Enhanced photosensitizing properties of protein bound curcumin

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Marco Cozzolino[1,2,3,&], Pietro Delcanale[4,&], Chiara Montali[5], Massimiliano Tognolini*[6], Carmine Giorgio[6], Miriam Corrado[6], Luigi Cavanna[7], Paolo Bianchini[1,2], Alberto Diaspro[1,2,3], Stefania Abbruzzetti*[5], Cristiano Viappiani[5]

[1]Nanoscopy, Istituto Italiano di Tecnologia, via Morego 30, Genoa 16163, Italy

[2] Nikon Imaging Center, Istituto Italiano di Tecnologia, via Morego 30, Genoa 16163, Italy

[3] Department of Physics, University of Genoa, via Dodecaneso 33, Genoa 16146, Italy.

[4] Institute for Bioengineering of Catalonia (IBEC), the Barcelona Institute of Science and Technology (BIST), Barcelona, Spain

[5] Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco area delle Scienze 7/A, 43124 Parma, Italy

[6] Dipartimento di Scienze degli Alimenti e del Farmaco, Università di Parma, Parco area delle Scienze 27/A, 43124 Parma, Italy

[7] Dipartimento di oncologia-ematologia. Azienda USL di Piacenza. Via Taverna, 49, 29121 Piacenza, Italy

[&] These authors contributed equally to this work.

*Corresponding authors:

Stefania Abbruzzetti Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università di Parma, Parco area delle Scienze 7/A, 43124 Parma, Italy, email: stefania.abbruzzetti@unipr.it

Massimiliano Tognolini Dipartimento di Scienze degli Alimenti e del Farmaco, Università di Parma, Parco area delle Scienze 27/A, 43124 Parma, Italy email: massimiliano.tognolini@unipr.it

**Abstract**

The naturally occurring compound curcumin has been proposed for a number of pharmacological applications. In spite of the promising chemotherapeutic properties of the molecule, the use of curcumin has been largely limited by its chemical instability in water. In this work, we propose the use of water soluble proteins to overcome this issues in perspective applications to photodynamic therapy of tumors. Bovine serum albumin binds curcumin with moderate affinity and solubilizes the hydrophobic compound preserving its photophysical properties for several hours. Cell viability assays demonstrate that when bound to serum albumin, curcumin is an effective photosensitizer for HeLa cells, with better performance than curcumin alone. Confocal fluorescence imaging reveals that when curcumin is delivered alone, it preferentially associates with mitochondria, whereas curcumin bound to bovine serum albumin is found in additional locations within the cell, a fact that may be related to the higher phototoxicity observed in this case. The higher bioavailability of the photosensitizing compound curcumin when bound to serum albumin may be exploited to increase the efficiency of the drug in photodynamic therapy of tumors.

43 List of Abbreviations

44 apoMb, apomyoglobin

45 BSA, Bovine Serum Albumin

46 DMSO, dimethyl sulfoxide

47 FWHM, full width at half maximum

48 HSA, Human Serum Albumin

49 Hyp, Hypericin

50 LED, light emitting diode

51 Mb, Horse heart myoglobin

52 MTT, Dimethyl thiazolyl diphenyl tetrazolium

53 PBS, Phosphate Buffered Saline

54 PDT, photodynamic therapy

55 PS, photosensitizer

56

## Introduction

Curcumin is a naturally-occurring pigment found in the root turmeric or *Curcuma longa*. Turmeric is a wide-spread spice, typical of Indian cooking, that also found use in traditional Indian and Chinese medicine. Curcumin is the main responsible for the yellow/orange color of this spice and it is widely studied for its therapeutic potential in the treatment of a large number of diseases. [1] Curcumin has been used in traditional medicine for the treatment of several respiratory disorders and certain tumors. [2, 3] The compound is reported to modulate a variety of signaling molecules. [4] Although a number of preclinical and clinical studies have indicated the chemotherapeutic potential of curcumin, also in cancer, [5-7] its use remains controversial. Neither curcumin nor curcumin derivatives appear to possess the properties required for a good drug candidate since they have poor chemical stability, low water solubility, low absorption from the gut, high intestinal degradation, fast metabolism and rapid plasma elimination, finally resulting in poor bioavailability and low toxicity (it was “generally recognized as safe” by FDA). [8]

