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REVIEW

TRANSLATIONAL BIOPHOTONICS

Photosensitizing proteins for antibacterial photodynamic inactivation

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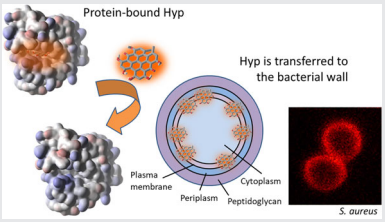
Abstract



We review recent applications of water-soluble proteins as carriers for photosensitizing molecules in antimicrobial photodynamic therapy (aPDT). The low water solubility of photosensitizers and their tendency to aggregate often impair their photophysics, thus resulting in lower bioavailability of the compounds. Taking advantage of the spontaneous interaction between water soluble proteins endowed with hydrophobic cavities and the otherwise insoluble photosensitizing compounds, it is possible to obtain efficient delivery systems to be applied inaPDT. These self-assembled structures are endowed with good photosensitizing and fluorescence emission properties (comparable to those of monomerized photosensitizers) with high biocompatibility and warrant good bioavailability of the photoactive drug. The proteins used so far comprise serum albumins, β-lactoglobulin, and apomyoglobin, while photosensitizers considered up to now have been hypericin, curcumin, Zn-protoporphyrin IX, and Zn-phthalocyanine. Furthermore, mutants of fluorescent proteins derived from GFP-like systems or from flavin binding proteins complete the array of available photosensitizing proteins for aPDT.

KEYWORDS
Photodynamic effect, singlet oxygen, drug delivery, protein carrier, fluorescence

1 | INTRODUCTION

Antimicrobial photodynamic therapy (aPDT), also termed photodynamic inactivation (PDI), is a versatile antimicrobial approach that has received renewed interest in recent years, since it relies on photooxidative effects to which microbial cells are not expected to develop resistance. The method consists in the combined use of otherwise harmless molecules (called photosensitizers, PS), visible light, and oxygen to produce reactive oxygen species (ROS; mostly singlet oxygen, $^1O_2$) that lead to cell death. [1–3]

A number of photosensitizing compounds have been developed with enhanced spectral properties, singlet

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oxygen yield, and improved targeting capabilities, tailored to specific environments. Most of these efforts, however, have been devoted to cancer applications. [4–8]

The use of photodynamic effects was recently proposed for food decontamination [9–12], exploiting photosensitizers from natural sources, such as curcumin, [13] chlorophyllin, [14] or hypericin. [11, 15] These compounds offer the advantage that they are approved as food additives. [16] Chemical modifications of the compounds allowed to address low water solubility issues. [9, 13]

Hydrophobicity of several of the existing photosensitizing molecules is a known general limitation. Low water solubility induces formation of aggregates when the PSs are dissolved in aqueous environments, a fact that impairs their photophysics and reduces their bioavailability. The above solubility problems can be circumvented by employing suitable carriers that preserve the monomeric state of the photosensitizers by embedding them within a suitable environment, such that the photophysics of the PS is fully maintained. [4–8] Photosensitizing molecules were successfully bound to several classes of water soluble compounds, [4] but only in a few cases have protein-bound photosensitizers been used against bacteria. [5–7]

Small size, water soluble proteins endowed with hydrophobic binding sites are promising candidates as biocompatible carriers for aPDT. They form spontaneously non covalent complexes with hydrophobic photosensitizing molecules of suitable size and shape, thus becoming efficient photosensitizing protein systems. Their use appears even more appealing when combined with naturally occurring PS molecules, whose use against bacteria has been reviewed. [1]

In this short review, we summarize recent work on the use of water soluble proteins (apomyoglobin, bovine and human serum albumins, and β-lactoglobulin B) as carriers for naturally occurring photosensitizing molecules (hypericin, curcumin, and flavins). [1] In addition, we also describe the use of fluorescent proteins (FPs; either mutants of proteins related to the green fluorescent protein (GFP) from *Aequorea victoria*, or mutants of LOV-domain, flavin-binding proteins), that show appreciable photosensitizing properties. At present, only applications to planktonic bacterial suspensions have been demonstrated for these molecular systems, but it is expected that the photosensitizing protein complexes will prove functional in a number of practical cases.

Given the full compatibility of the protein-based photosensitizing materials with a range of food processing materials and possibly even foodstuff, it is evident the potential interest for antibacterial photodynamic applications in food industry.

