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Serum albumins are efficient delivery systems for the photosensitizer hypericin in photosensitization-based treatments against *Staphylococcus aureus*

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KEYWORDS: natural photosensitizers, protein carrier, antibacterial photosensitization-based treatment, singlet oxygen, *Staphylococcus aureus*

Abstract

The naturally occurring photosensitizer hypericin can be effectively delivered to bacterial cells using serum albumins as biocompatible carriers. Using a combination of spectroscopic methods we demonstrate that the photophysics of hypericin is fully preserved when bound to these proteins. Thanks to the excellent transport capabilities of serum albumins, that deliver hypericin to Gram-positive *S. aureus*, an efficient antibacterial action was observed, with a reduction of up to 8 log in the number of colony-forming units. The photoactive material is fully compatible with the use in food processing environments, and may be exploited as a viable method for decontamination from *S. aureus* and other Gram-positive bacteria.

1. Introduction

Increasing antimicrobial resistance is of great concern for public health because of the decreasing number of available effective antimicrobials, and the insufficient rate of development of new alternatives. (Boucher et al., 2009)

Formation of resistant genes in bacteria is mostly driven by selective pressure, exerted by the excessive use of antimicrobials for therapeutic use in humans or veterinary activities, e.g., growth promotion or disease prevention of livestock. The problem is exacerbated by the use of sub-therapeutic doses. (Laxminarayan et al., 2013)

Within this context, *Staphyloccocus aureus*, a Gram-positive microorganism of the Micrococcaceae family, is of special interest. It is a commensal and opportunistic pathogen responsible for a wide spectrum of infections in humans, ranging from superficial skin diseases to invasive and potentially life-threating illnesses. (Lowy 1998; Tong et al., 2015) This ubiquitous microorganism can cause nosocomial and community-acquired infections, as well as food-borne diseases. (Kadariya, Smith & Thapaliya 2014) It can colonize human nasal mucosa and skin as well as environmental surfaces and clothing resulting in a significant potential to contaminate food products during production and handling. (le Loir, Baron & Gautier 2003) Furthermore, *S. aureus* is commonly found in a wide variety of food-producing animals, and its transfer to food may occur from dairy animals in case of mastitis (Hennekinne, De Buyser & Dragacci 2011) or contact with live animals by food handlers. Its ability to grow in a wide range of temperatures (7 to 48.5 °C, with optimum 30 to 37 °C), pH (4.2 to 9.3, with optimum 7 – 7.5) and high sodium chloride concentration (up to 15%) makes *S. aureus* able to survive in a large variety of foods and in dry environments. (Chaibenjawong & Foster 2011) (le Loir, Baron & Gautier 2003)

S. aureus food-borne disease is one of the most common worldwide and results from the ingestion of staphylococcal enterotoxins (SEs) preformed in foods by enterotoxigenic strains of the microorganism. More than 20 SEs have been recognized and all of them have superantigenic activity, whereas nearly half of them have emetic properties which are hazardous for consumers. (Balaban & Rasooly 2000) (Hennekinne, De

55 Buyser & Dragacci 2011) Improper food handling practices, favourable food composition for *S. aureus* growth
56 and toxinogenesis, favourable temperatures and time for *S. aureus* growth and ingestion of sufficient
57 amounts of toxin to provoke symptoms are the conditions required to evoke a staphylococcal food-borne
58 disease. (Hennekinne, De Buyser & Dragacci 2011)
59 In the last decades, the emergence of *S. aureus* strains resistant to beta-lactams (Methicillin-resistant *S.*
60 *aureus;* MRSA) caused nosocomial and community-acquired infections characterized by high morbidity
61 worldwide. (Bukharie et al., 2001) (King et al., 2006) Recently, MRSA strains have been isolated from farm
62 animals and their meat, and were recognised as a novel pathogen associated with human infections (Price
63 et al., 2012) (Lassok & Tenhagen 2013) (Larsen et al., 2016) (Kinross et al., 2017) thus representing a new
64 One Health issue for human and veterinary medicine.
65 The development of alternative strategies for preventing and treating infectious animal diseases of bacterial
66 origin is therefore of great interest, (Trevisi et al., 2014) and the application of novel methods for
67 decontamination of food processing and handling environment is a current topic in food science. (Demirci &
68 Ngadi 2012; Kairyte, Lapinskas, Gudelis & Luksiene 2012; Luksiene & Brovko 2013; Tortik et al., 2016; Glueck,
69 Schamberger, Eckl & Plaetzer 2017)

70 Antibacterial photosensitization-based treatment is a promising approach that relies on the combined action
71 of otherwise nontoxic molecules (called photosensitizers, PS), visible light, and oxygen to induce formation
72 of reactive oxygen species, particularly singlet oxygen, that result in cellular phototoxicity. Among limitations
73 of the approach is the fact that several of the known photosensitizing molecules are highly hydrophobic and
74 therefore require a delivery vehicle, that is biocompatible with the target environment. Additional issues
75 arise from the lack of cell specificity of the dyes, that require chemical engineering of targeting moieties into
76 the photoactive structure. Several strategies to address both issues have been proposed and were recently
77 reviewed. (Planas et al., 2014)

