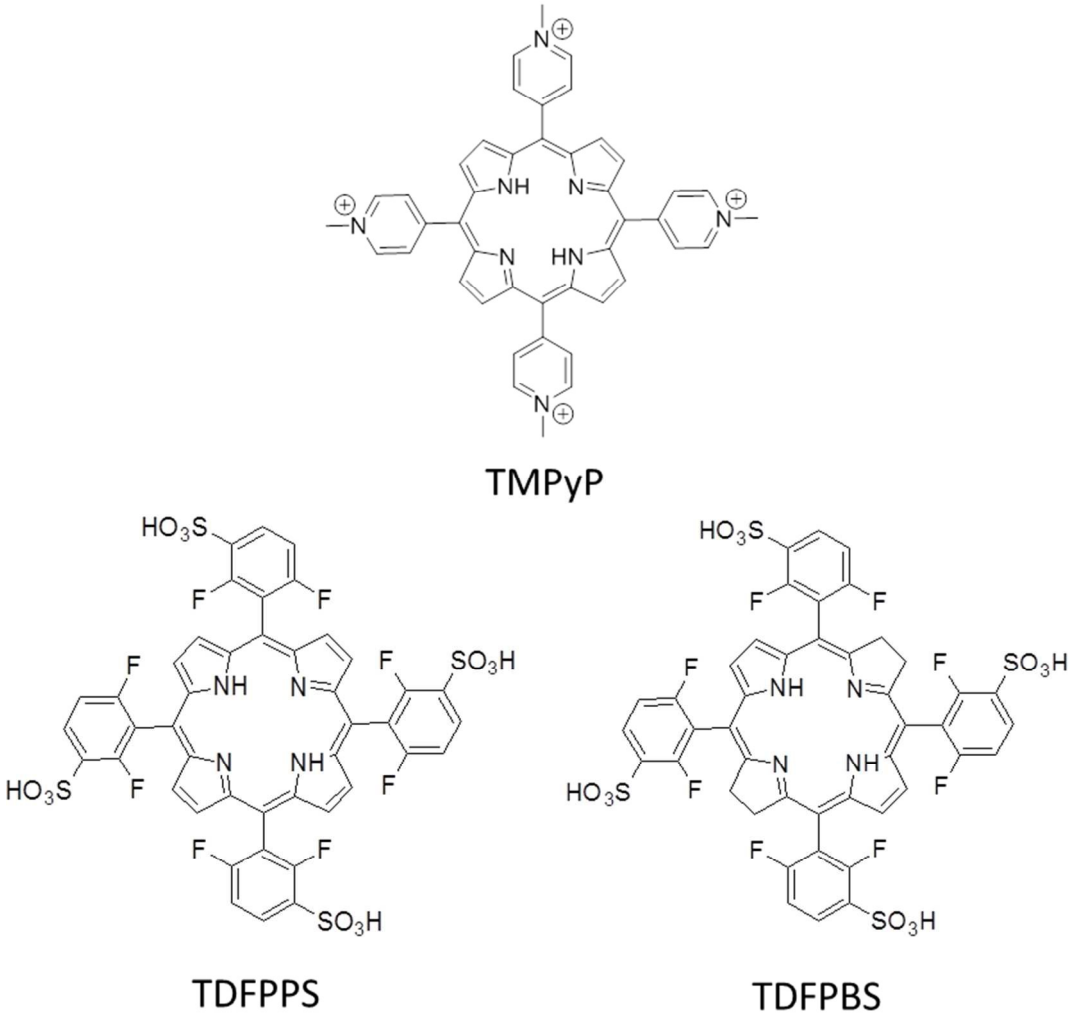

Fig. 2. Porphyrin-based singlet oxygen sensitizers examined in this study.

BP. The polycyclic aromatic molecule BP has an absorption profile that extends to wavelengths longer than 500 nm (Figure 3). It has a weak fluorescence ($\Phi_F \sim 0.004$ in toluene) with a band maximum at ~ 560 nm.[74] This comparatively small quantum yield of fluorescence, Φ_F, can be attributed to the carbonyl group which facilitates $S_1 \rightarrow T_1$ intersystem crossing. The latter is manifested in a quantum yield of BP-sensitized singlet oxygen production that is close to unity.[74]

On the basis solely of the chemical structure of BP, one might expect it to be resistant to singlet-oxygen-mediated degradation. Indeed, BP is reasonably stable upon prolonged irradiation at 400 nm in oxygenated solutions of toluene or benzene (Figure 3). On the other hand, appreciable photoinitiated changes rapidly occur when BP is dissolved in DMSO (Figure 3), a phenomenon that is reflected in the comparatively large photobleaching quantum yield of 1.3×10^{-2}. The isosbestic points in the spectra shown in Figure 3 suggest that, over the time period examined, only one photoproduct of BP is formed in each case. However, both the absorption and emission spectra (see Supporting Information) indicate that the BP photoproduct formed in DMSO may be different from that in benzene. These solvent-dependent differences may reflect the fact that, as a polar aprotic solvent, DMSO may selectively increase the rate of an oxidative degradation reaction that involves polar intermediates.[84]

Although BP is hydrophobic, it is soluble in aqueous solutions that contain a small amount (~ 0.25% by volume) of DMSO. In PBS, as well as PBS containing BSA, BP is indeed sensitive to irradiation (see Supporting Information). On the basis of these solution phase results, one might expect that BP would not be particularly stable when incorporated into a cell. The data obtained are indeed consistent with this expectation (Figure 4).

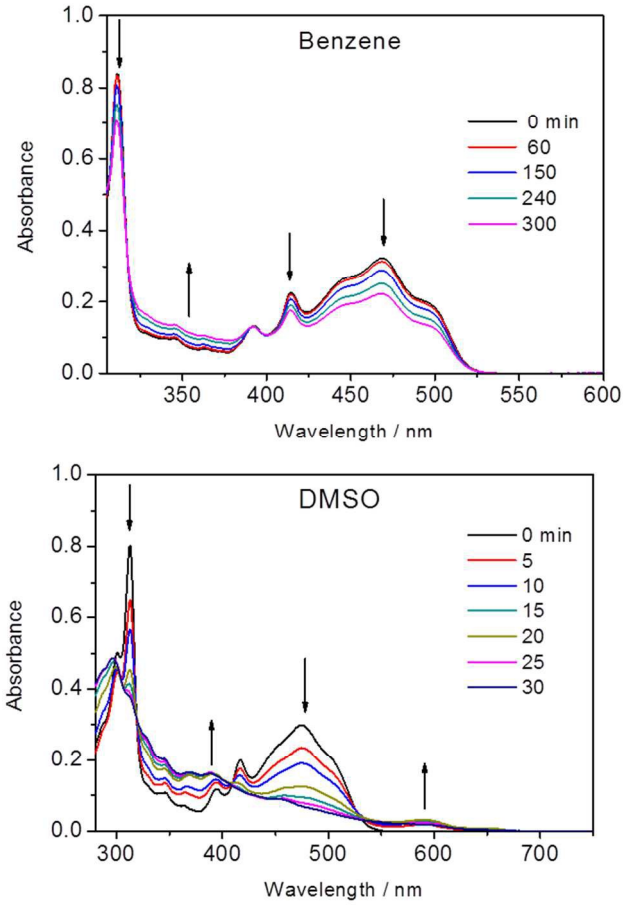

Fig. 3. Absorption spectra of BP as a function of the elapsed irradiation time in benzene (1 kHz fs laser irradiation at 400 nm with 57 mW/cm^2) and DMSO (irradiation at 420 nm with 43 mW/cm^2). In benzene, data were recorded over an elapsed irradiation period of 300 min, whereas in DMSO the data were recorded only over an elapsed irradiation period of 30 min. Arrows show the direction of irradiation-induced changes in the spectra.