Curcumin is a relatively hydrophobic molecule, readily soluble in organic solvents like ethanol, methanol, or DMSO. Its photophysical properties are strongly dependent on the characteristics of the solvent (polarity, H-bond donating or accepting properties, and pH). Curcumin usually displays an absorption band around 420 nm and a large Stokes shift, with fluorescence emission in the 450-550 nm region of the spectrum. [9] The decay of the excited state preferentially occurs with non-radiative processes, mainly related to an inter- or intra-molecular proton transfer. As a consequence, fluorescence emission is characterized by a relatively low quantum yield (usually lower than 0.1) and a short lifetime (a few hundred ps). [10] Inter-system crossing also occurs with low yield, leading to a triplet state that can be quenched by molecular oxygen. [9, 11]

Curcumin is one of the most exploited photosensitizers for antibacterial photosensitization-based treatment (aPDT), [12-14] especially for the treatments of oral diseases. [15] Its use has indeed

several advantages like ready availability, low cost, efficacy against several kinds of micro-organisms and negligible dark toxicity (curcumin is pharmacologically safe at a dose of 8 g/day). [10]

Despite its wide-spread use as photosensitizer in photodynamic therapy (PDT), the mechanisms of curcumin's photo-toxicity are quite unclear. Both singlet oxygen and superoxide anion are known to be photo-generated by energy and charge transfer processes, respectively. [16-19] Despite showing oxygen-dependent photo-toxicity, curcumin is also known to be an antioxidant and even a singlet oxygen quencher under certain conditions. [20, 21] Thus, curcumin's photo-reactivity appears to be strongly dependent on the environment and it is likely that several mechanisms, such as photo-generation of different ROS and other photo-products, contribute to its overall photo-toxicity in a biological system. [18]

A crucial issue regarding the use of curcumin for PDT applications is the stability of the molecule in aqueous environments. [8] Curcumin is indeed known to rapidly degrade in aqueous buffers at alkaline or physiological pH. [22-24] Photo-degradation also occurs, particularly under irradiation with UV light. [25, 26] It is thus convenient, if not necessary, to combine the photosensitizer with a delivery vehicle in order to increase its stability and its water-solubility. Plasma proteins like albumins or fibrinogen, [8, 27-29] liposomes, [29] micelles, [30] nanoparticles [31] and cyclodextrins [32] are some examples of systems used to stabilize the molecule in aqueous solutions.

In this work, we explore the capability of two water-soluble proteins, apomyoglobin (apoMb) and bovine serum albumin (BSA), to act as delivery systems for curcumin. We show that when curcumin is bound to either protein, it becomes more stable. The enhancement in stability is particularly relevant in the case of BSA, which is able to stabilize curcumin for hours, long enough to achieve a high and reproducible phototoxic effect when used to treat cultured tumor cells. We have

previously demonstrated that both proteins are very effective in transporting the naturally occurring photosensitizer hypericin (Hyp), and allow to obtain highly bioavailable photosensitizing compounds for PDT of tumors [33] and aPDT against, e.g., *Staphylococcus aureus*. [34-36] Hyp-apoMb proved very effective also when tumor spheroids of HeLa cells were treated. [33] Water soluble proteins may thus represent interesting delivery means to minimize drug degradation and loss in herbal medicine, and to increase drug bioavailability and accumulation in tissues to be treated. [37]

## Materials and Methods

Horse heart myoglobin (Mb), bovine serum albumin (BSA), and curcumin were from Sigma-Aldrich. Apomyoglobin (apoMb) was prepared from myoglobin by acid acetone extraction, using standard biochemical procedures. [38, 39] Phosphate Buffered Saline (PBS) was prepared as 0.2g/l KCl, 8.0g/l NaCl, 0.2g/l $KH_2PO_4$, 1.65g/l $Na_2HPO_4$, pH 7.4.

### Molecular docking

Formation of the ApoMb-curcumin and BSA-curcumin complexes was modelled by SwissDock (http://www.swissdock.ch/), a web service that predicts the molecular interactions that may occur between a target protein and a small molecule. [40, 41] Target structures for BSA and apoMb were PDB code 3v03 and PDB code 1bvc, respectively. The representative best pose was selected in both cases.

### Cell cultures

All culture media and supplements were purchased from Euroclone. Dimethyl thiazolyl diphenyl tetrazolium (MTT) was purchased from Applichem. Cells were maintained in a humidified atmosphere of 95% air, 5% $CO_2$ at 37°C.