78 One of the most effective photosensitizers is hypericin. Hypericin (Hyp) is a naturally occurring PS
79 (Brockmann, Haschad, Maier & Pohl 1939; Duràn & Song 1986; Karioti & Bilia 2010) that has been proposed
80 in the treatment of cancer, (Wang et al., 2010; Agostinis et al., 2011; Couldwell et al., 2011) as an antiviral,

(Jacobson et al., 2001; Kubin et al., 2005) antibacterial, (Kairyte, Lapinskas, Gudelis & Luksiene 2012; Yow, Tang, Chu & Huang 2012; Comas-Barceló et al., 2013; Nafee et al., 2013; Rodríguez-Amigo et al., 2015) and antifungal agent. (Rezusta et al., 2012) The molecule emits an intense fluorescence in polar organic solvents (Φ_F = 0.35 in ethanol (Duràn & Song 1986; López-Chicón et al., 2012) and in DMSO (English et al., 1997)), and sensitizes singlet oxygen with high yield (Φ_Δ = 0.32 in ethanol, (Darmanyan, Burel, Eloy & Jardon 1994) 0.39 ± 0.01 in methanol, (Roslaniec et al., 2000) and 0.28 ± 0.01 in DMSO (Losi 1997; Delcanale et al., 2015)). Due to the hydrophobic character of the molecule, aggregates are formed in aqueous solutions, characterized by a much weaker fluorescence (Yamazaki, Ohta, Yamazaki & Song 1993) and singlet oxygen yields.

Binding of Hyp to several proteins prevents aggregation of the otherwise insoluble PS in aqueous media. (Miskovsky et al., 1998; Das et al., 1999; Hritz, Kascakova, Ulicny & Miskovsky 2002; Gbur et al., 2009; Roelants et al., 2011) Proteins are highly biocompatible and warrant good bioavailability of the photoactive drug. We recently proposed the use of proteins such as apomyoglobin (apoMb) and β-lactoglobulin as carriers to deliver Hyp to bacterial cells for antimicrobial photodynamic applications. (Comas-Barceló et al., 2013; Delcanale et al., 2015; Rodríguez-Amigo et al., 2015; Delcanale et al., 2016; Delcanale et al., 2017)

Serum albumins are interesting additional candidates, given their known capability of binding a variety of endogenous molecules as well as drugs. (Fasano et al., 2005; Varshney et al., 2010) A well-studied example is human serum albumin (HSA), which represents the most prominent protein in plasma. HSA binds different classes of ligands at distinct sites which can affect the pharmacokinetics of many drugs and influence the reactivity of bound compounds. At the same time, HSA can act as carrier in the mechanism of disposal of potentially harmful molecules. (Fanali et al., 2012)

The interaction of Hyp with serum albumins has been described in the literature, in an attempt to devise a photosensitizing agent suitable for tumor or antiviral PDT. (Senthil, Longworth, Ghiron & Grossweiner 1992; Miskovsky 2002) Early competition experiments suggested that the binding site of Hyp is in the IIA subdomain of the protein, (Falk & Meyer 1994; Köhler et al., 1996) a fact later confirmed by Resonance Raman and surface-enhanced Raman spectroscopy. (Miskovsky et al., 1998) A subsequent thorough investigation where

vibrational spectroscopies and molecular modeling were employed, allowed to draw a structural model for the binding site and characterize specific interactions with amino acid residues for human and bovine (BSA) serum albumins. (Miskovsky et al., 2001) Interestingly, the complex between HSA and Hyp appears to be remarkably stable towards photobleaching upon prolonged exposure to visible light. (Uzdensky, Iani, Ma & Moan 2002) Hyp was studied in the presence of different biological systems: bound to human serum albumin, in cultured human adenocarcinoma WiDr cells and in the skin of nude mice. Hypericin was reported to be more photostable than photosensitizers like mTHPC and Photofrin that are commonly used in PDT.

Serum albumins represent an interesting starting material for the development of nanostructured macromolecular assemblies endowed with specific functionalities. HSA constitutes about 50% of the protein present in the plasma of normal healthy individuals, (Evans 2002) corresponding to a concentration ranging between 33 and 52 g L^{-1}. (Boldt 2010) Similarly, BSA is the most abundant protein in bovine plasma with a typical concentration of 50 g L^{-1}. Purified bovine albumin is used to help replenish blood or fluid loss in animals. It is used in testing for the Rh factor in human beings, and as a stabilizer for vaccines. It is also used in antimicrobial sensitivity tests. (Jayathilakan, Sultana, Radhakrishna & Bawa 2012) One remarkable advantage of BSA is the low production cost, as large amounts of the protein can be easily purified from bovine blood, which is a byproduct of the cattle industry.