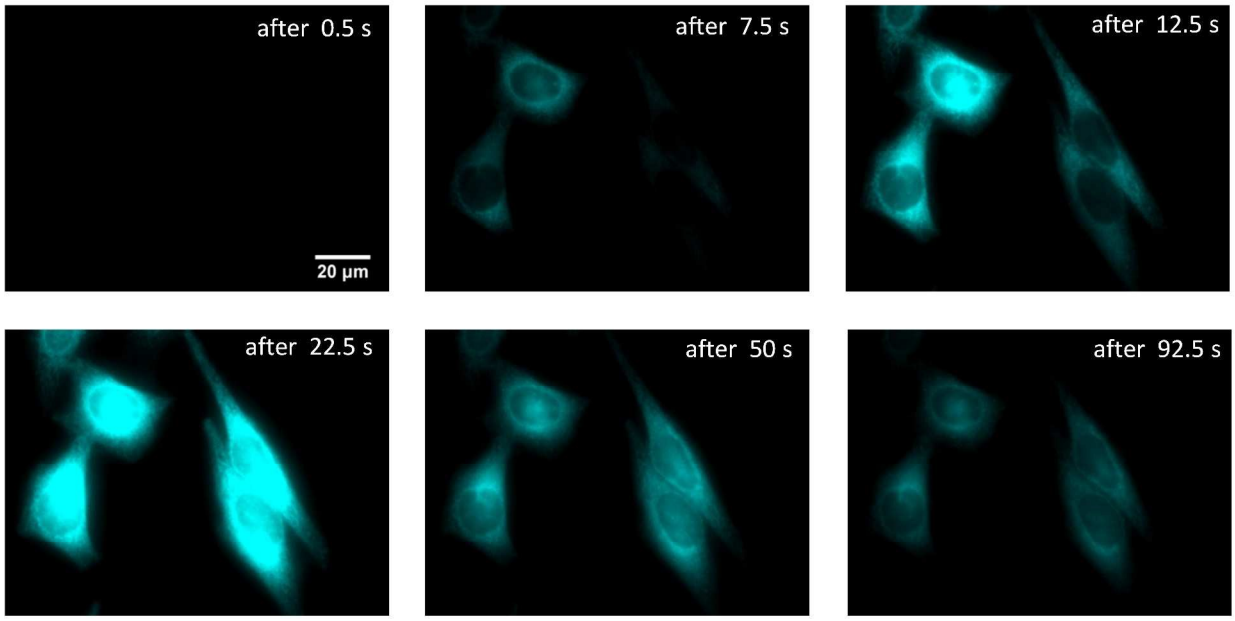

Fig. 4. Images of HeLa cells that had been incubated for 4 h in normal growth medium containing 10 µM BP. The images are based on emission detected at wavelengths longer than 500 nm upon excitation at 480 nm, and separate images were recorded as a function of the elapsed irradiation time at 480 nm (4.1 mW/cm^2 from a *cw* metal halide lamp). The data are consistent with a process where a nascent product of BP photodegradation emits light, but then this compound further evolves upon prolonged irradiation to produce non-emissive molecules and/or molecules that no longer absorb at 480 nm. The corresponding emission spectra are not the same as those seen in the solution phase experiments (see Supporting Information), suggesting that (a) the emitting molecules are not the same as those in the solution phase experiments, and/or (b) the local environment appreciably perturbs the spectra.

In conclusion, BP provides a nice example of a molecule that, *a priori*, would be expected to be reasonably stable against singlet-oxygen-mediated degradation. However, under intracellular conditions, BP is quite labile, most likely as a consequence of other protein-dependent oxidative processes that can more readily play a role, perhaps involving peroxide-

spawned radicals. All of this is consistent with the observation that irradiation of intracellular BP results in cell death.

BBB. The discussion on BP is likewise applicable to BBB. This latter compound was the focus of an earlier solution phase study which showed that replacing the conjugating alkenyl groups in phenylene vinylene oligomers with alkynyl groups appreciably increased the stability of the molecule to singlet-oxygen-mediated degradation.[52] Upon incorporating BBB into cells, however, where it presumably localizes in protein-containing lipophilic domains, it becomes extremely labile to irradiation (see Supporting Information).

Thus, on the basis of our BP and BBB data, we conclude that caution must indeed be exercised with respect to all-inclusive statements about the stability of a given singlet oxygen sensitizer based solely on the reactivity of functional groups in that sensitizer to singlet oxygen; the local environment of the sensitizer clearly plays an important role, certainly when this environment contains molecules (*e.g.*, proteins, lipids) that can propagate other reactions (*e.g.*, peroxide-initiated radical processes[64, 85]).

DMP-C_{60}. The fullerene C_{60} sensitizes the production of singlet oxygen with a quantum efficiency of ~ 1.0 and has a structure that renders it stable to singlet-oxygen-mediated degradation.[86-88] Although C_{60} itself is hydrophobic and insoluble in many media, this limitation can often be overcome using derivatives of C_{60}.[86, 89, 90] The derivative we opted to study here, DMP-C_{60}, can be incorporated into a cell and can initiate cell death upon irradiation.[91]

The quantum yield of singlet oxygen production, ϕ_Δ, sensitized by DMP-C_{60} is reported to be 0.27 ± 0.02 in CH_3OD (with 1% added DMSO).[91] In a D_2O-based PBS solution (with 4%

added DMSO), these same investigators were not able to detect a $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence signal upon irradiation of DMP-C_{60} implying that, under these latter conditions, ϕ_Δ is appreciably less than 0.27.[91] Although the D_2O data may partly reflect the aggregation of DMP-C_{60},[91] these observations also contribute to the suggestion that, under physiological conditions, irradiation of C_{60} and its derivatives may lead to cell death as a consequence of the production of oxygen-related radicals, not singlet oxygen.[82, 91, 92] We can nevertheless still use DMP-C_{60} to address aspects of the photoinitiated oxygen-dependent degradation of intracellular sensitizers.

Irradiation of DMP-C_{60} at 355 nm in a solution of H_2O/DMSO (9-to-1, by volume) shows a modest degradation-dependent change in the absorption spectrum (Figure 5A). Upon irradiation of a corresponding solution containing 0.75 mM BSA, more pronounced changes in the absorption spectrum are observed (Figure 5B). There is a clear irradiation-dependent increase in the absorbance at ~ 310 nm, which is consistent with the oxidation of selected amino acids in a protein.[77, 93] The irradiation-induced change in the spectrum at ~ 350 nm, however, is somewhat puzzling and could reflect a number of phenomena. Although BSA does not absorb in this spectral region, the pronounced irradiation-induced "dip" in the spectrum at ~350 nm that appears to be correlated with the absorbance increase at ~310 nm could reflect a decrease in the extent to which the solution scatters light as BSA is oxidized. [Note: Our choice of using BSA at 0.75 mM or 2.0 mM (*vide infra*) is intended to mimic the expected average intracellular concentration of a protein: ~ 0.5 – 5 mM.[94, 95]] Alternatively, this change could reflect a BSA-enhanced, spectrally-localized "bleach" of DMP-C_{60} that is not strongly manifested at wavelengths longer than ~400 nm (see corresponding changes in Figure 5A). Thus, one might

indeed be able to exert some control in a cell-based experiment by irradiating DMP-C_{60} at wavelengths longer than 400 nm.

