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

3

4 Denise Pezzuoli, ^{#,&} Marco Cozzolino, ^{#,§} Chiara Montali, [#] Lorenzo Brancaleon, [⊥] Paolo Bianchini, [£] Marta
5 Zantedeschi, [#] Silvia Bonardi, [⊗] Cristiano Viappiani^{#,*}, Stefania Abbruzzetti[#]

6

7 [#]Dipartimento di Scienze Matematiche, Fisiche e Informatiche, Università degli Studi di Parma, Parco Area
8 delle Scienze 7/A, 43124 Parma, Italy

9 [⊥]Department of Physics and Astronomy, University of Texas at San Antonio, San Antonio , TX, USA

10 [£]Nanoscopy, Fondazione Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

11 [⊗]Dipartimento di Scienze Medico-Veterinarie, Università degli Studi di Parma, Strada del Taglio 10, 43126
12 Parma, Italy

13 [&]Present address: Dipartimento di Fisica Università degli Studi di Genova, Via Dodecaneso 33, 16146
14 Genova, Italy

15 [§] Present address: Dipartimento di Fisica Università degli Studi di Genova, Via Dodecaneso 33, 16146
16 Genova, Italy, and Fondazione Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

17 ^{*}Corresponding author: Cristiano Viappiani, Dipartimento di Scienze Matematiche, Fisiche e Informatiche,
18 Università degli Studi di Parma, Parco Area delle Scienze 7/A, 43124 Parma, Italy. Email:
19 cristiano.viappiani@unipr.it

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

22 **Abstract**

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

30 1. Introduction

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

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

38 Within this context, *Staphylococcus aureus*, a Gram-positive microorganism of the Micrococcaceae family, is
39 of special interest. It is a commensal and opportunistic pathogen responsible for a wide spectrum of
40 infections in humans, ranging from superficial skin diseases to invasive and potentially life-threatening illnesses.
41 (Lowy 1998; Tong et al., 2015) This ubiquitous microorganism can cause nosocomial and community-
42 acquired infections, as well as food-borne diseases. (Kadariya, Smith & Thapaliya 2014) It can colonize human
43 nasal mucosa and skin as well as environmental surfaces and clothing resulting in a significant potential to
44 contaminate food products during production and handling. (le Loir, Baron & Gautier 2003) Furthermore, *S.*
45 *aureus* is commonly found in a wide variety of food-producing animals, and its transfer to food may occur
46 from dairy animals in case of mastitis (Hennekinne, De Buyser & Dragacci 2011) or contact with live animals
47 by food handlers. Its ability to grow in a wide range of temperatures (7 to 48.5 °C, with optimum 30 to 37
48 °C), pH (4.2 to 9.3, with optimum 7 – 7.5) and high sodium chloride concentration (up to 15%) makes *S. aureus*
49 able to survive in a large variety of foods and in dry environments. (Chaibenjawong & Foster 2011) (le Loir,
50 Baron & Gautier 2003)

51 *S. aureus* food-borne disease is one of the most common worldwide and results from the ingestion of
52 staphylococcal enterotoxins (SEs) preformed in foods by enterotoxigenic strains of the microorganism. More
53 than 20 SEs have been recognized and all of them have superantigenic activity, whereas nearly half of them
54 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,