2 | GLOBULAR PROTEINS AS CARRIERS FOR PHOTOSENSITIZERS

The use of small size, water soluble protein carriers for hydrophobic photosensitizers has remarkable advantages. Besides enhancing water solubility of the PS, the protein carriers are inherently biocompatible and enjoy the benefits of a nano-metric size object, which improves bioavailability of the compound. Easily obtained water soluble proteins like apomyoglobin (ie, myoglobin where the prosthetic group heme has been removed, apoMb), β-lactoglobulin (βLG), or serum albumins (eg, human, HSA, or bovine, BSA) provide binding sites that are suitable for several hydrophobic PS. These proteins proved to be able to bind, more or less strongly, naturally occurring photosensitizers like hypericin and curcumin. A list of values for the dissociation constant (K_d) of these photosensitizers from protein-based carriers is reported in Table 1.

The main advantage of these noncovalent and mostly hydrophobic interactions is that they provide a favorable environment to keep the PS in the monomeric, photoactive state. Low polarity of the hydrophobic binding pockets and protection from the solvent are key points to increase quantum yields of $^1O_2$ photosensitization (Φ_Δ) and fluorescence emission (Φ_F), and hence to realize the full potential of the PSs.

2.1 | Hypericin, a versatile and reliable photosensitizing molecule, binds to several globular proteins

Hypericin (Hyp) is one of the most effective PS present in Nature. [17–19] Hyp was proposed for cancer PDT [20–22], and as an antiviral [23, 24], antibacterial [15, 25–29], and antifungal agent. [30]

TABLE 1 Equilibrium dissociation constants K_d values of Hyp and curcumin from protein carriers in phosphate buffer solutions at pH ~7.4

	K_d (M)	
Protein carrier	**Hypericin**	**Curcumin[a]**
apoMb[27, 77]	$(4.2 \pm 0.2) \times 10^{-6}$	1×10^{-3}
βLG[28, 104]	$(0.7 \pm 0.1) \times 10^{-6}$	1×10^{-5}
BSA[29, 77]	$(2.1 \pm 0.2) \times 10^{-5}$	2×10^{-5}
HSA[29]	$(1.1 \pm 0.5) \times 10^{-5}$	1×10^{-5}

Abbreviations: apoMb, apomyoglobin; βLG, β-lactoglobulin; BSA, bovine serum albumin; HSA, human serum albumin.

[a]Due to the limited chemical stability of curcumin in water, these values should be considered as an indicative estimate.

Hyp sensitizes singlet oxygen with high yield in organic polar solvent with $\Phi_\Delta = 0.32$ in ethanol,[31] 0.39 ± 0.01 in methanol [32], and 0.28 ± 0.01 in dimethylsulfoxide (DMSO). [33, 34] Besides being an efficient photosensitizer, the molecule is also a bright fluorophore, and emits orange fluorescence ($\lambda_{em} = 600$ nm) with $\Phi_F = 0.35$ in ethanol [19, 35] and in DMSO [36].

Hyp is not soluble in water, where it forms aggregates that lose the above photophysical properties [37]. Thus, suitable carriers have to be employed, in order to preserve the photosensitizing ability of the compound in aqueous environments. Hyp was shown to bind to several proteins, thereby preventing the aggregation of the PS in aqueous media [38–42]. We have explored the use of a few proteins, such as apoMb [27, 33, 43], β-lactoglobulin [28, 44], and serum albumins [29] as carriers to deliver Hyp to bacterial cells for aPDT.

2.1.1 | Apomyoglobin

Myoglobin (153 amino acids, ~17 kDa) is a promising transport system, which is easily available in large quantity as it is readily purified from heart or skeletal muscles (eg, from equine), or expressed as recombinant protein (in the case of human myoglobin). The protein holds the cofactor heme (Fe-protoporphyrin IX) inside a mostly hydrophobic cavity, partly shielded from the solvent, and exposing the carboxylic acids. The Fe ion coordinates the N atom of a His residue (usually referred to as proximal His) that provides stabilization to the cofactor. When the heme is removed through standard biochemical procedures, [45] the apo-protein (apoMb) is left with a cavity which can bind several hydrophobic molecules.

Hyp binds to apoMb with moderate affinity (dissociation constant $K_d = 4.2 \pm 0.8$ μM) and is accommodated in the heme cavity. It is important to note that no binding is observed to myoglobin, where the inner cavity is occupied by the heme [27]. Computer modeling confirmed the theoretical feasibility of the binding. [27] The nature of the binding site provides a very good environment to preserve the photophysics of Hyp. When bound to apoMb, Hyp is characterized by relatively high singlet oxygen ($\Phi_\Delta = 0.14 \pm 0.03$)), triplet ($\Phi_T = 0.13 \pm 0.04$), [46] and fluorescence ($\Phi_F = 0.14 \pm 0.02$) yields [33]. Additional proofs of binding to the protein come from fluorescence anisotropy. When dissolved in DMSO, the fluorescence anisotropy is negligible across the whole excitation spectrum, due to the fast rotational averaging of the molecule. The anisotropy becomes appreciable for the complex with apoMb, with anisotropy on the order of 0.25 in the 550 to 600 nm range, and a negative value (~ −0.1) between 400 and 475 nm [33]. Due to the larger size of this complex, rotational diffusion is slower and averaging is consequently incomplete. Finally, complex formation is easily detectable by fluorescence correlation spectroscopy (FCS), a technique that allows the direct determination of diffusion coefficients of fluorescent species. The diffusion coefficient of the complex between Hyp and apoMb is $D = 120 \pm 20$ μm^2 s^{-1} [33], coincident with the one of myoglobin, an indication that Hyp is bound to the protein.