Curcumin was delivered to the cell cultures using two different protocols. Concentrated stock solutions in ethanol were then diluted to the desired concentrations either in the presence of apoMb or BSA. Attempts to deliver curcumin as a PBS buffered solution resulted in lower phototoxicity and scattered results, due to instability of the compound in these conditions.

**Viability assay.**

MTT was used to evaluate HeLa viability as previously described. [33] Cells were seeded in 96-well cell culture plates at the density of $3x10^5$ cells/ml, the following day they were starved and treated with increasing concentrations of curcumin either conjugated with apoMb or not, or control (1.2% ethanol for curcumin). Curcumin was manipulated in the dark. Cells were exposed to light 30 minutes after treatment with the compound and incubated for 24 hours in standard conditions, without light exposure. MTT was finally added at the concentration of 1 mg/ml and incubated for 2 hours. The resulting formazan crystals were solubilized with DMSO and the absorbance was measured at 550 nm using an ELISA plate reader (Sunrise, TECAN, Switzerland).

**Irradiation of cultured cells.**

Cells were irradiated using a LED light source (LED par 64 short, Show Tec (Highlite International B.V., Kerkrade, The Netherlands) for which the blue output at 460 nm (30 nm FWHM, 30.7 $mW/cm^2$) was selected.

The irradiance was homogeneous on the whole surface of a 96 well plate. Exposure of cultured cells was performed for 0, 2, 3, and 5 minutes, corresponding to light fluences of 0, 3.6, 5.5, and 9.2 J/cm$^2$, respectively.

**Fluorescence microscopy**

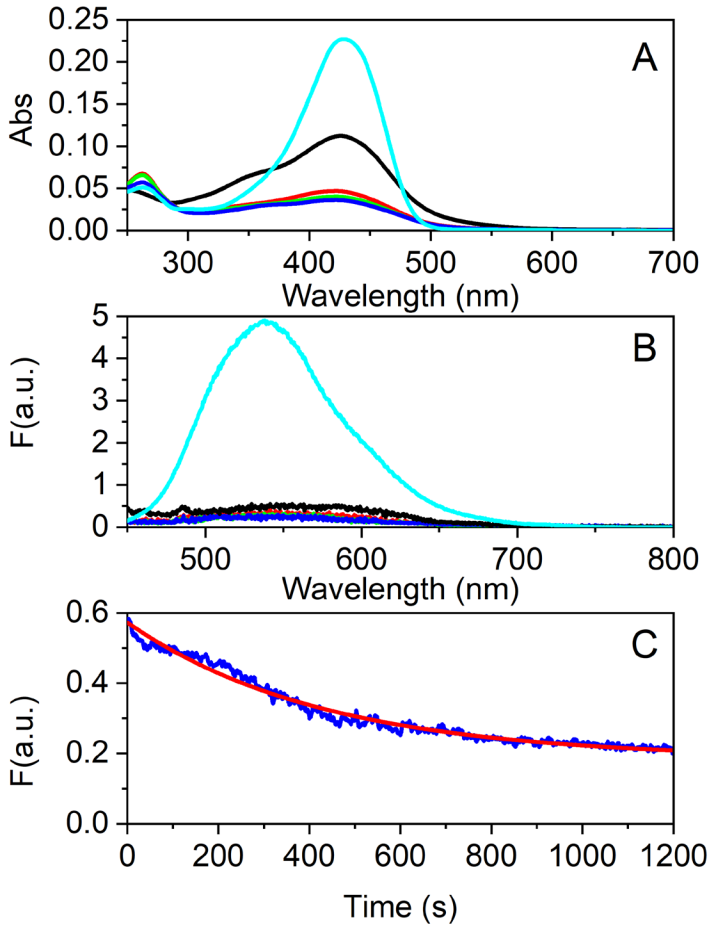
Spinning disk confocal microscopy allows high-speed three-dimensional imaging of living cells; it utilizes multiple pinholes that project a series of simultaneously excitation light beams onto the specimen in a multiplexed pattern, that is subsequently detected from a camera after fluorescence emission has passed through the same pinholes. Since such a parallelization requires a low laser power intensity at the specimen to excite fluorescence, photobleaching and phototoxicity are minimized. The microscope is composed of a TiE inverted Microscope, four laser sources (405nm, 488nm, 561nm, 640nm) and a Yokogawa CSU-X1 spinning disk confocal head, which comprises two disks of about 20,000 pinholes and the relative microlenses. The fluorescence light is collected by an Andor EMCCD camera, Ixon3 897.