81 (Jacobson et al., 2001; Kubin et al., 2005) antibacterial, (Kairyte, Lapinskas, Gudelis & Luksiene 2012; Yow,
 82 Tang, Chu & Huang 2012; Comas-Barceló et al., 2013; Nafee et al., 2013; Rodríguez-Amigo et al., 2015) and
 83 antifungal agent. (Rezusta et al., 2012) The molecule emits an intense fluorescence in polar organic solvents
 84 ($\Phi_F = 0.35$ in ethanol (Duràn & Song 1986; López-Chicón et al., 2012) and in DMSO (English et al., 1997)),
 85 and sensitizes singlet oxygen with high yield ($\Phi_\Delta = 0.32$ in ethanol, (Darmanyan, Burel, Eloy & Jardon 1994)
 86 0.39 ± 0.01 in methanol, (Roslaniec et al., 2000) and 0.28 ± 0.01 in DMSO (Losi 1997; Delcanale et al., 2015)).
 87 Due to the hydrophobic character of the molecule, aggregates are formed in aqueous solutions,
 88 characterized by a much weaker fluorescence (Yamazaki, Ohta, Yamazaki & Song 1993) and singlet oxygen
 89 yields.
 90 Binding of Hyp to several proteins prevents aggregation of the otherwise insoluble PS in aqueous media.
 91 (Miskovsky et al., 1998; Das et al., 1999; Hritz, Kascakova, Ulicny & Miskovsky 2002; Gbur et al., 2009;
 92 Roelants et al., 2011) Proteins are highly biocompatible and warrant good bioavailability of the photoactive
 93 drug. We recently proposed the use of proteins such as apomyoglobin (apoMb) and β -lactoglobulin as
 94 carriers to deliver Hyp to bacterial cells for antimicrobial photodynamic applications. (Comas-Barceló et al.,
 95 2013; Delcanale et al., 2015; Rodríguez-Amigo et al., 2015; Delcanale et al., 2016; Delcanale et al., 2017)
 96 Serum albumins are interesting additional candidates, given their known capability of binding a variety of
 97 endogenous molecules as well as drugs. (Fasano et al., 2005; Varshney et al., 2010) A well-studied example
 98 is human serum albumin (HSA), which represents the most prominent protein in plasma. HSA binds different
 99 classes of ligands at distinct sites which can affect the pharmacokinetics of many drugs and influence the
 100 reactivity of bound compounds. At the same time, HSA can act as carrier in the mechanism of disposal of
 101 potentially harmful molecules. (Fanali et al., 2012)
 102 The interaction of Hyp with serum albumins has been described in the literature, in an attempt to devise a
 103 photosensitizing agent suitable for tumor or antiviral PDT. (Senthil, Longworth, Ghiron & Grossweiner 1992;
 104 Miskovsky 2002) Early competition experiments suggested that the binding site of Hyp is in the IIA subdomain
 105 of the protein, (Falk & Meyer 1994; Köhler et al., 1996) a fact later confirmed by Resonance Raman and
 106 surface-enhanced Raman spectroscopy. (Miskovsky et al., 1998) A subsequent thorough investigation where

107 vibrational spectroscopies and molecular modeling were employed, allowed to draw a structural model for
108 the binding site and characterize specific interactions with amino acid residues for human and bovine (BSA)
109 serum albumins. (Miskovsky et al., 2001) Interestingly, the complex between HSA and Hyp appears to be
110 remarkably stable towards photobleaching upon prolonged exposure to visible light. (Uzdensky, Iani, Ma &
111 Moan 2002) Hyp was studied in the presence of different biological systems: bound to human serum albumin,
112 in cultured human adenocarcinoma WiDr cells and in the skin of nude mice. Hypericin was reported to be
113 more photostable than photosensitizers like mTHPC and Photofrin that are commonly used in PDT.
114 Serum albumins represent an interesting starting material for the development of nanostructured
115 macromolecular assemblies endowed with specific functionalities. HSA constitutes about 50% of the protein
116 present in the plasma of normal healthy individuals, (Evans 2002) corresponding to a concentration ranging
117 between 33 and 52 g L⁻¹. (Boldt 2010) Similarly, BSA is the most abundant protein in bovine plasma with a
118 typical concentration of 50 g L⁻¹. Purified bovine albumin is used to help replenish blood or fluid loss in
119 animals. It is used in testing for the Rh factor in human beings, and as a stabilizer for vaccines. It is also used
120 in antimicrobial sensitivity tests. (Jayathilakan, Sultana, Radhakrishna & Bawa 2012) One remarkable
121 advantage of BSA is the low production cost, as large amounts of the protein can be easily purified from
122 bovine blood, which is a byproduct of the cattle industry.

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

128

129 **2. Materials and Methods**

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

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

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

137

138 **2.1 General spectroscopic instrumentation**

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

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

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

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

156 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 ($\Phi_T = 0.32$ (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

170 **2.4 STED nanoscopy**

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

182

183 **2.5 Microbial strains and growth conditions**

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

186

187 **2.6 Bacterial photoinactivation**

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

191 Irradiation of bacterial colonies was conducted using a RGB LED light source (LED par 64 short, Show Tec
192 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/cm² 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/cm², 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

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

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

267 The fluorescence quantum yield for the complexes can be estimated using Hyp-apoMb as a reference ($\Phi_F =$
 268 0.14 (Delcanale et al., 2015)) and is readily evaluated as $\Phi_F = 0.11$ and $\Phi_F = 0.12$ for Hyp-BSA and Hyp-HSA,
 269 respectively.