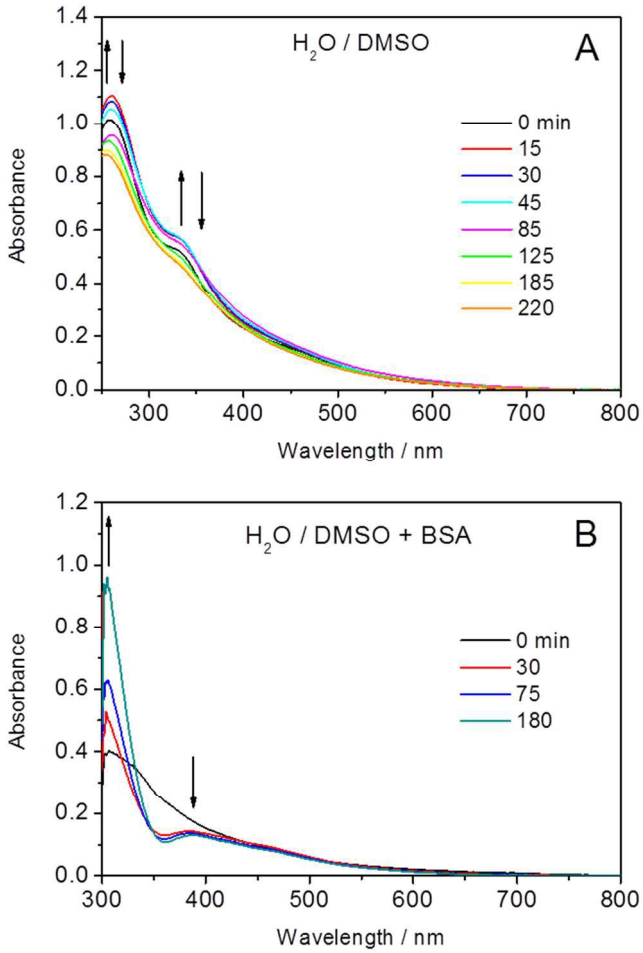

Fig. 5. (A) Absorption spectra of DMP-C_{60} in H_2O/DMSO (9-to-1, by volume) as a function of the elapsed time of 355 nm irradiation (10 Hz ns laser at 45 mW/cm^2). As noted, the elapsed irradiation period was 220 min. (B) Data were recorded under similar conditions over an elapsed irradiation period of only 180 min from a solution that also contained 0.75 mM BSA. Arrows show the direction of irradiation-induced changes in the spectra.

Upon incubation of HeLa cells with DMP-C_{60}, this dye was readily incorporated into the cells (Figure 6). Images obtained of these cells based on the fluorescence of DMP-C_{60} imply that the principal sites of intracellular localization are lysosomes, although a non-negligible amount of the dye appears to be randomly dispersed in the cytoplasm (Figure 6). Upon elapsed irradiation of these cells at 450 nm, only slight changes in the broad-band intensity of emitted light were observed, with much of the change occurring at early irradiation times (Figure 6). Most notably, there appears to be minimal irradiation-induced relocalization of the dye suggesting that lysosomes are stable to the reactive oxygen species produced by DMP-C_{60}. This latter observation is in stark contrast to what is observed upon irradiation of TMPyP, a singlet-oxygen-producing sensitizer that likewise initially localizes in lysosomes but that appears to facilitate lysosome rupture upon irradiation (*vide infra*). Thus, it appears that DMP-C_{60} might indeed be a sufficiently stable sensitizer for selected oxygen-dependent intracellular experiments, albeit those that do not involve singlet oxygen.

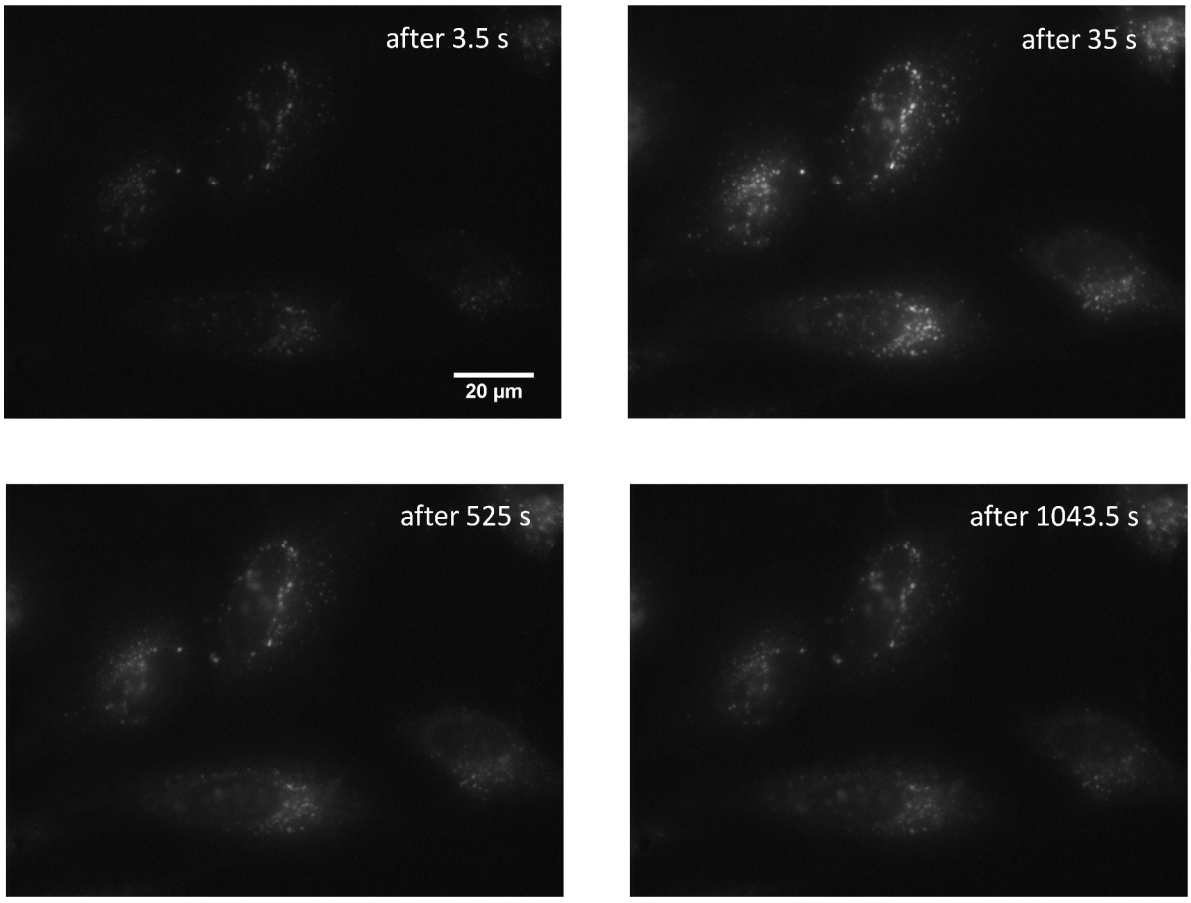

Fig. 6. Fluorescence images of HeLa cells that had been incubated with 25 µM DMP-C_{60} recorded as a function of the elapsed irradiation time at 450 nm (8.2 mW/cm^2 from *cw* metal halide lamp). Emission was detected at wavelengths longer than 600 nm.