From a photodynamic point of view, the complex is effective against Gram-positive *Staphylococcus aureus*, leading to a reduction in CFU of up to 6 to 8 log units for light fluence above 15 J cm^{-2} and concentration of 1 to 10 μM. A lower efficiency was observed against Gram-positive *Bacillus subtilis* (3 log units), while no reduction in CFU was evident for Gram-negative *Escherichia coli* [27, 29, 33]. These results are consistent with the differences in cell wall structure between Gram-positive and Gram-negative bacteria, which contain a double membrane in the latter strain that confers a higher resistance to photooxidative damage [47]. Since it is a constituent of muscles, apoMb is a good candidate as a transport system for industrial applications in meat handling and processing factories.

2.1.2 | β-lactoglobulin

βLG is a small, homodimeric protein (162 amino acids, ~18.4 kDa), member of the lipocalin family [48, 49]. βLG it is the most abundant protein in the whey of cow's milk. [50] The protein was suggested as a candidate for drug delivery as it can bind small hydrophobic ligands and can be recognized by surface receptors [48]. While some lipocalins show very high specificity toward ligands, βLG can accommodate different types of molecules in its binding sites, with dissociation constants around 1 μM. Binding of Hyp to βLG was demonstrated using fluorescence spectroscopy and FCS [28].

Unlike the case of apoMb, multiple Hyp binding sites are predicted for βLG. Theoretical analysis of the docking poses suggested binding of Hyp, mostly to a narrow cleft at the dimer interface, energetically more favorable than binding to a wide cleft, also located within the dimer contact areas [28]. The small separation between the walls of the narrow cleft allows to occlude Hyp from the solvent. On the contrary, binding to the second, wider cleft leaves one of the two faces of Hyp exposed to the solvent. The advantage of this transport system is that more than one copy of the photosensitizer is carried by the same protein, although the spectral properties of the bound Hyp indicate incomplete protection from the solvent.

The efficiency of the Hyp-βLG complex in bacterial photoinactivation was tested against Gram-positive *S aureus* and compared to the efficiency of lone Hyp. No dark

toxicity was observed for the complex, and a 5 to 6 logs decrease in CFU was observed, similar to the one recorded for the pure Hyp. Although the bacterial phototoxicity of the protein bound Hyp was similar to that of the lone compound, a major advantage of the complex is the possibility of administering it using a buffered aqueous solution. The full compatibility of the protein carrier with dairy industry processes prompts for potential applications as an effective disinfectant for food manufacturing and handling materials for these industrial activities, including milking.

In spite of the encouraging performance of the complex Hyp-βLG as a photosensitizing agent, the spectral properties and the low fluorescence ($\Phi_F = 0.03 \pm 0.01$), triplet ($\Phi_T = 0.050 \pm 0.002$), and singlet oxygen ($\Phi_\Delta = 0.065 \pm 0.010$) quantum yields point to less than ideal environmental conditions for the binding site(s) of Hyp. In particular, the absorption spectrum suggests that the molecule is not fully monomeric when bound to the protein, a fact that may result in lower photoactivity.

In an attempt to improve the photophysical properties of the bound Hyp, DMSO, known to coat βLG, [51] was used as a cosolvent to provide a better environment to the PS [44].

Indeed, the results confirm that DMSO leads to an improvement in the fluorescence ($\Phi_F = 0.06 \pm 0.01$) and singlet oxygen ($\Phi_\Delta = 0.12 \pm 0.05$) yields of βLG bound Hyp.

Nevertheless, in the presence of 20 % DMSO the complex Hyp-βLG turned out to be less efficient in the photoinativation of *S aureus*, for which a 3 (instead of 6) logs decrease in CFU was obtained. This was attributed to a change in the interaction of the βLG bound Hyp and the cellular constituents, which hampers the capability of the PS to reach more photosensitive regions within the bacterial wall. The apparently contradictory result points out that higher $^1O_2$ photosensitization efficiency may not necessarily result in higher photodynamic efficiency against bacteria.