In this work we have studied the interactions between Hyp and bovine as well as human serum albumins, to assess the possibility of using the complex between albumins and Hyp as a photosensitizing agent in antibacterial photodynamic applications for decontamination of food processing materials and possibly foodstuff. *Staphylococcus aureus* ATCC 25923 was used as target microorganism, in view of its importance as human and food-borne pathogen.

2. Materials and Methods

Hypericin was from HWI Analytik GmbH (Ruelzheim, Germany). Bovine serum albumin (BSA), Human serum albumin (HSA) and Bovine Albumin–fluorescein isothiocyanate conjugate were from Sigma Aldrich (St. Louis, MO). Samples were used as received.

For spectroscopic investigations, protein concentration was always in large excess of Hyp (typically tenfold), so that the concentration of free Hyp is essentially negligible.

BSA and HSA were labeled with Fluorescein 5-maleimide (FMA, Sigma-Aldrich), a Fluorescein derivative that is selectively reactive towards Cys residues, following an established protocol.

2.1 General spectroscopic instrumentation

Absorption spectra were measured with a Jasco V-650 (Jasco Europe) spectrophotometer. Fluorescence excitation, emission and anisotropy spectra were collected with a Perkin Elmer LS50 spectrofluorometer (PerkinElmer, Waltham, MA).

Hypericin fluorescence decays were recorded by a FLS920 time-correlated single photon counting system (TCSPC) (Edinburgh Instruments, UK) with pulsed LED excitation at 365 nm (EPLED, Edinburgh Instruments, UK, operated at 10 MHz repetition rate) and detection at 600 nm.

Fluorescence lifetime measurements for FRET experiments were recorded with a 5000U (Horiba JobinYvon, Edison,NJ) TCSPC system. Measurements were carried out using a pulsed LED at 457 nm (N-457, Horiba Sci., Edison, NJ). The LED operates at a fixed repetition rate of 1 MHz and pulse width of ~ 1 ns. The emission decay was recorded at 520 ± 4 nm corresponding to the region of the maximum of FMA emission. The decay data were analyzed using the deconvolution software DAS 6.2 (IBH, Glasgow, U.K.), which yields the value of the fluorescence lifetimes (τ_i) and their fractional amplitude (α_i). The quality of the fitting was evaluated through i.) the value of the reduced $\chi 2$ (~1.0-1.5), ii.) the visual inspection of the residuals, and iii.) the value of the Durbin-Watson parameter (~1.8-2.0).

Fluorescence quantum yields were determined using a comparative method. (Lakowicz 2006) The reference compound was Hyp-apoMb, for which the fluorescence quantum yield was previously established (Φ_F = 0.14 (Delcanale et al., 2015)).

All experiments were performed at 20 °C.

157 **2.2 Laser Flash Photolysis**

158 Triplet state decay of Hyp were monitored at 520 nm after photoexcitation with the second harmonic (532
159 nm) of a nanosecond Nd:YAG laser (Spectron Laser) using a previously described setup. (Abbruzzetti et al.,
160 2006) Triplet quantum yields were determined using a comparative method, adopting Hyp in ethanol as a
161 reference compound (Φ_T = 0.32 (Darmanyan, Burel, Eloy & Jardon 1994)).

162 **2.3 Fluorescence Correlation Spectroscopy (FCS)**

163 FCS experiments were performed using a Microtime 200 system from PicoQuant, based on an inverted
164 confocal microscope (Olympus IX70) and equipped with two SPADs (Single Photon Avalanche Diodes) used
165 in the cross-correlation mode. Excitation was achieved by a 475 nm picosecond diode laser. Fluorescence
166 emission by Hyp was collected through a bandpass filter and split with a 50/50 splitter between the two
167 detection channels. Hyp concentration was kept in the nM range, so that only a few molecules were detected
168 in the confocal volume.

169

2.4 STED nanoscopy

Stimulated emission depletion (STED) nanoscopy has been performed using a custom made setup equipped with a supercontinuum pulsed laser source (ALP-710-745-SC, Fianium LTD, Southampton, UK) described elsewhere. (Delcanale et al., 2015) We selected the excitation wavelength by means of an AOTF, while the STED wavelength is predefined by the laser outputs, in particular the 715 nm output is in resonance with a vibronic transition in the emission spectrum. (Comas-Barceló et al., 2013) The laser has a repetition frequency of 20MHz and a pulse width of about 100ps. In all the experiments we used 566 nm for excitation and 715nm for STED. The doughnut shape of the STED beam is realized by a vortex phase plate (RPC photonics inc., Rochester, NY, USA). The beams are scanned on the sample by galvanometer mirrors (Till-photonics, FEI Munich GmbH, Germany), focused by a HCX PL APO CS 100x 1.4NA oil (Leica Microsystems, Mannheim, Germany) objective and fluorescence is collected by an avalanche photodiode (SPCM-AQRH-13-FC, Excelitas Technologies, Vaudreuil-Dorion, Quebec, Canada) in the spectral window 670-640nm. (Bianchini et al., 2015)

2.5 Microbial strains and growth conditions

S. aureus ATCC 25923 was grown overnight in sterile Luria Bertani medium (LB) at 37°C. Stock inoculum suspensions were prepared in sterile PBS and adjusted to an optical density of 0.4 at 600 nm.