TMPyP. Over the years, the cationic porphyrin TMPyP has been extensively used as a singlet oxygen sensitizer in a wide range of experiments. It is readily incorporated into cells, efficiently produces singlet oxygen with a quantum yield of 0.77 ± 0.04,[96] and is sufficiently fluorescent to allow for imaging experiments to ascertain its localization.[26] Indeed, these properties of TMPyP have made it a popular sensitizer in experiments to record singlet oxygen phosphorescence from cells.[17, 23, 26]

Unfortunately, TMPyP arguably provides one of the better examples for photoinduced relocalization of an intracellular sensitizer.[26, 32-34] Upon incubation of a cell with TMPyP, this molecule first localizes in lysosomes.[26] Upon irradiation of this localized TMPyP, the singlet oxygen produced presumably facilitates lysosome rupture. Depending on the experimental conditions, the TMPyP thus released may then localize in the nucleus, binding to the DNA, and/or it may cross the plasma membrane into the extracellular medium.[26, 34] Moreover, intracellular TMPyP bleaches upon prolonged irradiation.[34] All of these processes are manifested in TMPyP-sensitized singlet oxygen phosphorescence signals.[23, 26, 34] Thus, despite its frequent use, TMPyP is far from an ideal intracellular sensitizer.

In the context of the present report, it is sufficient to show that, in a model study using TMPyP dissolved in a buffered solution containing BSA, photoinduced bleaching is readily apparent (Figure 7). As with other sensitizers, the quantum yield of TMPyP photobleaching in the presence of BSA (1.2×10^{-5}) is appreciably larger than that in a PBS solution lacking BSA (3.3×10^{-6}), again with the caveat that the respective mechanisms involved are likely different.

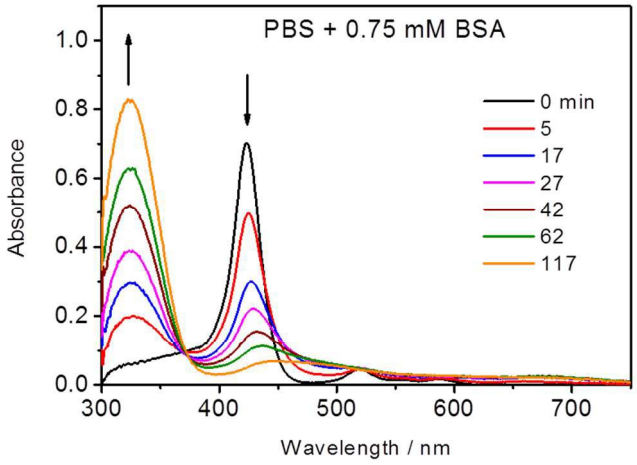

Fig. 7. Absorption spectra of TMPyP dissolved in a buffered aqueous solution containing 0.75 mM BSA. Data were recorded as a function of the elapsed time of irradiation at 420 nm (1 kHz fs laser, 50 mW/cm^2); the irradiation time covered the range 0 to 117 min. Arrows indicate the direction of irradiation-induced changes in the spectra recorded.

TDFPPS. Fluorine and chlorine substituents in the *ortho* positions of the *meso*-phenyl rings of tetraphenylporphyrin, TPP, can increase the oxidation potentials of these derivatives up to 0.3 V with respect to TPP itself.[97] Moreover, interactions between these *ortho* substituents and the H atoms in the *β*-pyrrole positions increase the twist angle between the pendant phenyl ring and the macrocycle and this, in turn, diminishes the tendency of the porphyrins to aggregate.[98] Substituents other than hydrogen in these *ortho* positions likewise appear to provide a hindrance to the attack of reactive species to the macrocycle and thereby impart extra stability.[58] Finally, halogen substituents in the *meso*-phenyl rings can also provide an internal heavy atom effect that increases the quantum yield of triplet state production without appreciably reducing the triplet state lifetime which, in turn, should be reflected in the corresponding yield of sensitized singlet oxygen production.[99] With these points in mind, TPP derivatives that are *ortho*-halogenated in

the *meso*-phenyl ring have been synthesized for use as singlet oxygen sensitizers in PDT.[100] For the present study, we set out to examine TDFPPS which is a fluorinated sulfonic acid derivative of TPP (Figure 2). This choice partly reflects the facts that TDFPPS is highly soluble in water, sensitizes the production of singlet oxygen in good yield ($\phi_\Delta = 0.71 \pm 0.09$), and is expected to be comparatively photostable. Spectra of TDFPPS solutions recorded as a function of elapsed irradiation (Figure 8) give rise to the following quantum yields of photobleaching: ϕ_{pb}(PBS) = 4.2×10^{-6} and ϕ_{pb}(DMSO) = 1.3×10^{-5}. The quantum yield of TDFPPS photobleaching in PBS is indeed smaller than that of other porphyrins commonly used as singlet oxygen sensitizers in PDT,[49, 79] although it is approximately the same as that for TMPyP (*vide supra*). The effect of DMSO is consistent with that observed upon irradiation of BP (*vide supra*, Figure 3).

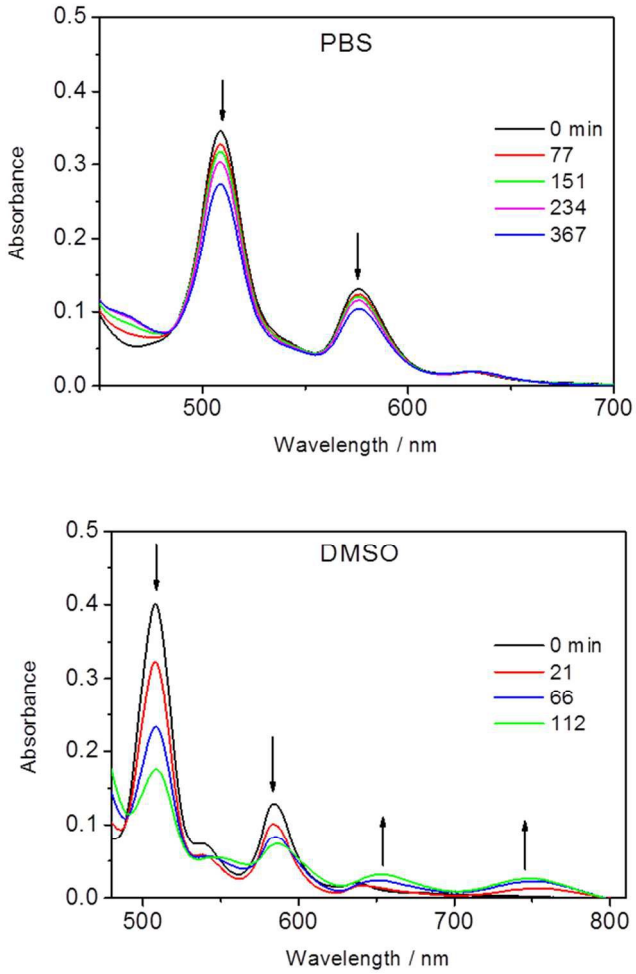

Fig. 8. Absorption spectra of TDFPPS in PBS (top panel) and DMSO (bottom panel) recorded after different periods of elapsed irradiation at 508 nm (77 mW/cm^2). Spectra in PBS were recorded at elapsed irradiation times that covered the range 0 to 367 min, whereas the data in DMSO were recorded over the range 0 to 112 min. The arrows indicate the direction of irradiation-induced spectral changes.