2.1.3 | Serum albumins

The interaction of Hyp with serum albumins, the most abundant plasma protein, has been reported in connection to the development of photosensitizing agents appropriate for tumor or antiviral PDT [52, 53].

Serum albumins are known to bind a variety of endogenous molecules as well as drugs [54, 55]. In particular, human serum albumin (HSA), which is the most important plasma protein, binds different classes of ligands to different sites that can influence the pharmacokinetics of many drugs and tune the reactivity of bound compounds. At the same time, HSA can act as a vector in the mechanism of elimination of potentially harmful molecules [56].

Mechanistic experiments, based on competition binding and Resonance Raman and surface-enhanced Raman spectroscopy, proposed that Hyp binds to a site located in the IIA subdomain of the protein [39, 57, 58]. The use of vibrational spectroscopies and molecular modeling allowed to draw a structural model for the binding site and identify the main interactions with amino acid residues for BSA and HSA.[59] The HSA-bound Hyp was reportedly stable against photobleaching [60]. Binding of Hyp to serum albumins is confirmed by fluorescence anisotropy and FCS experiments. The dissociation constants from HSA and BSA are in the order of 10 μM [29].

The binding sites of HSA and BSA preserve the photophysics of Hyp, and fluorescence emission yield is $\Phi_F = 0.11$ for Hyp-BSA and $\Phi_F = 0.12$ for Hyp-HSA, comparable to the one observed for apoMb. Similarly, the triplet yields for Hyp-BSA ($\Phi_T = 0.13$) and Hyp-HSA ($\Phi_F = 0.10$) indicate a favorable environment for the bound Hyp.

Given the above promising photophysical properties, Hyp bound to BSA and HSA were tested as photosensitizing agents against *S aureus* [29]. The compounds were able to induce 8-logs (Hyp-HSA) or 6-logs (Hyp-BSA) decrease in CFU.

While further studies are needed to evaluate the efficacy of Hyp-BSA and Hyp-HSA on contaminated food processing material to assess their industrial applicability, we wish to emphasize that these materials have some inherent important advantages in view of their nature. Proteins like albumins, which are natural constituents of food of animal origin, are not considered food additives by Regulation EC N° 1333/2008 [61]. Moreover, albumin exhibits excellent gelling and water binding capacity and it is often used to improve texture, sliceability, and yield losses of processed meat products [62].

2.2 | Hypericin is a fluorescent reporter suitable for super-resolution microscopy

The relatively intense fluorescence emission by Hyp when embedded in proteins like apoMb (Φ_F ~0.15) has been exploited to track the uptake of the PS by bacteria. Moreover, Delcanale et al. showed that Hyp-apoMb undergoes stimulated emission when excited in the near IR with femtosecond laser pulses [33]. This property was exploited to collect STimulated Emission Depletion (STED) nanoscopy images of bacteria loaded with Hyp-apoMb, thus allowing a precise sub-diffraction localization of the PS in living cells [33]. The fluorescence emission of Gram-positive (*S aureus* and *B subtilis*) and Gram-negative (*E coli*) bacteria treated with Hyp or Hyp-

apoMb came clearly from the wall and not from the cytoplasm. It was proposed that the compound is localized in the membrane within the wall. In spite of the similar location, the photoinactivation capability of the compound was very different for the Gram-positive *S aureus* and the Gram-negative *E coli*, again possibly due to the presence of a double membrane in the latter strain.

Similar results were obtained when BSA bound Hyp was applied to *S aureus*, indicating that the albumin carrier is not hampering the photophysical properties of Hyp at the basis of the STED nanoscopy [29].

2.3 | Binding to proteins protects curcumin against oxidation

Besides providing improved photophysics, protection of the bound PS from the solvent may also result in enhanced chemical stability of the compound. This is the case for curcumin, one of the most exploited photosensitizers for aPDT, [9, 10, 13] especially for the treatments of oral diseases [63]. Advantages of the compound include ready availability, low cost, efficacy against several kinds of microorganisms and negligible dark toxicity.[64] However, the known instability of curcumin in aqueous environments severely limits its applicability [65]. Curcumin rapidly degrades in aqueous buffers at alkaline or physiological pH also in the dark, [66–68] and photo-degradation has been reported under UV irradiation [69, 70]. To overcome this issue, carriers have been proposed to improve water solubility, and prevent interaction with the solvent.