2.6 Bacterial photoinactivation

Cell suspensions in sterile PBS were incubated for 30 min in the dark at room temperature with the PS. The final concentration of the PS in the cell suspensions was 10 µM. Then, 0.3 mL of the suspensions was placed in 96-well plates.

Irradiation of bacterial colonies was conducted using a RGB LED light source (LED par 64 short, Show Tec Highlite International B.V., Kerkrade, The Netherlands), equipped with 19, 3W, RGB LEDs. The green output

193 at 515 nm (40 nm FWHM) was chosen given the good overlap with the absorption spectrum of Hyp (Figure
194 1A). The irradiance at the surface of a 96 well plate was homogeneous and corresponds to 16 mW/cm^2 in the
195 green. Exposure of cultured cells was performed for 0, 5, 15 and 30 minutes which correspond to light doses
196 of 0, 4.9, 14.7, and 29.4 J/cm^2, respectively.
197 The bacterial suspensions were serially diluted, seeded on tryptic soy agar, and incubated in the dark for 24
198 h at 37 °C. Colony-forming units (CFUs) were counted to calculate the survival fraction.

199

245 **3. Results and discussion**

246 **3.1 Interaction between Hyp and serum albumins**

247 Binding of Hyp to serum albumins is readily detected from the changes in the absorption and in the
248 fluorescence emission spectra that occur when Hyp is in the presence of the proteins. (Miskovsky et al., 1998;
249 Miskovsky et al., 2001; Hritz, Kascakova, Ulicny & Miskovsky 2002) As shown in **Figure 1A**, the absorption
250 spectrum of Hyp in PBS buffer (green curve) is characterized by broad absorption bands, due to the formation
251 of aggregates. (Miskovsky et al., 1998) In the presence of serum albumins, the absorption bands become
252 more intense and structured (red and black curves), as previously reported. (Miskovsky et al., 2001) Due to
253 the interaction with the protein, Hyp is kept in a hydrophobic environment that prevents aggregation and
254 mimics the conditions met in good organic solvents like DMSO and ethanol, where absorption bands are
255 narrow and more intense. (Comas-Barceló et al., 2013) When bound to serum albumins, absorption maxima
256 for Hyp-HSA are observed at 553 nm and 596 nm, whereas for Hyp-BSA peaks are located at 551 nm and 593
257 nm.

258 **Figure 1B** reports the fluorescence excitation and emission spectra for Hyp-BSA and Hyp-HSA. The
259 monomeric state allows the bound Hyp to emit a relatively intense fluorescence, with prominent bands at
260 596 nm and 609 nm for Hyp-BSA and Hyp-HSA, respectively. Minor peaks are observed at 645 nm for both
261 compounds. It is worth recalling that, due to aggregation, Hyp in aqueous solutions is essentially not
262 fluorescent. Fluorescence excitation and emission occur at slightly different wavelengths for the two

proteins, in agreement with the different environment of the binding sites for Hyp in HSA and BSA. (Miskovsky et al., 2001)

The observed effects are similar to those reported for Hyp binding to apomyoglobin (apoMb) (Comas-Barceló et al., 2013) and β-lactoglobulin. (Rodríguez-Amigo et al., 2015; Delcanale et al., 2017)

The fluorescence quantum yield for the complexes can be estimated using Hyp-apoMb as a reference (Φ_F = 0.14 (Delcanale et al., 2015)) and is readily evaluated as Φ_F = 0.11 and Φ_F = 0.12 for Hyp-BSA and Hyp-HSA, respectively.

The increase in fluorescence upon binding allows to determine the association constant. (Comas-Barceló et al., 2013) Binding of Hyp to albumins occurs with moderate affinity. The dissociation constant for HSA-Hyp is $K_d = (1.1 \pm 0.2)\times10^{-5}$ M and for BSA-Hyp is $K_d = (2.1 \pm 0.2) \times10^{-5}$ M. The value we retrieved for the dissociation constant from HSA is lower (about 8-fold) than a previous determination. (Senthil, Longworth, Ghiron & Grossweiner 1992)

Binding of Hyp to the studied proteins leads to a strong increase in fluorescence anisotropy. **Figure 1C** compares the anisotropy measured for Hyp-HSA and for Hyp-BSA solutions, that is similar to the one determined for Hyp-apoMb. (Delcanale et al., 2015) When Hyp is bound to albumins, rotational depolarization is a much slower process and emission occurs before the polarization selected upon photoexcitation is lost.