HeLa cells readily incorporate TDFPPS, and images of these cells based on the TDFPPS emission suggest that the site of first localization may be lysosomes (Figure 9), much like what is observed upon incorporation of TMPyP into cells.[26] Upon irradiation of these cells at a

wavelength strongly absorbed by TDFPPS (*i.e.*, 425 nm), the images indicate that, in the least, TDFPPS tends to re-localize (Figure 9), a process that may be concomitant with morphological changes associated with cell death. This observation is likewise consistent with that observed using other dyes.[26]

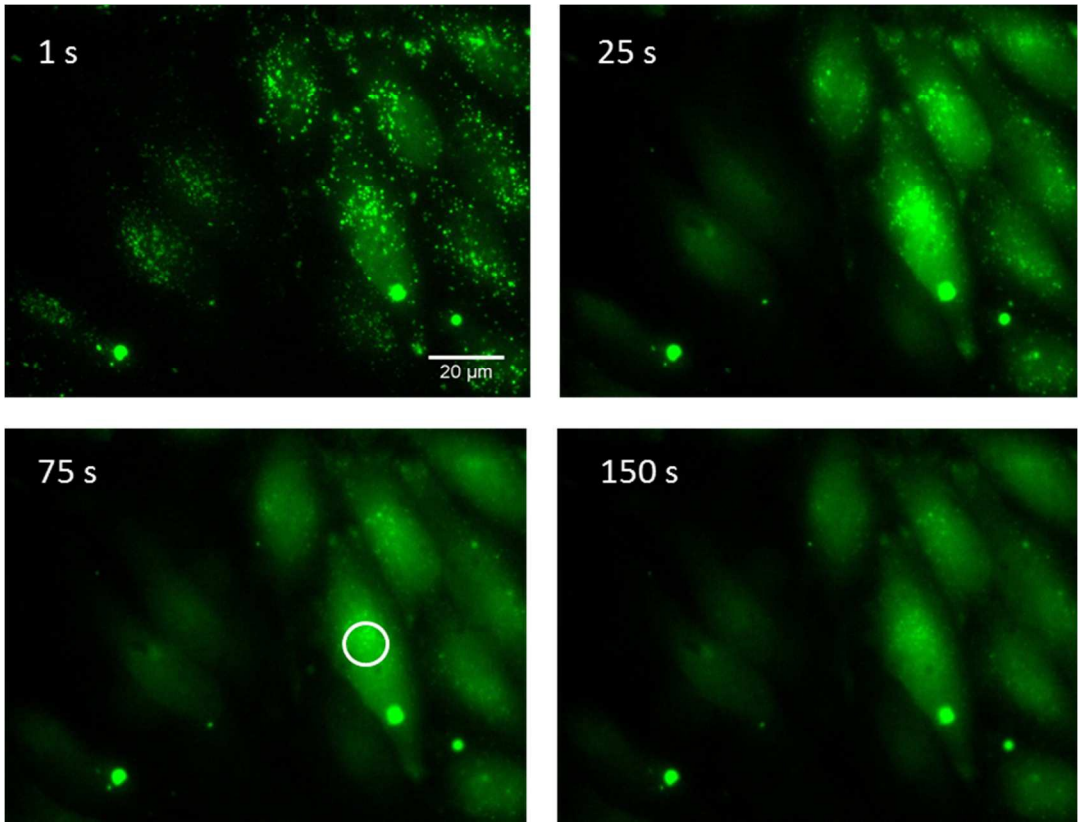

Fig. 9. Images of HeLa cells based on TDFPPS emission at 650 nm as a function of elapsed irradiation at 425 nm (44 mW/cm^2 from a *cw* metal halide lamp). The elapsed irradiation time is shown on each panel. The white circle superimposed on the third panel represents the area from which the fluorescence spectra shown in Figure 10 were recorded.

The irradiation-dependent intracellular re-localization of TDFPPS, and perhaps even the related diffusion of this dye out of the cell into the surrounding medium, makes it difficult to use

the fluorescence intensity of TDFPPS as an accurate measure of TDFPPS bleaching in a cell. However, emission spectra recorded from these cells as a function of elapsed irradiation may yield some insight in this regard. Specifically, and as shown in Figure 10, there is an apparent change in the relative intensity of two vibronic bands in the TDFPPS spectrum upon irradiation which, in turn, provides an internally-calibrated measure of a photo-initiated change in the system. Although the exact origin of this spectral change is uncertain (*e.g.*, it possibly reflects the superposition of the spectrum of a photoproduct onto the TDFPPS spectrum), this observation nevertheless allows one to better quantify the apparent photo-initiated degradation of intracellular TDFPPS. In contrast, data recorded from cells containing TMPyP, for example, indicate that re-localization of the dye in and out of the cell appears to occur much faster than a bleaching reaction (see Supporting Information as well as cell images in Silva, *et al.*[26]).

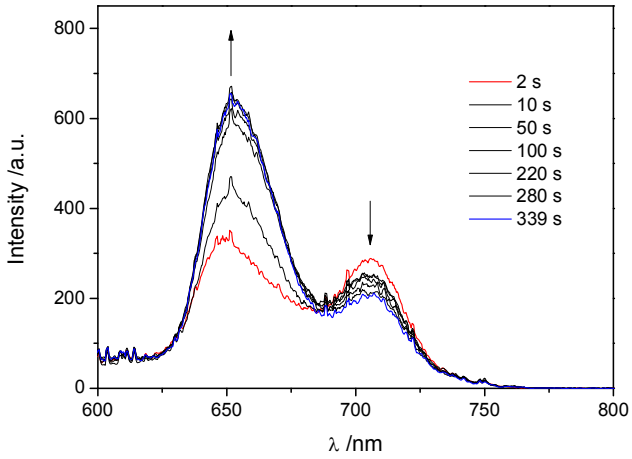

Fig. 10. Emission spectra that correspond to the data shown in Figure 9 for HeLa cells that had been incubated with TDFPPS. The spectra shown were recorded as a function of elapsed irradiation at 425 nm (44 mW/cm^2 from a *cw* metal halide lamp).

TDFPBS. To complement the TDFPPS study, we set out to examine the behavior of the corresponding bacteriochlorin, TDFPBS, well aware that, under our experimental conditions, bacteriochlorins are not expected to be as stable as porphyrins.[49, 81, 101] Indeed, the quantum yield of TDFPBS photobleaching in PBS ($\phi_{pb} = 2 \times 10^{-4}$)[101] is appreciably larger than the corresponding quantum yield for TDFPPS photobleaching in PBS (4.2×10^{-6}, *vide supra*), despite the fact that TDFPBS sensitizes the production of singlet oxygen ($\phi_{\Delta} = 0.44 \pm 0.05$)[56] less efficiently than TDFPPS ($\phi_{\Delta} = 0.71 \pm 0.09$, *vide supra*).