## Results and discussion

Curcumin is unstable in water and its spectral properties undergo major changes within minutes from preparation of the solutions. Immediately after preparing a solution in PBS buffer, the compound shows a prominent absorbance band at 420 nm and a very weak (almost negligible), structure-less fluorescence emission (black curves in **Figures 1A** and **1B**). **Figures 1A** and **1B** also show the changes observed in the absorption and fluorescence emission spectra of curcumin in PBS buffer at selected delays after dilution from a concentrated ethanol solution. **Panel C** reports the kinetics of changes in the absorption spectrum monitored at 420 nm over the first 20 minutes. On the contrary, ethanol solutions of curcumin show much stronger absorption and fluorescence emission (cyan curves in **Figures 1A** and **B**), and their spectral properties are stable over days.

The spectral instability observed in PBS solutions poses strong limitations to applications in PDT of tumors and in antibacterial photosensitization-based treatment.

Oxidation of the compound may be prevented, or at least made less efficient, if a delivery agent is used to solubilize curcumin in water. Among available carriers, we have decided to test a few water soluble proteins endowed with hydrophobic cavities, that proved to be efficient carriers with other hydrophobic photosensitizers. Specifically, we selected apoMb and BSA, that were found capable of transporting hypericin and other hydrophobic dyes. [34-36]

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185

186 **Figure 1.** Absorption (**panel A**) and fluorescence emission spectra (**panel B**) of curcumin (12 μM) in
187 PBS buffer at pH = 7.4, collected at increasing delays after dilution from a concentrated ethanol
188 solution (black, 0 min; red, 10 min; green, 20 min; blue, 90 min). For comparison, the cyan curves
189 represent the absorption (panel A) and fluorescence emission (panel B, on a /10 scale) from
190 curcumin in ethanol at the same concentration. **Panel C.** Progress curve (blue curve) for changes in
191 Fluorescence emission. The red solid curve is the result of a fit with a single exponential decay
192 model, with lifetime 7±1 min. T = 20 °C.

193

194 ApoMb was found to bind curcumin, although with quite low affinity. ApoMb is endowed with a
195 rather large hydrophobic cavity, normally hosting the heme in the holoprotein. In the presence of
196 apoMb, the absorption spectrum of curcumin undergoes changes in shape and the fluorescence
197 emission of curcumin increases with increasing apoMb concentration, indicating binding of the
198 compound to the protein with a relatively low association constant ($K_a$~$10^3$ $M^{-1}$).
199
200 The stability of absorption and fluorescence emission spectra of curcumin is significantly improved
201 when bound to apoMb in PBS at pH 7.4 (**Figures 2A** and **2B**), resulting in a much slower (~ 25-fold)
202 degradation of the spectral properties.