Fluorescence emission decays for Hyp-HSA and for Hyp-BSA solutions were measured by collecting the emission at 600 nm after pulsed excitation at 365 nm. Sample curves are reported in **Figure 1D**. Unlike the case of ethanol or DMSO solutions, where fluorescence emission decays with a single exponential relaxation, (López-Chicón et al., 2012) (Comas-Barceló et al., 2013) (Delcanale et al., 2015) the decay for albumin-bound Hyp is best described by a biexponential function. The retrieved lifetimes are τ_1 = 2.98 ns (45 %) and τ_2 = 5.52 ns (55 %) for Hyp-HSA and τ_1 = 3.13 ns (23 %) and τ_2 = 6.11 ns (77 %) for Hyp-BSA. In both cases Hyp

concentration was 4.7 μM while albumin was 47 μM. Decay parameters are similar to those reported in the literature. (Das et al., 1999) The observed lifetimes are comparable to those previously reported when Hyp is bound to apoMb (Comas-Barceló et al., 2013) or to dimeric β-lactoglobulin. (Delcanale et al., 2017) Conversely, Hyp in PBS buffer is aggregated and fluorescence decays through a complex multiexponential process with a dominant, short lived component, indicative of strong quenching of the excited state and scattering from the aggregates. (Lenci et al., 1995; López-Chicón et al., 2012)

3.2 Labeling of albumins with FMA

BSA and HSA were selectively labeled with FMA, a fluorescent probe that binds selectively to Cys34, which is the only Cys residue which is not involved in a disulfide bond. The labeling provides a local fluorescent probe and a spatial reference in each protein that can be exploited to further assess interaction of Hyp with the proteins. Hyp binds in the IIA subdomain of the protein nearby Trp214, (Miskovsky et al., 1998; Miskovsky et al., 2001) located at about 30 Å from Cys34. The fluorescence emission spectrum of FMA strongly overlaps to the absorption spectrum of Hyp. It is thus expected that FRET may occur between FMA acting as the donor, and Hyp as the acceptor. Due to the spectral properties of the two fluorophores, it is difficult to detect the energy transfer from FMA to Hyp based on changes in emission intensity. We have thus monitored the fluorescence lifetime of the FMA donor.

The average lifetime of the donor is 3.58 ns for HSA-FMA and 4.47 ns for BSA-FMA. In the presence of Hyp, these values decrease to 3.44 ns and 4.21 ns, respectively. The FRET efficiency can be calculated as 0.039 for HSA-FMA and 0.058 for BSA-FMA. From the spectral data it is possible to estimate the Förster radius as R_0 = 1.54 nm. Using the FRET efficiencies reported above, the distance between FMA and Hyp is estimated as ~2.5 nm BSA-FMA and ~2.6 nm for HSA-FMA, in keeping with the expected values.

It is worth observing that the affinity for Hyp is not affected by the presence of FMA. The added value of albumin co-labeling with FMA is that the presence of this second fluorophore may be exploited as a second

311 fluorescence readout in fluorescence imaging applications. This is expected to minimize the photoinduced
312 generation of cytotoxic reactive oxygen species by hypericin.

313

314 **3.3 Triplet state**

315 Hyp bound to the hydrophobic pockets in albumins is shielded from molecular oxygen in the buffer. As a
316 consequence, the triplet state of the protein-bound Hyp is much longer lived than the triplet states of solvent
317 exposed PS molecules. **Figure 2A** shows the triplet decay detected through the absorption changes at 510
318 nm. The triplet lifetime of HSA-bound Hyp, τ_T = (40 $\pm$ 6) μs, is longer than the one of BSA-bound Hyp, τ_T = (17
319 $\pm$ 3) μs, suggesting that the binding site is located deeper in the protein matrix and more protected from
320 dissolved molecular oxygen. The triplet lifetime for albumin-bound Hyp is sensibly longer than the one
321 observed for Hyp bound to apomyoglobin (11.6$\pm$ 0.1 μs) (Delcanale et al., 2015) and β-lactoglobulin (10$\pm$ 2)
322 μs, (Delcanale et al., 2017) where shielding from the solvent appears less effective.

323 The triplet yields for HYp-BSA (Φ_T = 0.13) and Hyp-HSA (Φ_T = 0.10) were readily calculated using Hyp in ethanol
324 as a reference, for which Φ_T = 0.32.