HeLa cells appear to readily incorporate TDFPBS, and the TDFPBS-emission-based images recorded of these cells likewise suggest that the site of first localization may be lysosomes (Figure 11). However, upon irradiation of these cells at a wavelength absorbed by TDFPBS (500 nm), it appears that events resulting in TDFPBS bleaching dominate events that result in the re-localization of this dye in the cell (Figure 11). This observation is certainly consistent with the expectation that the bacteriochlorin will be more labile than the corresponding porphyrin with respect to self-sensitized photo-oxygenation.

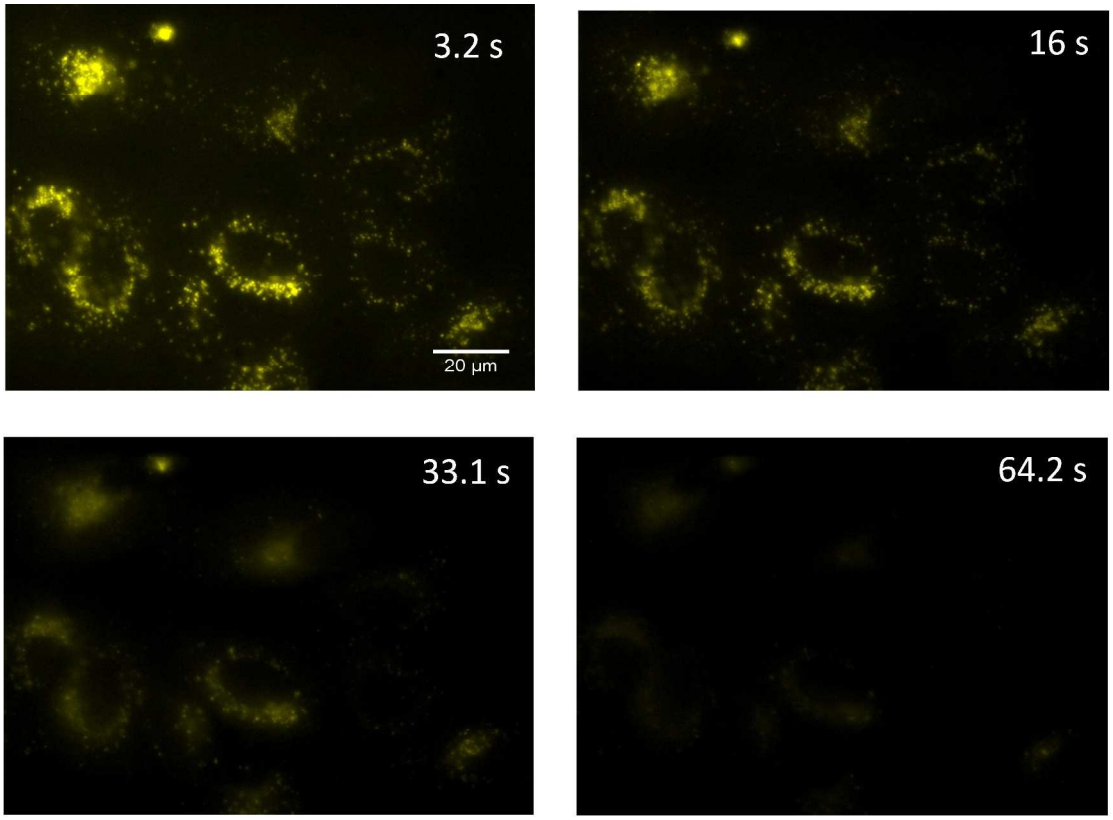

Fig. 11. Images of HeLa cells based on TDFPBS emission at 750 nm as a function of elapsed irradiation at 500 nm (4 mW/cm^2 from a *cw* metal halide lamp). The elapsed irradiation time is shown on each panel.

Emission spectra recorded from these cells containing TDFPBS (Figure 12) provide useful data that corroborate our interpretation of the irradiation-dependent phenomena observed with TDFPPS (*vide supra*, Figure 10). Specifically, the relative intensities of bands centered at 750 and 660 nm change upon irradiation in a way that is consistent with a photoinitiated reaction; in this case, the spectral data suggest that the product of TDFPBS irradiation may be a chlorin.[102]

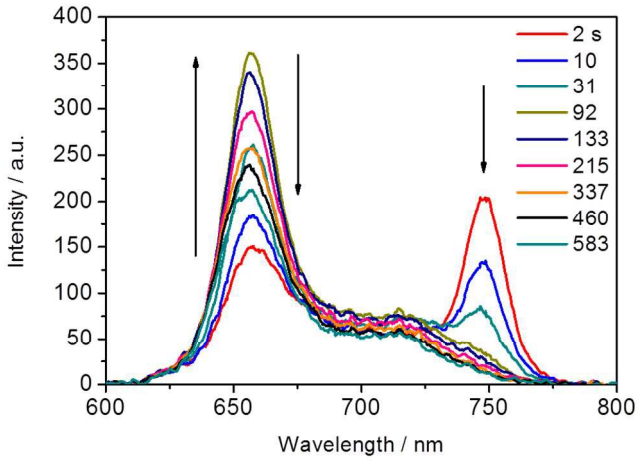

Fig. 12. Emission spectra that correspond to the data shown in Figure 11 for HeLa cells that had been incubated with TDFPBS. The spectra shown were recorded as a function of elapsed irradiation at 500 nm (4 mW/cm^2 from a *cw* metal halide lamp). Arrows indicate the direction of irradiation-induced changes in the spectra. Note that the intensity of the band at ~ 660 nm first increases and then decreases upon elapsed irradiation. This band is due to the presence of the corresponding chlorin as a "contaminant" in the TDFPBS sample, and the intensity changes reflect the initial oxidation of TDFPBS to the chlorin, followed by the slower bleaching of the chlorin.[101, 102]

FMN and protein-encased FMN. The use of enclosures to control the properties of a singlet oxygen sensitizer has several appealing features.[17] First, one can ideally maintain a constant local environment around the chromophore which, in turn, ensures that key photophysical parameters do not change over time. As a consequence of this controlled local environment, solution phase experiments will presumably better represent intracellular behavior. Second, a protein-based enclosure facilitates, through genetic engineering, the localization of the chromophore in a specific subcellular domain.[103] Finally, a protective shield around the chromophore could be used to inhibit bimolecular reactions that lead to bleaching.[31] With these

points in mind, we examined FMN dissolved in a buffered aqueous solution and, independently, a buffered solution of FMN encased in a LOV2-derived protein called "miniSOG" (for mini Singlet Oxygen Generator[76]); see Figure 1. MiniSOG has already been used as a localized intracellular sensitizer in a number of experiments.[29, 76, 104]

When considering miniSOG, or any other protein-encased sensitizer, an important caveat is that photo-induced electron transfer reactions involving the protein kinetically compete with the desired energy transfer from the sensitizer to produce singlet oxygen. This competition is indeed significant for FMN encased in the miniSOG protein;[77, 78] FMN dissolved in an aqueous medium has a comparatively large quantum yield of singlet oxygen production ($\phi_\Delta = 0.65 \pm 0.05$ in D_2O[77]), whereas the quantum yield of miniSOG-sensitized singlet oxygen production is quite small ($\phi_\Delta = 0.03 \pm 0.01$).[77, 78, 105]

With these points in mind, any attempt to use a protein enclosure to retard the bleaching of a singlet oxygen sensitizer must be complemented by the concomitant use of rational protein design, for example, to mitigate the undesired electron-transfer reactions that adversely influence singlet oxygen production. As an aside, we have addressed the latter in the development of a miniSOG mutant called SOPP (for "Singlet Oxygen Photosensitizing Protein").[77] However, in order to make salient points about the effect of a protein enclosure on sensitizer photobleaching, it is sufficient to limit ourselves here to a discussion of miniSOG.