The delivery systems include liposomes, [71] micelles, [72] nanoparticles, [73] and cyclodextrins [74]. Proteins like albumins, fibrinogen or apoMb have been also reported [65, 71, 75–77]. The enhancement in curcumin stability is dependent on the specific protein. While apoMb preserves the spectral properties of curcumin for tens of minutes, bovine serum albumin is able to keep curcumin fully functional for several hours.[77] This enabled PDT studies on HeLa cells, where the BSA-bound curcumin demonstrated a higher phototoxicity than curcumin alone [77]. Applications of the complex between albumin and curcumin in aPDT have not been reported yet.

2.4 | Proteins can act as passive carriers and deliver their cargo to the bacterial wall

The PS molecules discussed above show a relatively weak interaction with the hydrophobic protein cavities of apoMb, HSA, BSA, or βLG. The dissociation constants of Hyp show values in the order of 1 to 10 μM, [27–29, 44] while curcumin has even lower affinity, with a dissociation constant from apoMb and BSA in the order of 100 μM [77]. This property may limit the usefulness of these particular complexes for systemic administration, since Hyp can translocate to other abundant proteins like, for example, serum proteins, or the lipid phases of cellular membranes in real biological systems. Transfer from the protein carrier to the bacterial wall was demonstrated using FCS and a two-color detection system [29]. Hyp was bound to BSA labeled with FITC, and FCS signals in the green (from the BSA bound FITC) and in the red (from Hyp) were collected.

Analysis of the autocorrelation function for the two separate colors for Hyp bound to FITC-BSA provided the same diffusion coefficient for the fluorescence emitting species (Hyp in the red and FITC in the green), which was coincident with that of BSA (D~60 $\mu m^2\ s^{-1}$).

When in the presence of bacteria, the fluorescence intensity time-traces (MCS traces) monitored in the red showed large intensity peaks. The corresponding MCS trace for the green emission was totally devoid of the large spikes. The autocorrelation function for the green emission revealed a diffusion coefficient for FITC typical of BSA (D~60 $\mu m^2\ s^{-1}$), showing that the protein is not bound to the bacteria. On the contrary, analysis of the autocorrelation function for the red emission afforded a diffusion coefficient D~0.3 $\mu m^2\ s^{-1}$, corresponding to objects the size of bacteria (~700 nm), indicating that Hyp was associated with the bacteria. STED nanoscopy allowed localizing the position of Hyp on the bacterial wall [29]. Figure 1 shows schematically the mechanism of Hyp transport by apoMb.

With a similar approach, transfer of Hyp from apoMb to the plasma membrane was demonstrated with confocal microscopy. ApoMb was labeled with FITC and bound to Hyp. After incubation with HeLa cells, red emission from Hyp was detected from the cellular structure whereas the green emission from FITC only came from the surrounding medium, indicating that the protein did not interact with the cellular structure and released the PS in the presence of the lipid phase [78].

A general conclusion that can be drawn is that PS transported by water soluble proteins through noncovalent interactions are eventually downloaded to the cellular components for which the PS has higher affinity, either for their chemical nature or for their large concentration. The scenario may be different if the protein carrier has specific interactions with cellular components.

In light of the above discussed mechanisms of interaction between the PS loaded proteins and bacteria, it is interesting to discuss a related photosensitizing system obtained by reconstituting apoMb with Zn protoporphyrin IX (Zn Mb). This system was recently proposed by

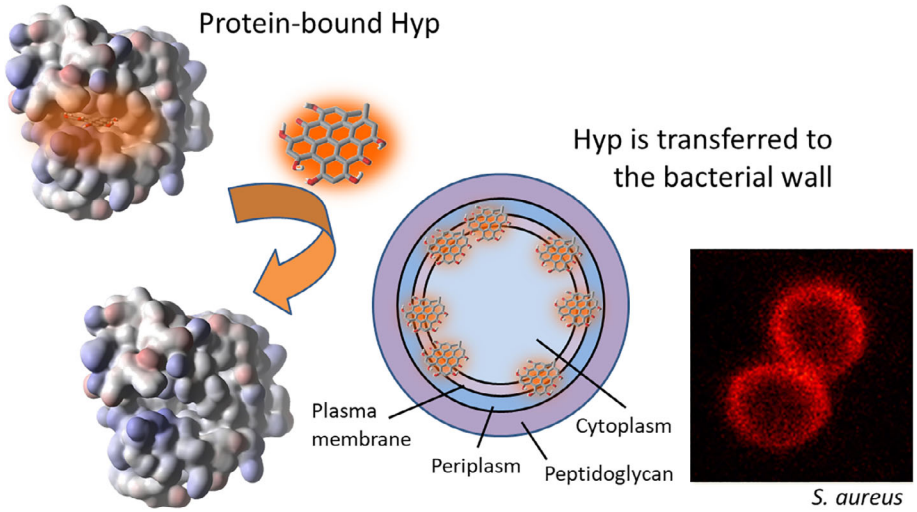

FIGURE 1 The PS Hyp is transported by apoMb through non covalent interactions. In the presence of bacteria, Hyp is released by apoMb and is bound to the plasma membrane in the bacterial wall (central scheme). The image of *S aureus* (on the right) loaded with Hyp-apoMb was collected with STED nanoscopy. [33] The fluorescence from Hyp is only observed on the bacterial wall