270 The increase in fluorescence upon binding allows to determine the association constant. (Comas-Barceló et
 271 al., 2013) Binding of Hyp to albumins occurs with moderate affinity. The dissociation constant for HSA-Hyp is
 272 $K_d = (1.1 \pm 0.2) \times 10^{-5}$ M and for BSA-Hyp is $K_d = (2.1 \pm 0.2) \times 10^{-5}$ M. The value we retrieved for the dissociation
 273 constant from HSA is lower (about 8-fold) than a previous determination. (Senthil, Longworth, Ghiron &
 274 Grossweiner 1992)

275

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

281 Fluorescence emission decays for Hyp-HSA and for Hyp-BSA solutions were measured by collecting the
 282 emission at 600 nm after pulsed excitation at 365 nm. Sample curves are reported in **Figure 1D**. Unlike the
 283 case of ethanol or DMSO solutions, where fluorescence emission decays with a single exponential relaxation,
 284 (López-Chicón et al., 2012) (Comas-Barceló et al., 2013) (Delcanale et al., 2015) the decay for albumin-bound
 285 Hyp is best described by a biexponential function. The retrieved lifetimes are $\tau_1 = 2.98$ ns (45 %) and $\tau_2 = 5.52$
 286 ns (55 %) for Hyp-HSA and $\tau_1 = 3.13$ ns (23 %) and $\tau_2 = 6.11$ ns (77 %) for Hyp-BSA. In both cases Hyp

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

293

294 **3.2 Labeling of albumins with FMA**

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

304 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,
305 these values decrease to 3.44 ns and 4.21 ns, respectively. The FRET efficiency can be calculated as 0.039 for
306 HSA-FMA and 0.058 for BSA-FMA. From the spectral data it is possible to estimate the Förster radius as $R_0 =$
307 1.54 nm. Using the FRET efficiencies reported above, the distance between FMA and Hyp is estimated as ~ 2.5
308 nm BSA-FMA and ~ 2.6 nm for HSA-FMA, in keeping with the expected values.

309 It is worth observing that the affinity for Hyp is not affected by the presence of FMA. The added value of
310 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, $\tau_T = (40 \pm 6) \mu s$, is longer than the one of BSA-bound Hyp, $\tau_T = (17$
319 $\pm 3) \mu s$, 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 ($11.6 \pm 0.1 \mu s$) (Delcanale et al., 2015) and β -lactoglobulin (10 ± 2)
322 μs , (Delcanale et al., 2017) where shielding from the solvent appears less effective.

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

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 $20 \mu s$) 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.

335

336 **3.4 Interaction with bacterial cells**

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

343 The fluorescence intensity time-traces (MCS traces) monitored in the red show large intensity peaks (**Figure**
344 **3A**), which correspond to bacteria going through the confocal volume of the microscope, over a small
345 background. The low intensity background reflects the presence of a very small fraction of unbound Hyp-BSA
346 molecules. The very slow diffusing species at low (\sim 5 nM) concentration, giving rise to the spikes, is identified
347 with bacteria, decorated with several copies of Hyp-BSA. When the green emission is monitored instead
348 (**Figure 3B**), the MCS trace is devoid of the large spikes. **Figure 3C** shows an expanded view on selected
349 portions of the MCS traces reported in **Figures 3A** (in a region corresponding to bacteria passing through the
350 confocal volume) and **3B**.

351 The corresponding cross-correlation functions show dramatically different shapes. Analysis of the cross-
352 correlation curve calculated on the full MCS trace for red fluorescence (red curve in **Figure 3E**) led to an
353 estimate of the diffusion coefficient D as \sim 0.3 μ m²/s. According to the Stokes-Einstein equation for spherical
354 particles, this value corresponds to diffusing species of radius \sim 0.7 μ m, roughly in keeping with the expected
355 size of the investigated *S. aureus* cells. On the other hand, the cross-correlation curve monitored in the green
356 (green curve in **Figure 3E**) is very similar to the one measured for Hyp-BSA alone (blue curve in **Figure 3E**).
357 The model used to describe the green curve comprises a triplet state (of about 20 μ s lifetime) and a diffusion
358 coefficient of 60 μ m²/s, indicating that the diffusing species emitting green fluorescence is albumin freely
359 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 ($\tau_1 = 2.69$ ns (38 %) and $\tau_2 = 5.94$ 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 μ s for Hyp-BSA, indicating a similar degree of protection of the triplet state from molecular oxygen after binding to the bacteria.