325 The presence of the triplet state is further confirmed by Fluorescence Correlation Spectroscopy (FCS)
326 experiments. The autocorrelation functions for Hyp-BSA (blue curve in **Figure 3D**) and Hyp-HSA (not shown)
327 are very similar. For both compounds, the best fit was obtained using a model comprising a triplet state (with
328 lifetime of about 20 μs) and a diffusive species. The diffusion coefficient of the fluorescent compound is about
329 60 $\mu m^2/s$, in agreement with literature values for serum albumins. (Raj & Flygare 1974) This indicates that
330 the molecular weight of the observed fluorescent species coincides with that of albumin, confirming
331 formation of the complex between Hyp and the proteins.

332 The above data show that binding of Hyp to BSA or HSA occurs with an appreciable affinity and preserves the
333 photophysics of the compound. The resulting nanostructure is thus endowed with triplet and fluorescence
334 yields that are similar to those previously reported for complexes with other, smaller size globular proteins.

3.4 Interaction with bacterial cells

Evidence for the existence of spontaneous interactions between Hyp transported by albumins and bacterial cells was provided by FCS measurements. *S. aureus* suspensions were incubated for 30 minutes with Hyp-BSA (to final concentrations [Hyp] = 100 nM and [BSA] = 30 μM). The suspension contained also BSA labeled with fluorescein isothiocyanate at 100 nM concentration. This provided us with two fluorescent probes that enable monitoring separately the interaction with bacteria of Hyp, through its red emission, and of the protein, through the green emission of the covalently attached label.

The fluorescence intensity time-traces (MCS traces) monitored in the red show large intensity peaks (**Figure 3A**), which correspond to bacteria going through the confocal volume of the microscope, over a small background. The low intensity background reflects the presence of a very small fraction of unbound Hyp-BSA molecules. The very slow diffusing species at low (~5 nM) concentration, giving rise to the spikes, is identified with bacteria, decorated with several copies of Hyp-BSA. When the green emission is monitored instead (**Figure 3B**), the MCS trace is devoid of the large spikes. **Figure 3C** shows an expanded view on selected portions of the MCS traces reported in **Figures 3A** (in a region corresponding to bacteria passing through the confocal volume) and **3B**.

The corresponding cross-correlation functions show dramatically different shapes. Analysis of the cross-correlation curve calculated on the full MCS trace for red fluorescence (red curve in **Figure 3E**) led to an estimate of the diffusion coefficient D as ~0.3 $\mu m^2/s$. According to the Stokes-Einstein equation for spherical particles, this value corresponds to diffusing species of radius ~0.7 μm, roughly in keeping with the expected size of the investigated *S. aureus* cells. On the other hand, the cross-correlation curve monitored in the green (green curve in **Figure 3E**) is very similar to the one measured for Hyp-BSA alone (blue curve in **Figure 3E**). The model used to describe the green curve comprises a triplet state (of about 20 μs lifetime) and a diffusion coefficient of 60 $\mu m^2/s$, indicating that the diffusing species emitting green fluorescence is albumin freely diffusing in solution and not attached to the bacteria. If the complex between Hyp and BSA was stable, the

diffusion behavior would have been the same at both wavelengths. This finding suggests that Hyp is downloaded from the protein to the bacterial wall, where it is most likely embedded in the membrane. Red emission arising from bacterial wall is indeed observed for *S. aureus* loaded with Hyp (**Figure 3F**). The STED image shows that the compound is localized on the bacterial wall, with little, if any, internalization. (Delcanale et al., 2015) The same distribution of fluorescence emission is observed when *S. aureus* is incubated with Hyp-apoMb (**Figure 3G**), Hyp-BSA (**Figure 3H**), or Hyp-HSA (not shown). This finding means that the protein is only providing a temporary docking site ensuring good solubilization of the compound in the buffered solution. When the complexes are in presence of bacteria, Hyp is exchanged to the bacterial wall, where it finds a better environment within the membrane.

Analysis of the TCSPC photon histogram built from the full MCS trace measured for the red emission in the presence of *S. aureus* (**Figure 3D**) afforded biexponential decays similar to those observed for Hyp-BSA in PBS solutions (**Figure 1D**). The retrieved lifetimes and weights are a bit different when the TCSPC histogram is calculated over a range where no large peaks are detected (reproducing the parameters observed for Hyp-BSA), or when restricting the estimate to a high intensity peak (τ_1 = 2.69 ns (38 %) and τ_2 = 5.94 ns (62 %)). The change in lifetime may be due to different interaction experienced by the photosensitizing compound with the bacterial wall.

No substantial changes in the triplet state decay of Hyp-BSA could be observed when the compound was in the presence of *S. aureus* cells (**Figure 2B**). The triplet lifetime τ_T in the presence of *S. aureus* was 21±1 µs for Hyp-BSA, indicating a similar degree of protection of the triplet state from molecular oxygen after binding to the bacteria.