Lepeshkevich and coworkers [79] as a biocompatible photosensitizer with an exceptionally high singlet oxygen yield ($\Phi_\Delta = 0.9 \pm 0.1$) and moderate fluorescence emission. Zn-Mb was applied to *S aureus* suspensions and turned out to be effective in the photoinactivation of the bacteria [43]. However, spectroscopy and fluorescence imaging revealed that Zn-Mb undergoes a very weak association with bacteria, possibly because no specific interaction exists between the protein and the bacterial wall. Unlike the case of self-assembled, noncovalent complexes between proteins and PS molecules, Zn protoporphyrin IX is expected to remain more tightly bound to apoMb. Thus, transfer of the PS molecule to the bacterial membrane is not likely to occur, which may result in lower photoinactivation efficiency.

3 | GENETICALLY ENCODABLE PHOTOSENSITIZERS

3.1 | Fluorescent proteins (FPs)

Although proteins belonging to the GFP family are mostly known for their fluorescence emission properties, it is by now well established that several variants undergo photochemical reactions, the most notable of which is the *cis-trans* photo-isomerization of the chromophore [80]. These photochromic FPs have largely contributed to the development and application in super-resolution microscopies such as FPALM [81] and RESOLFT [82].

The autocatalytically formed amino acid-based 4-hydroxybenzylidene-5-imidazolinone (HBDI) in some GFP mutants (like the E222Q mutant) is fluorescent in its *cis* state while it is dark in *trans* [83]. Thus, the E222Q substitution can restore the intrinsic photochromic behavior of the isolated HBDI chromophore [84]. The optical properties of a FP are not only determined by the inherent photochemistry of the chromophore, but also by the interactions it establishes with the protein residues in its vicinity, which finely tune the fluorescent properties observed. Some mutants have proved that even the "on-off" states of a FP can be inverted by only modifying the chromophore's surroundings [80].

FP mutants have not been limited only to surrounding modifications, but also of the chromophore itself, yielding to an expansion of the FP toolbox to all colors of the spectra [85].

It is much less known that several of these proteins generate ROS, although generally the yield of this de-excitation pathway is much less efficient than the fluorescence emission one. In fact, many FP mutants are discarded due to excess cytotoxicity due to ROS. Jiménez-Banzo et al. [86] showed that the GFP chromophore is able to photosensitize singlet oxygen with an appreciable singlet oxygen yield both in solution ($\Phi_\Delta = 0.003 \pm 0.001$) and encased in the protein, (eg, $\Phi_\Delta = 0.004 \pm 0.001$ for enhanced-GFP). Variants of the GFP family have since been explored and the role of oxygen access has been elucidated [86, 87] Using TagRFP, Ruiz-González et al. were able to demonstrate killing of bacteria by purely endogenous singlet oxygen [88]. The variant "KillerRED" [89, 90] showed further improved photosensitizing properties. Bulina et al. reported an inactivation cell death of *E coli*-transformed bacteria with "KillerRed" greater than 3-log (>99.9%) after 216 J cm^{-2} of green light illumination. Studies performed further on demonstrated that this protein mainly actuates via electron-transfer mechanism (Type I, via radical reaction) [91].

However, to date, the yield of these GFPs remains much lower than that observed for most popular organic photosensitizers.

3.2 | Flavin binding proteins

The scenario changed when R. Tsien proposed to use a LOV domain, flavin binding protein, to develop a photosensitizing protein that he named "mini Singlet Oxygen Generator" (miniSOG). [92] miniSOG is a fluorescent flavoprotein engineered from *Arabidopsis thaliana* phototropin 2 with a much smaller size than GFPs and an improved singlet oxygen yield ($\Phi_\Delta = 0.03 \pm 0.01$) [93, 94]. Thanks to the mutations introduced, fundamentally by substituting the cysteine involved in the LOV cycle, the native photochemistry of the flavin is inhibited and generation of ROS facilitated. The goal for which, at first, miniSOG was developed was for correlated light and electron microscopy (CLEM) in order to generate an osmiophilic polymer inside cell structures by oxidizing diaminobenzidine by photosensitized singlet oxygen.