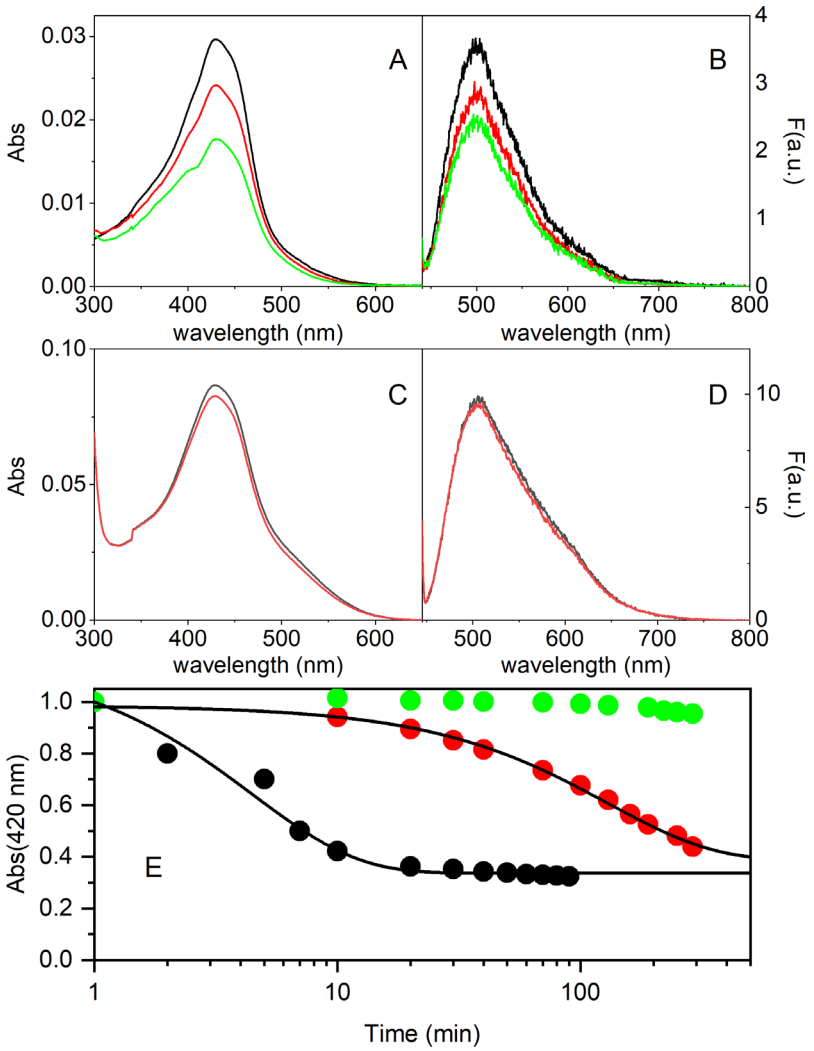


**Figure 2.** Absorption (**panel A**) and fluorescence emission spectra (**panel B**) of curcumin (2 μM) bound to apoMb (55 μM) in PBS pH = 7.4, at selected increasing delays (black 0 min, red 40 min, green 130 min) after preparation of the complex. Absorption (**panel C**) and fluorescence emission spectra (**panel D**) of curcumin (3 μM) bound to BSA (30 μM) in PBS pH = 7.4, at selected increasing delays (black 0 min, red 290 min) after preparation of the complex. **Panel E** compares the normalized absorbance at 420 nm after dilution/preparation of the complex at increasing delays for curcumin in PBS (black circles), curcumin (2 μM) bound to apoMb (55 μM) in PBS pH = 7.4 (red circles), and curcumin (3 μM) bound to BSA (30 μM) in PBS pH = 7.4 (green circles). All experiments

were conducted at 20 °C. Black solid lines are the fits with a single exponential decay. The retrieved lifetimes are 5±1 min for curcumin in water and 130±10 min for curcumin bound to apoMb. No change was appreciable for curcumin bound to BSA in the investigated time window (5 hours).

A slightly stronger binding was observed for BSA (~$5\times10^4$ $M^{-1}$). A similar value was obtained also for HSA, for which an association constant of $6.1\times10^4$ $M^{-1}$ was reported. [29] **Figures 2C** and **2D** demonstrate that when bound to BSA, the spectral properties of curcumin are stable over at least 5 hours, which corresponds to a dramatic increase in stability (lifetime $\geq$ 1000 s) also in comparison to the complex with apoMb.

A previous investigation reported curcumin stabilization by HSA already at low protein concentration (roughly 13 μM). The rate of degradation under those conditions was found to be reduced only by tenfold, [27], much less than the effect reported in Figure 2, possibly due to the lower protein concentration employed in that work.

The increased stability of curcumin in the presence of apoMb or BSA is considered to arise from the protection against hydrolysis provided to the compound by the macromolecule scaffold. The hydrophobic pocket, which is occupied by the heme in the holoprotein myoglobin, in principle provides a suitable binding site for curcumin. Similarly, BSA is endowed with several docking sites for hydrophobic compounds, which could potentially host curcumin. A qualitative estimate of the theoretical feasibility of fitting curcumin into the hydrophobic cavities of apoMb or BSA was assessed through the computational macromolecular docking software Swissdock (www.swissdock.ch/). **Figure 3 A** shows the lowest energy pose for curcumin bound to the heme cavity in apoMb. Other binding sites are present on the protein, but their energy scores appear more unfavorable. The existence of other binding sites on myoglobin was suggested also by

previous experimental investigations. [42] **Figure 3 B** reports the lowest energy pose for curcumin bound to BSA.