As the elapsed period of miniSOG irradiation increases, the yield of miniSOG-sensitized singlet oxygen production correspondingly increases, reflecting protein degradation reactions that influence the competition between the electron and energy transfer reactions.[77, 78] Thus, to some extent, our current miniSOG experiments fall into the same category as our DMP-C_{60}

experiments discussed above; although the pertinent ROS initially produced may not be singlet oxygen, we can still comment on the relative effects of the encasing protein on reactions that degrade the sensitizer. Carrying this latter point further, and keeping in mind (a) the experiments of Krieg and Whitten,[63] and (b) our studies using BSA described herein, we must remain aware that oxidation of the protein enclosure could contribute to an accelerated bleaching reaction of the chromophore.

Although the miniSOG protein enclosure changes the absorption spectrum of FMN slightly (*i.e.*, vibronic structure becomes more apparent),[76, 78, 106, 107] the data in Figure 13 show that this protein enclosure also imparts stability to FMN with respect to photobleaching. Specifically, upon irradiation at 420 nm, under the relatively "mild" conditions shown in Figure 13, the change in the absorbance of the FMN band centered at ~ 450 nm is greater in an aqueous solution than in the protein. The only prominent irradiation-dependent change in the spectra of protein-encased FMN recorded under these conditions occurs at ~ 340 nm, and this likely reflects the photo-oxygenation of the tryptophan residue found in miniSOG.[108-110]

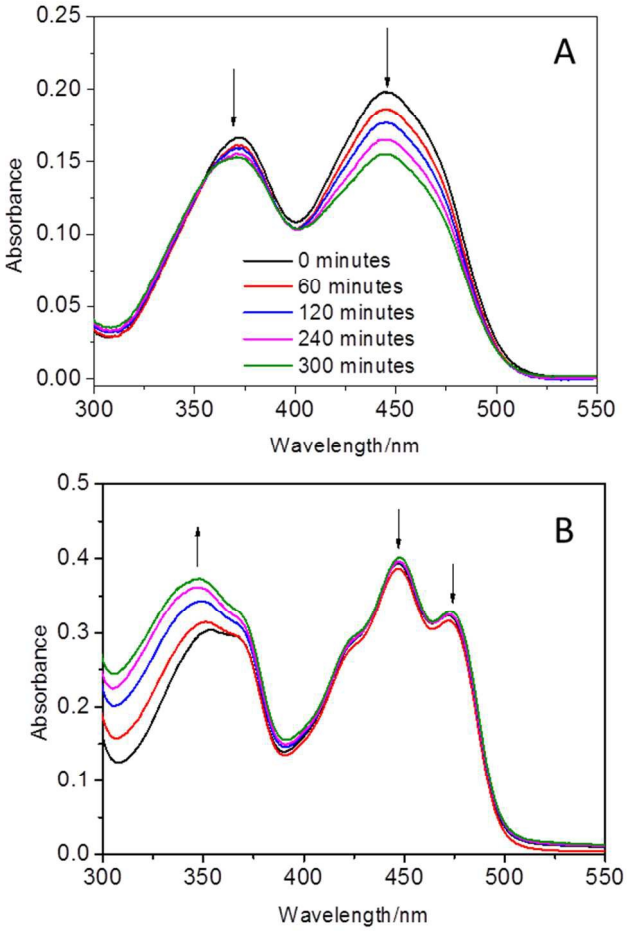

Fig. 13. (A) Absorption spectra of FMN dissolved in phosphate buffered D_2O recorded as a function of elapsed irradiation at 420 nm (*cw* Xe lamp, 0.32 mW/cm^2). A total of 1.7 J were absorbed after 300 min irradiation. (B) Absorption spectra of miniSOG recorded over the same elapsed time period and with the same actinic irradiance as used for the FMN experiments shown in panel A. Note that, even though our solution of FMN in miniSOG has a slightly higher absorbance at 420 nm, and hence absorbs a greater number of photons (*i.e.*, a total of 2.7 J were absorbed after 300 min irradiation), this protein-encased FMN still bleaches less than FMN dissolved in PBS. However, as discussed in the text, the differences in photobleaching observed here likely reflect a pronounced difference in the yield of singlet oxygen produced in these respective experiments. We previously published these data in a different form.[78]

Upon examining the data in Figure 13, however, care must be exercised not to misinterpret the effect of the protein enclosure on the degradation of FMN. Specifically, if singlet oxygen is the reactive species principally responsible for the photobleaching of FMN, then the difference between the data shown in Figures 13A and 13B most likely reflect the simple fact that singlet oxygen is produced in high yield when FMN is dissolved in water, whereas, under "mild" irradiation conditions, it is produced in low yield when FMN is encased in the miniSOG protein (*vide supra*). To support this point, we have shown that the photo-initiated spectral changes in miniSOG occur more rapidly in D_2O-based solutions than in H_2O-based solutions, suggesting that singlet oxygen is indeed the pertinent intermediate in the degradation reactions (*i.e.*, the data reflect the established fact that the lifetime of singlet oxygen is longer in D_2O than in H_2O; hence, the probability of a reaction between singlet oxygen and a given substrate is greater in a D_2O-based solution).[77] To further support our perspective, we have shown that pronounced photobleaching of FMN in the miniSOG protein can indeed be observed when one is at the limit where, upon prolonged elapsed irradiation and the absorption of appreciable energy, photoinduced changes in the surrounding protein result in a higher yield of photosensitized singlet oxygen production.[77, 78]

In conclusion, the data in Figure 13 show that chromophore/sensitizer bleaching is not always accelerated in the presence of a protein. We address this point further in the next section.

MiniSOG and added BSA. In light of the results presented in preceding sections, we examined the effect of added BSA on the rate of miniSOG photobleaching. These experiments extended into the domain where an appreciable amount of energy had been absorbed by FMN and the

quantum yield of singlet oxygen production was greater than the lower limit of $\phi_\Delta = 0.03 \pm 0.01$ obtained under "mild" irradiation conditions (*vide supra*). Recall that, in general, for a sensitizer dissolved in a given solvent, the addition of BSA accelerated the rate of sensitizer degradation/bleaching. However, data obtained using miniSOG clearly show that, when present in the surrounding solution, BSA imparts a protective effect on FMN enclosed in the miniSOG protein (Figure 14). These results imply a number of things. First, upon irradiation of FMN, any ROS produced diffuse through the miniSOG protein and interact with BSA, thereby mitigating any "back reaction" of that ROS with the miniSOG protein and FMN. BSA can certainly quench the singlet oxygen that escapes into the solution.[31] The miniSOG protein may also shield FMN from the effects of protein peroxides, for example, formed on the BSA in the surrounding solution. Any photo-produced intermediate in the miniSOG protein that contributes to FMN degradation (*e.g.*, the radical ion of a specific amino acid residue) could be trapped/quenched by the BSA in the surrounding medium. Finally, one might infer that, by adding BSA to a solution of miniSOG, one disrupts any potential miniSOG-miniSOG "aggregation-based" interactions that might contribute to FMN bleaching. With this in mind, it is important to note that, under our conditions, it is likely that miniSOG always remains as a dissolved monomeric species.[76]