After this seminal work, several contributions have led to improved singlet oxygen yields for flavin binding protein variants. In 2014, Torra et al. reported the photochemical behavior of the FP mutant Pp2FbFP, extracted from *Pseudomonas putida* [95]. In this case, not only one, but two excited triplet states were formed due to the presence of, mainly, two different flavin-derived prosthetic groups (FMN and flavin adenine dinucleotide [FAD]), resulting in an overall $\Phi_\Delta = 0.09 \pm 0.01$, being threefold than that of miniSOG. Later on, through a systematic and rational approach involving mutations to a LOV2 protein that binds the chromophore flavin mononucleotide (FMN), Westberg et al. were able to obtain a "singlet oxygen photosensitizing protein" (SOPP) with $\Phi_\Delta = 0.25 \pm 0.03$ [96]. Further mutations were introduced, rendering SOPP3, which presented a $\Phi_\Delta = 0.61 \pm 0.06$ in D_2O-based phosphate buffer saline, a value comparable to the one observed for the FMN chromophore in solution [97].

The initial microscopic approach has expanded into using these proteins to induce photo-oxidative damage to bacterial cells which have been transformed with the encoding plasmid. To prove this concept, Ruiz-Gonzalez et al. hinted in this direction publishing the inactivation of *E coli*-expressing miniSOG in 2013, inducing more than 5-log cell death with 70 J cm^{-2} of blue light while leaving unharmed *E coli* overexpressing TagRFP under green light [93]. In 2018, Endres et al. compared eleven LOV-based photosensitizing proteins expressed in *E coli* and found that 23 J cm^{-2} of blue light inactivated up to 2-log of the initial microbial population. Each different construct presented different capabilities of generating both singlet oxygen and hydrogen peroxide, yields of which at first did not correlate with the cell death induced to bacteria, but was finally achieved by correcting for the bleaching of some of the FPs [98]. Being miniSOG the first genetically-encoded LOV-based FP, its Φ_Δ is far from that of other improved mutants, but it is still capable of photoinactivating bacteria to a large extent. This mismatch between the quantum yield and the inactivation outcome has been recently explained as being due to the phototransformation of the protein, namely of the proteic residues and of the FMN chromophore, forming lumichrome, which favors oxygen quenching of the flavin triplet state and thus has a tenfold larger Φ_Δ than the native protein [99].

Furthermore, and most recently published, Hilgers et al. studied seven other GFP and LOV derived FPs, including DsFbFp, SOPP3, KillerOrange, and SuperNova, as genetically encoded photosensitizers against different Gram-positive and Gram-negative bacteria [98, 100]. FPs genetically encoded or externally added to the system resulted in the inactivation of both Gram-positive and negative bacteria, for which the internally produced PS needed less amount of light than the other approach since ROS are produced directly inside its target. In order to improve the performance of the exogenously applied FPs, DsFbFp was fused to lectin LecB (which specifically binds to *P aeruginosa* sugars' on the outer wall), externally added to a *P aeruginosa* culture and demonstrated an enhancement in the cell death induced in comparison with the FP lacking the targeting agent. Another important conclusion met is that LOV derived FPs inactivate bacteria more efficiently than one derived from GFP.

The flavin-based photosensitizing proteins further confirmed their superior performance as photosensitizing systems over GFP derived proteins also for the recently reported variant of the protein Mr4511 from *Methylobacterium radiotolerans*, for which the single mutation of the reactive cysteine responsible for the photocycle (Cys71) in the native protein results in a $\Phi_\Delta = 0.20$ [101]. Also, this novel LOV derivative

TABLE 2 Summary of Φ_Δ for genetically-encoded protein photosensitizers

Genetically encodable PS		Φ_Δ
Fluorescent proteins	GFP	0.003 ± 0.001[87]
	EGFP	0.004 ± 0.001[87]
	Killer Red	Radical reactions[91]
Flavin binding proteins	miniSOG	0.03 ± 0.01[93, 94]
	Pp2FbFP	0.09 ± 0.01[95]
	SOPP	0.25 ± 0.03[96]
	SOPP3	0.61 ± 0.06[a][97]
	Mr4511	0.20[101]

Abbreviations: EGFP, enhanced GFP; FbFP, FMN-binding fluorescent protein; GFP, green fluorescent protein; miniSOG, mini-singlet oxygen generator; SOPP, singlet oxygen photosensitizing protein.
[a]Obtained in D_2O-based phosphate buffer saline.