The superior stability of curcumin spectral properties when bound to BSA suggests that this delivery system may be safely and reliably employed in photodynamic investigations. We have therefore performed cell viability assays after having treated cultured cells with curcumin-BSA and exposed them to blue light.

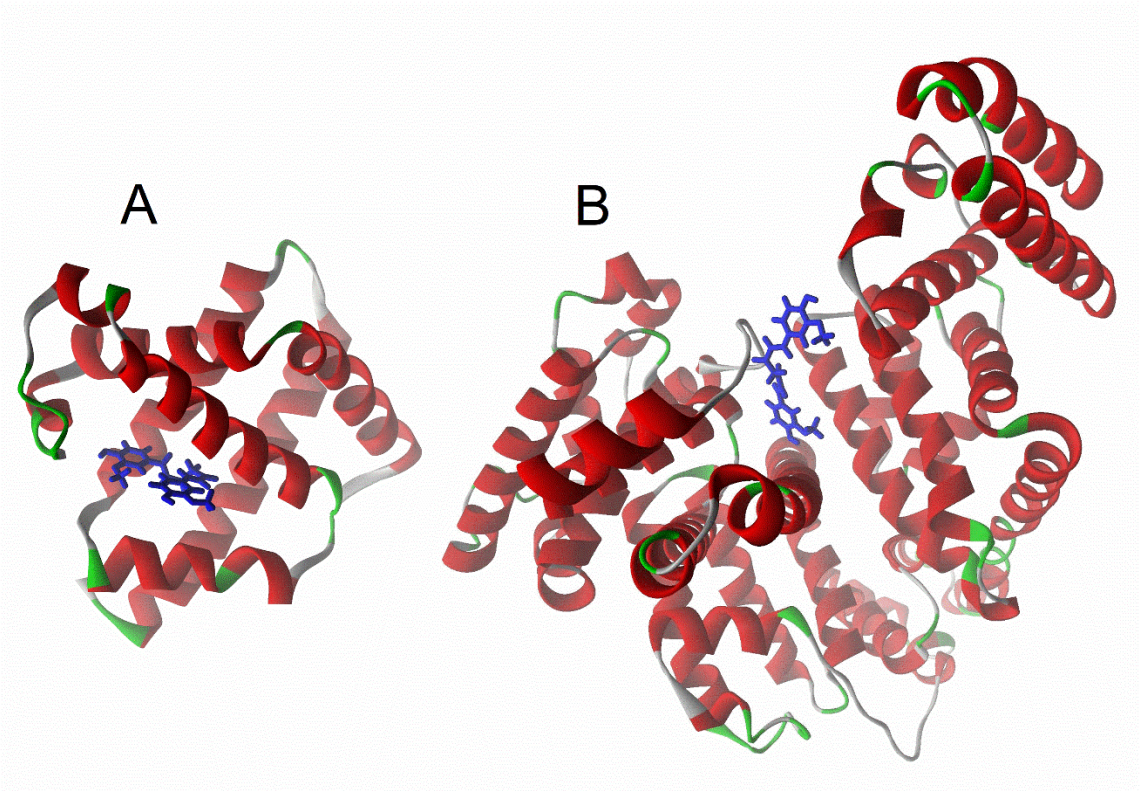


**Figure 3.** Best poses for curcumin bound to apoMb (PDB code 1wr) and BSA (PDB code 3v03) as determined by Swissdock (http://www.swissdock.ch/).

**Phototoxicity of curcumin-BSA**

Cell viability of HeLa cells in the presence of curcumin or curcumin-BSA was determined as a function of PS concentration and illumination time, i.e. light fluence. The plots (**Figure 4**) evidence

a clear concentration-dependent cytotoxic effect, with can be observed at all irradiation times (2, 3 and 5 minutes) when cells are treated with either curcumin or curcumin-BSA.

When curcumin-BSA is used, phototoxicity is observed at lower concentrations as reported in **Figure 4B** for 2 min illumination. Under these conditions, the concentration at which a 50% reduction in cell viability is observed is about 3-times lower when curcumin-BSA is used in comparison with curcumin. Increasing light-exposure time to 5 minutes decreases the difference in cytotoxicity for curcumin and curcumin-BSA. The lower phototoxicity observed when curcumin is delivered without BSA may correlate with the instability of curcumin in PBS solutions discussed above.