3.5 Bacterial photoinactivation

The use of hypericin in antimicrobial photodynamic therapy is well established, (Rezusta et al., 2012; Yow, Tang, Chu & Huang 2012) and the advantages of using a protein as a biocompatible delivery vehicle have been proven. (Comas-Barceló et al., 2013; Rodríguez-Amigo et al., 2015; Delcanale et al., 2017) To assess the

efficacy of the constructs Hyp-BSA and Hyp-HSA as photosensitizing agents we have tested the compounds in photoinactivating the Gram-positive *Staphylococcus aureus*. The results obtained with Hyp-BSA and Hyp-HSA are compared with those obtained for free Hyp and for Hyp-apoMb under the same experimental conditions. **Figure 4** reports the change in *Staphylococcus aureus* population at increasing light dose in the presence of the different photosensitizing compounds.

The absence of dark-toxicity for all complexes is evident from the lack of reduction in population for bacteria kept in the dark (points corresponding to light dose = 0 J/cm^2 in **Figure 4**). All compounds become phototoxic when exposed to visible light and lead to a dramatic reduction of population upon increasing the applied light dose. At a dose of 15 J/cm^2, the drop in population corresponds to 5 $\log_{10}$ units for Hyp-BSA, a performance similar to the one observed for Hyp-apoMb. At the same light dose, Hyp and Hyp-HSA show an even higher bacterial eradication reaching the value of nearly 8 $\log_{10}$ units. The same performance is observed for Hyp-apoMb at a light dose of roughly 30 J/cm^2, whereas for Hyp-BSA the drop in CFUs remains stable at 5 $\log_{10}$ units also at this light dose.

While the bacterial phototoxicity of complexes of Hyp with serum albumins is not much different from that of free Hyp, a fundamental advantage of the formulation exploiting the proteins as carriers stems from the fact that the photosensitizing molecule is delivered to the cell suspension using a buffered aqueous solution. This is a remarkable improvement over administration of free Hyp in an organic solvent (either DMSO or ethanol) because it is more compatible with the use in a food processing environment and hence holds the potential for industrial applications.

It is worth pointing out the higher efficiency of the complex Hyp-HSA over Hyp-BSA. This finding suggests the possible presence of a specific interaction mechanism, leading to a higher degree of damage inflicted to the microbial cells when Hyp is delivered using HSA. Thus, the use of albumins as drug carriers may bear some interest since specific interactions between albumin and bacterial components, presented on the exterior of the cell wall, may be exploited to improve targeting of bacterial species. One example is protein G-like albumin binding module (GA module), that is found in a family of surface proteins of different bacterial

species. Protein PAB from the anaerobic bacterium *Finegoldia magna* (formerly *Peptostreptococcus magnus*) represents one of these proteins. Protein PAB contains a domain of 53 amino acid residues known as the GA module. GA homologs are also found in protein G of group C and G streptococci. It has been reported that the GA module binds near a cleft located between domain IIA and IIIB of HSA. (Lejon et al., 2004) Interestingly, Hyp binds within domain IIA of HSA, near the interaction site with the GA module. Many Gram-positive bacteria express surface proteins with ability to bind serum proteins. (Navarre & Schneewind 1999) The surface proteins typically contain tandemly repeated serum protein-binding domains with one or several specificities, which often include albumin binding. (Kronvall, Simmons, Myhre & Jonsson 1979; Myhre & Kronvall 1980; Nilvebrant & Hober 2013) It remains to be established whether these specific interactions may be exploited in selectively addressing bacterial contaminations. Unfortunately, STED imaging experiments did not provide a clue to a possible difference in the interaction between albumins and the bacterial wall.

Finally, in view of the potential application in food industry, it is worth noting that Regulation EC N° 1333/2008 on food additives does not consider proteins like albumins, which are natural constituents of food of animal origin, to be food additives. (European Commission 2008) Albumin, the most abundant plasma protein, exhibits excellent gelling and water binding capacity and it is often used to improve texture, sliceability and yield losses of processed meat products. (Parés, Toldrà, Saguer & Carretero 2014) In our study, we demonstrate the use of serum albumins to transport photosensitizers and preserve their antimicrobial action on bacterial suspensions. Future studies aimed at evaluating the efficacy on contaminated food processing material will provide further assessment on industrial applicability.

6. Conclusions

The complex between Hyp and serum albumin represents a highly biocompatible nanostructure preserving bioavailability of the photoactive compound. The photosensitizing properties of the compound allow to obtain a decrease in *Staphylococcus aureus* ATCC 25923 population between 5- and 8- orders of magnitude upon exposure to visible light.

While hypericin shows a photodynamic action that is comparable to those observed for the complexes between hypericin and serum albumins, the low water solubility of the photosensitizer requires the use of organic solvents that may not be acceptable in perspective applications. Thus, the use of a solubilizing agent that is fully biocompatible and easily removed by simple rinsing with water, introduces a remarkable advantage in terms of its practical use.

Moreover, the presence of potential binding sites on proteins exposed on the bacterial wall in several strains may be exploited to improve targeting of the microorganisms.