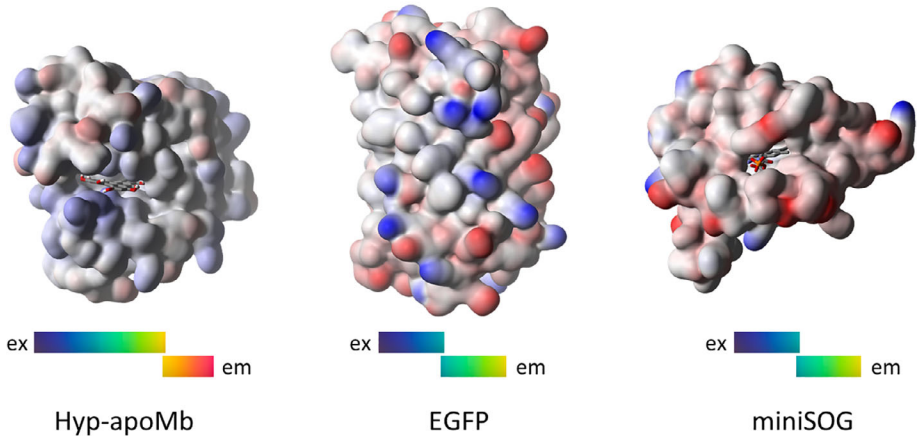

FIGURE 2 Representative structures (solvent accessible surface) of representative cases for the reviewed photosensitizing proteins. The color code in the protein surface has the following meaning: negatively charged groups are represented in red, positively charged groups are represented in blue, neutral groups are represented in white. The three dimensional model of Hyp-apoMb (left) shows that Hyp is docked inside the hydrophobic cavity normally hosting the heme. [27] The chromophore is in connection with the solvent to some extent, therefore oxygen can diffuse rather easily inside the cavity. In the case of EGFP (center), the chromophore is completely buried in the three dimensional structure (2y0g) and is almost inaccessible to the dissolved oxygen. The FMN chromphore in miniSOG (6gpu, right) is exposed to the solvent to some extent. The bottom colored bars represent qualitative excitation (left) and emission (right) spectral ranges

Mr4511 is endowed with a larger stability against denaturation in harsh urea 7.8 M conditions than other analogous proteins.

Reported values for Φ_Δ of the main genetically encodable photosensitizers are summarized in Table.2. Representative chromophore-structure arrangements of the photosensitizing proteins reviewed in this manuscript are compared in Figure 2.

It is worth pointing out that in the case of mutants of GFP-like proteins or flavin-binding proteins, the chromophore responsible for the photosensitization is either covalently or very tightly bound to the protein. Thus, no partitioning occurs to other molecular species or supramolecular structures, as discussed for Hyp.

The production of ROS affects a number of intracellular processes, but the details of these interactions are yet to be understood. It is expected that the availability of genetically encoded photosensitizers will provide the proper tools to probe the effect of ROS on a specific intracellular domain or molecular component [102].

4 | CONCLUSIONS

Small size, water-soluble globular proteins are effective delivery vehicles for otherwise insoluble photosensitizing compounds of natural origin and can be considered an effective approach to phototreatment of contaminated materials to remove microbial contamination.

These transport systems have several advantages that include the ease of assembly, full biocompatibility of formulations, and ease of removal of residual photosensitizing material after treatment. Moreover, specific carrier proteins can be selected to match requirements of the target environment, for instance βLG may be employed in dairy industry and myoglobin may find application in meat handling processes.

In general, self-assembled, PS-protein complexes dissociate in the presence of bacteria as the hydrophobic PS compounds are bound by the membranes in the bacterial wall. This appears as a generally applicable mechanism to all bacteria, although photoinactivation is observed only for Gram-positive ones.

The photophysical properties of Hyp bound to apoMb, βLG, and serum albumins make these photosensitizing structures the most effective and reliable systems against Gram-positive bacteria, among those tested so far. The protein of choice may be selected to meet specific environment requirements.

The efficacy of curcumin with similar protein-based transport systems remains poorly explored, even though the remarkable improvement of the chemical stability of this PS opens promising perspectives. Genetically-encoded PS are generally characterized by lower photosensitization yields and rational mutations are required for the effective application of these systems in antimicrobial applications. However, they are intrinsically water-soluble and can be directly expressed at intra-cellular level by bacteria, overcoming potential issues related to the stability of exogenous protein-PS systems in complex environments. So far, flavin binding proteins showed the best photosensitizing properties, obtained with just a few mutations, even though their

application as photo-antibacterial agents remains nearly unexplored.

Finally, beyond bacterial applications, it is worth mentioning that within the recently developed nanostructured drugs, a number of the approved compounds exploits proteins either as carriers or as targeting species [103]. In addition to their water solubility and biocompatibility, small size proteins can be extremely effective in systemic administration thanks to their stability, their lack of toxicity and the capacity of extravasation.

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CONFLICT OF INTEREST

There are no conflict to declare.

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