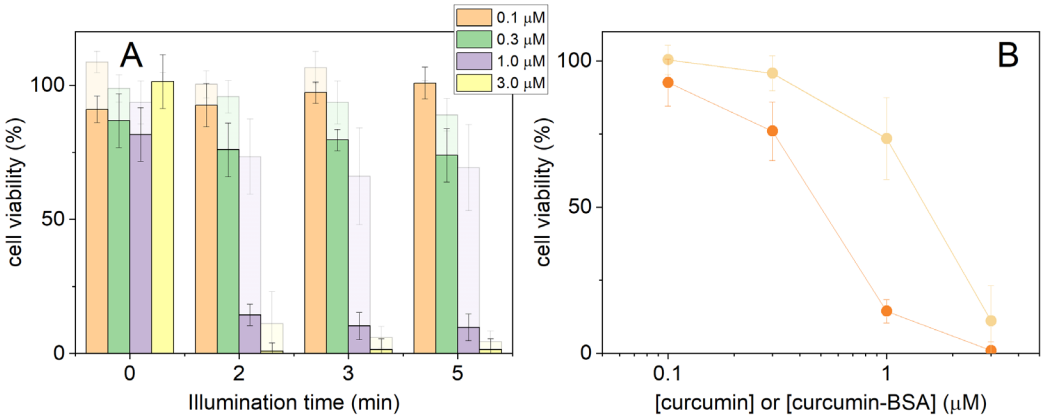


**Figure 4.** Cell viability for HeLa cells incubated with curcumin or curcumin-BSA and exposed to blue light. **Panel A.** Concentration-dependence (curcumin at 0.1, 0.3, 1, and 3 μM) and time-response (exposure for 0, 2, 3 and 5 minutes) for viability of HeLa cells treated with increasing concentrations of curcumin-BSA (dark bars) or curcumin (light bars) and exposed to increasing illumination times. Data are the means of at least three independent experiments. BSA was 30 μM. Viability controls were collected for cells grown in PBS, in the presence of 0.6% EtOH, in the presence of 30 μM BSA. **Panel B.** dose response for viability of HeLa cells treated with increasing curcumin (yellow) or curcumin-BSA (orange) concentrations for 2-minute illumination with blue light.

**Cellular uptake is different for curcumin and curcumin-BSA**

Taking advantage of the bright fluorescence emission of curcumin it is possible to follow the uptake of the compound by cells. When curcumin is delivered to cultured cells using a concentrated ethanol solution, fluorescence is observed from inside the cellular structures after just a few minutes of incubation. **Figure 5 (A-C)** shows that fluorescence emission from curcumin co-localizes with the fluorescence emission from MitoTracker red. Thus, accumulation of curcumin when delivered without a carrier appears to occur at the mitochondria, as previously reported. [43, 44] Curcumin and curcumin derivatives targeting mitochondria exhibited significant cytotoxicity towards several cancer cell lines, inducing significant ROS generation, cell-cycle arrest and apoptosis. [45]

**Figure 5 (D-F)** shows that when HeLa cells are incubated with curcumin-BSA, the observed distribution of the compound appears quite different from that observed for curcumin alone. Diffuse fluorescence from curcumin is observed in large areas of the cytoplasm, in addition to mitochondria. The more distributed loading of the cell appears to be a direct consequence of the higher bioavailability of the photoactive compound when delivered bound to BSA. The lack of aggregation and the dramatically increased stability of curcumin bound to BSA mean that the compound is bioavailable at higher concentration and for longer times, so that uptake from cells is more extensive. In the presence of HeLa cells, we expect that fully photoactive curcumin is efficiently transferred to the cell structures by the carrier protein, due to the higher affinity of the compound for lipids and possibly for other macromolecules endowed with hydrophobic binding sites. The process we envision for curcumin-BSA is similar to the one we recently demonstrated for the self-assembled hypericin-apomyoglobin construct. [33] In that case, the rapid and efficient transfer of monomeric apoMb-bound Hyp to the plasma membrane was found to speed up cell loading in comparison to the case when Hyp is added to the solution without the protein carrier.

Admittedly, the high affinity for membranes of the hydrophobic PS transported by the protein, held in place by non-covalent interactions, may limit the bioavailability of the photosensitizing compounds in systemic applications. Such applications have not yet been attempted.