380

381 **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

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

390 The absence of dark-toxicity for all complexes is evident from the lack of reduction in population for bacteria
391 kept in the dark (points corresponding to light dose = 0 J/cm² in **Figure 4**). All compounds become phototoxic
392 when exposed to visible light and lead to a dramatic reduction of population upon increasing the applied
393 light dose. At a dose of 15 J/cm², the drop in population corresponds to 5 log₁₀ units for Hyp-BSA, a
394 performance similar to the one observed for Hyp-apoMb. At the same light dose, Hyp and Hyp-HSA show an
395 even higher bacterial eradication reaching the value of nearly 8 log₁₀ units. The same performance is
396 observed for Hyp-apoMb at a light dose of roughly 30 J/cm², whereas for Hyp-BSA the drop in CFUs remains
397 stable at 5 log₁₀ units also at this light dose.

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

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

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

421 Finally, in view of the potential application in food industry, it is worth noting that Regulation EC N°
422 1333/2008 on food additives does not consider proteins like albumins, which are natural constituents of food
423 of animal origin, to be food additives. (European Commission 2008) Albumin, the most abundant plasma
424 protein, exhibits excellent gelling and water binding capacity and it is often used to improve texture,
425 sliceability and yield losses of processed meat products. (Parés, Toldrà, Saguer & Carretero 2014) In our study,
426 we demonstrate the use of serum albumins to transport photosensitizers and preserve their antimicrobial
427 action on bacterial suspensions. Future studies aimed at evaluating the efficacy on contaminated food
428 processing material will provide further assessment on industrial applicability.

429 **6. Conclusions**

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

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

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

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

444

445

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451

452

453 **Figure legends**

454 **Figure 1. A.** Absorption spectra of 5 μ M Hyp solutions in PBS buffer (green), and in the presence of HSA (50
455 μ M, black) or BSA (50 μ M, red). The optical path was 0.4 cm. **B.** Fluorescence excitation (solid, $\lambda_{em} = 646$ nm)
456 and emission (dashed, $\lambda_{ex} = 551$ nm) spectra for 5 μ M Hyp solutions in the presence of excess HSA (50 μ M,
457 black) or BSA (50 μ M, red). **C.** Fluorescence excitation anisotropy for 5 μ M Hyp PBS buffered solutions in the
458 presence of HSA (50 μ M, black) or BSA (50 μ M, red). For comparison, the anisotropy measured for Hyp-
459 apoMb is shown as the green curve. (Delcanale et al., 2015) $\lambda_{em} = 620$ nm in all cases. The optical path was
460 0.4 cm. **D.** Fluorescence decays measured for a 1.3 μ M DMSO solution (green), and for 2.6 μ M Hyp PBS
461 buffered solutions in the presence of HSA (50 μ M, black) BSA (50 μ M, red). Excitation was at 365 nm. The
462 blue line is the IRF of the pulsed LED and the gray lines are the results of the reconvolution fits.

463
464 **Figure 2. A.** Triplet-triplet absorption after excitation at 532 nm with a nanosecond pulsed laser for Hyp in
465 ethanol (black), Hyp-BSA (green), and Hyp-HSA (blue). [Hyp] = 10 μ M in all cases, [BSA] = 100 μ M, [HSA] =
466 100 μ M. Absorbance change was normalized to allow easier comparison of time traces. **B.** Triplet-triplet
467 absorption after excitation at 532 nm with a nanosecond pulsed laser for Hyp-BSA (green) in the presence of
468 *S. aureus*. [Hyp] = 10 μ M, [BSA] = 100 μ M. Red solid curves are the result of the fitting to a single exponential
469 decay function.

470
471 **Figure 3** Fluorescence emission in the red (**A**) and in the green (**B**) for a 600 s acquisition on a *S. aureus*
472 suspension loaded with Hyp bound to FITC-BSA. Time bins are 1 ms wide. (Hyp 100 nM, BSA 30 μ M, FITC-BSA
473 100 nM). **C.** Expanded view on selected portions of the MCS traces in A and B. **D.** TCSPC histograms calculated
474 for the full acquisition time in A (red curve) and B (green curve). The black curves are tail fits to a double
475 exponential decay (for the red curve) with lifetimes $\tau_1 = 2.96$ ns (40 %) and $\tau_2 = 6.10$ ns (60 %) or a triple
476 exponential decay (for the green curve) with lifetimes $\tau_1 = 0.48$ ns (60 %), $\tau_2 = 1.88$ ns (29 %) and $\tau_3 = 4.60$ ns
477 (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 $D = 0.3 \mu\text{m}^2\text{s}^{-1}$ (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 $D = 60 \mu\text{m}^2\text{s}^{-1}$, 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 $D = 60 \mu\text{m}^2\text{s}^{-1}$, 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, 2 μm . **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, 1 μm , 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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