The protein carrier and the natural product hypericin are fully compatible with applications in food processing environments, especially on food contact surfaces and equipment, and the nanostructure holds the potential to be introduced as an effective disinfectant for food manufacturing and handling materials.

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Figure legends

Figure 1. A. Absorption spectra of 5 μM Hyp solutions in PBS buffer (green), and in the presence of HSA (50 μM, black) or BSA (50 μM, red). The optical path was 0.4 cm. **B.** Fluorescence excitation (solid, λ_{em} = 646 nm) and emission (dashed, λ_{ex} = 551 nm) spectra for 5 μM Hyp solutions in the presence of excess HSA (50 μM, black) or BSA (50 μM, red). **C.** Fluorescence excitation anisotropy for 5 μM Hyp PBS buffered solutions in the presence of HSA (50 μM, black) or BSA (50 μM, red). For comparison, the anisotropy measured for Hyp-apoMb is shown as the green curve. (Delcanale et al., 2015) λ_{em} = 620 nm in all cases. The optical path was 0.4 cm. **D.** Fluorescence decays measured for a 1.3 μM DMSO solution (green), and for 2.6 μM Hyp PBS buffered solutions in the presence of HSA (50 μM, black) BSA (50 μM, red). Excitation was at 365 nm. The blue line is the IRF of the pulsed LED and the gray lines are the results of the reconvolution fits.

Figure 2. A. Triplet-triplet absorption after excitation at 532 nm with a nanosecond pulsed laser for Hyp in ethanol (black), Hyp-BSA (green), and Hyp-HSA (blue). [Hyp] = 10 μM in all cases, [BSA] = 100 μM, [HSA] = 100 μM. Absorbance change was normalized to allow easier comparison of time traces. **B.** Triplet-triplet absorption after excitation at 532 nm with a nanosecond pulsed laser for Hyp-BSA (green) in the presence of *S. aureus*. [Hyp] = 10 μM, [BSA] = 100 μM. Red solid curves are the result of the fitting to a single exponential decay function.

Figure 3 Fluorescence emission in the red (**A**) and in the green (**B**) for a 600 s acquisition on a *S. aureus* suspension loaded with Hyp bound to FITC-BSA. Time bins are 1 ms wide. (Hyp 100 nM, BSA 30 μM, FITC-BSA 100 nM). **C.** Expanded view on selected portions of the MCS traces in A and B. **D.** TCSPC histograms calculated for the full acquisition time in A (red curve) and B (green curve). The black curves are tail fits to a double exponential decay (for the red curve) with lifetimes τ_1 = 2.96 ns (40 %) and τ_2 = 6.10 ns (60 %) or a triple exponential decay (for the green curve) with lifetimes τ_1 = 0.48 ns (60 %), τ_2 = 1.88 ns (29 %) and τ_3 = 4.60 ns (19 %). **E.** Cross-correlation function (red curve) calculated for the trace in panel A. Best fit is obtained with a

diffusional model plus a triplet state decay. The diffusing species is characterized by a diffusion coefficient D = 0.3 $\mu m^2 s^{-1}$ (consistent with diffusing objects the size of *S. aureus*) and the triplet decay by a lifetime of about 20 μs. The green curve is the cross-correlation curve obtained for the trace in panel B. Best fit is obtained with a diffusional model plus a triplet state decay. The diffusing species is characterized by a diffusion coefficient D = 60 $\mu m^2 s^{-1}$, consistent with the expected value for BSA, and the triplet decay has a lifetime of about 20 μs. The blue curve is the cross-correlation curve obtained for Hyp-BSA in PBS buffer (Hyp 100 nM, BSA 30 μM) in the absence of bacteria, monitoring emission in the red. The diffusing species is characterized by a diffusion coefficient D = 60 $\mu m^2 s^{-1}$, indicating that Hyp is bound to BSA. **F.** Selected STED image of *S. aureus* cells in the presence of Hyp (1 μM) collected under excitation at 566 nm and detection at 605\70 nm. The STED beam was at 715 nm, power 30 mW and dwell time 0.05 ms. Scale bar, 2μm. **G.** Selected STED image of *S. aureus* cells in the presence of Hyp-apoMb ([Hyp] = 1 μM, apoMb = 3 μM). Conditions as in F. **H.** Selected STED image of *B. S. aureus* cells in the presence of Hyp (500nM) bound to BSA (5 μM) collected under excitation at 560 nm and detection at 570-670 nm. The STED beam was at 775 nm, power 130 mW and scan speed 8000 Hz. Scale bar, 1μm, 128 averages. Gating windows from 1 ns to 7 ns.

Figure 4. Light dose effects on *Staphylococcus aureus* photoinactivation by Hyp (green triangles, 10 μM) and the complexes between Hyp (10 μM) and BSA (100 μM, red circles), apoMb (30 μM, black squares), or HSA (100 μM, blue triangles).

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