Although the details of the uptake mechanism are beyond the scope of the present work, the wider distribution of the photoactive compound may be responsible for the more extensive damage induced by illumination of HeLa cells treated with curcumin-BSA.

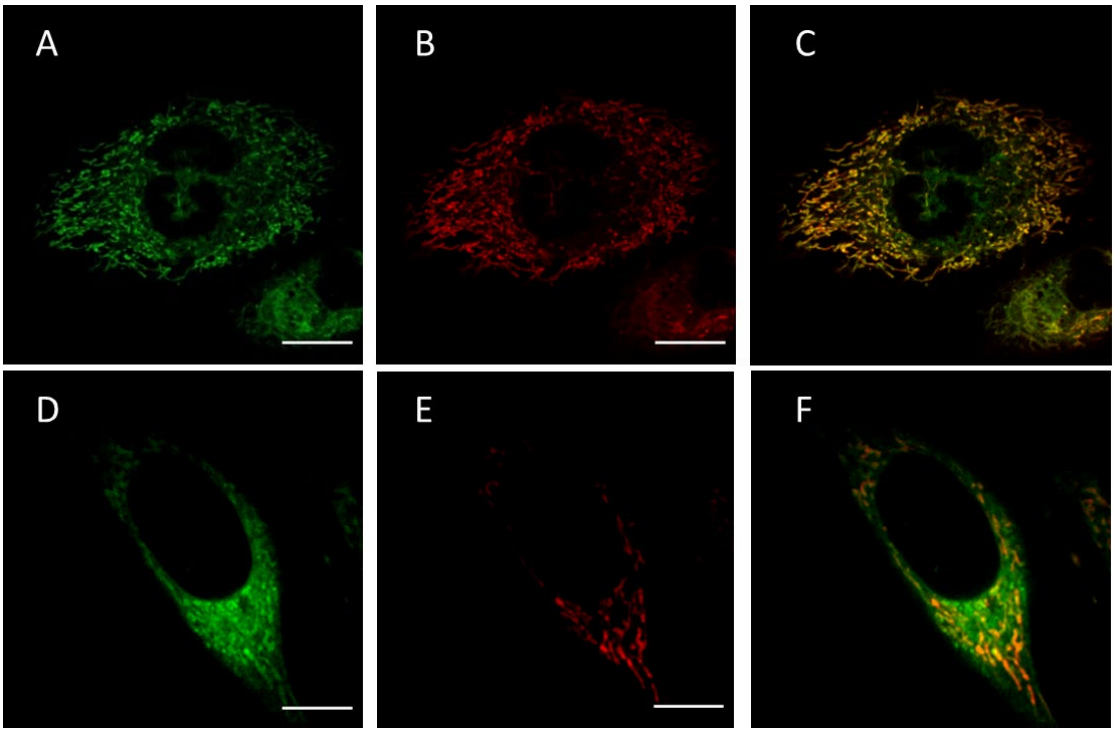


**Figure 5.** Fast confocal spinning disk fluorescence imaging of HeLa cells incubated 15 min with mitoTracker Deep red [0.3 μM], and 10 min with curcumin [15 μM] (**A-C**) or curcumin-BSA (**D-F**). A, D green channel (curcumin); B, E red channel (mitoTracker); C, F composite. Curcumin channel: excitation 405 nm, emission 460/60, exposure time 50 ms. MitoTracker channel: excitation 633 nm, emission 700/75 nm, exposure time 200 ms. For all the images the pixels area is 512x512, and pixel size is 0.16 μm. Scale bar 10 μm.

## Conclusions

The use of proteins, capable of binding curcumin with moderate affinity, allows to stabilize the photosensitizer and make it possible to use the compound in model studies without the interfering complications of its chemical degradation. In particular, when BSA is used as a delivery vehicle, the efficiency in reduction of cell viability upon illumination becomes substantially higher.

Delivery of curcumin with BSA has an impact on the intracellular distribution of the compound. When delivered without the protein carrier, curcumin is mainly localized in the mitochondria. On the other hand, when curcumin is bound to BSA, the cellular distribution appears more homogeneous, likely associated with a variety of target structures.

The above properties lead to higher phototoxicity of curcumin-BSA in comparison to curcumin alone, a fact that may be exploited to overcome the known limitations of this photosensitizing compound.

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## Competing Interests

The authors declare that no competing interest exists.

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