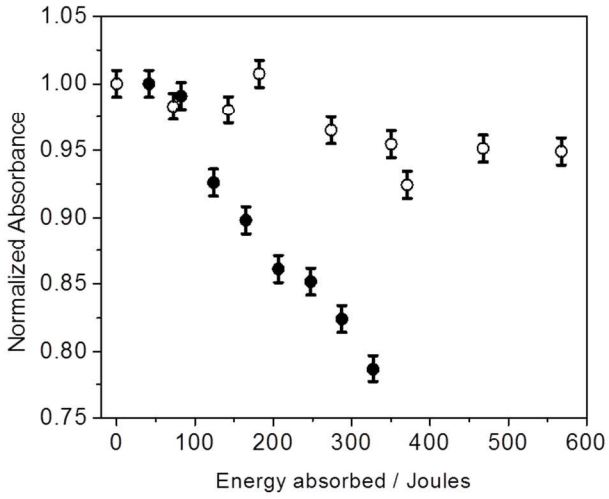

Fig. 14. Change in the absorbance of the miniSOG band at 447 nm recorded as a function of the total energy absorbed through irradiation at ~ 420 nm (1 kHz fs laser). Note that the total energy absorbed in these experiments far exceeds that for the data shown in Figure 13. Data were recorded in D_2O-based PBS in the presence of 2 mM BSA (open circles) and in the absence of BSA (filled circles). Absorbance changes in the respective data sets were normalized to facilitate comparison.

Relative photostability of miniSOG and TDFPPS. On the basis of our observations thus far, it would be useful to quantify the relative stabilities of two particular sensitizers to oxidative degradation: the fluorinated porphyrin TDFPPS and the protein-encapsulated FMN (miniSOG). Again, experiments were performed under conditions in which the total energy absorbed by FMN in miniSOG was large enough to result in a degradation-dependent increase in the singlet oxygen quantum yield above the value of $\phi_\Delta = 0.03 \pm 0.01$ recorded under "mild" irradiation conditions (*vide supra*).

The data shown in Figure 15A indicate that, under these conditions in aerated D_2O-based PBS, miniSOG is clearly less tolerant of prolonged irradiation than TDFPPS. This result speaks

well for TDFPPS because it makes singlet oxygen in high yield ($\phi_\Delta = 0.71 \pm 0.09$). However, given that these respective sensitizers respond differently to the presence of an added protein (*vide supra*), data recorded in the presence of BSA show a remarkable "inversion" effect (Figure 15B). Specifically, TDFPPS photobleaches more readily in the presence of BSA, whereas miniSOG becomes more photostable in the presence of BSA.

The data shown in Figure 15B provide compelling support for the continued development of protein-encased sensitizers for use in studies involving live cells.

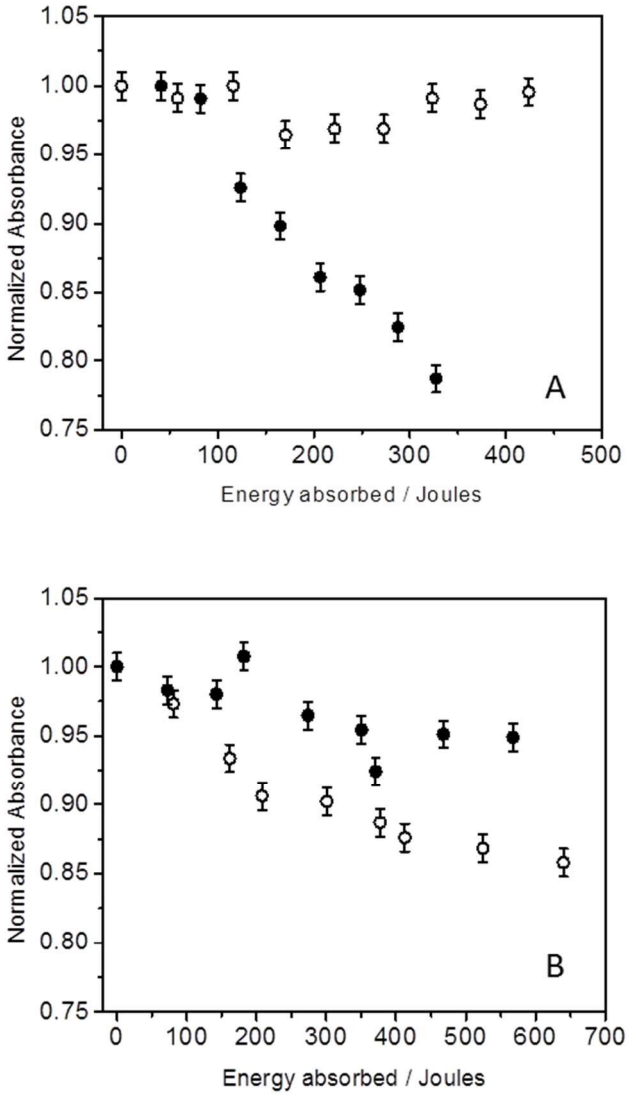

Fig. 15. Changes in the absorbance of the miniSOG band at 447 nm (filled circles) and TDFPPS at 407 nm (open circles) recorded as a function of the total energy absorbed through irradiation (~ 420 nm with a 1 kHz fs laser). (A) Data were recorded in D_2O-based PBS. (B) Data were recorded in D_2O-based PBS containing 2 mM BSA, and sample absorbance was monitored at a wavelength where any potential changes in the extent of light scattering due to BSA bleaching would not interfere (see discussion on Figure 5). In each case, absorbance changes in the respective data sets were normalized to facilitate comparison.

Conclusions

It remains challenging to use basic concepts of functional group reactivity to design a singlet oxygen sensitizer that is sufficiently stable under the photooxidative conditions currently employed to study singlet oxygen, and its effects, in mammalian cells. Reasonable predictions about the way a given sensitizer will behave in a cell can sometimes be made using model solutions (*e.g.*, solution phase experiments performed in the presence of a protein such as bovine serum albumin). However, the principal prohibitive complication that must be overcome is that the intracellular conditions to which a sensitizer is exposed present a plethora of oxidative reaction environments and reactive intermediates.

On the other hand, basic concepts of molecular architecture can be used to control encounter probabilities between a sensitizer and reactive intermediates that would otherwise contribute to a bleaching reaction. In this way a "physical" rather than "chemical" approach is exploited. Specifically, a viable solution appears to be the encapsulation of the sensitizing chromophore in a "shell" that allows singlet oxygen to escape but that also (1) precludes re-encounter between the sensitizer and singlet oxygen, and (2) inhibits collision-dependent interactions between the sensitizer and other reactive species that might be produced.

Finally, it is important to recognize that any mechanistic study of singlet oxygen behavior in a cell that relies on the photosensitized production of singlet oxygen will benefit appreciably through the use of a complementary and independent method by which this particular ROS can be selectively produced. To this end, our recent report that singlet oxygen can be produced simply by the direct excitation of oxygen at 765 nm in sensitizer-free systems[111] indicates that

yet another tool can be added to the arsenal used to elucidate the role(s) played by singlet oxygen in cell signaling.

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Electronic Supporting Information (ESI) Available: Additional absorption and emission spectra of the sensitizers and emission-based images of cells, all recorded as a function of elapsed irradiation; further details of the approach used to obtain photobleaching quantum yields.

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