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Zinc-substituted myoglobin is a naturally occurring photoantimicrobial agent with potential applications in food decontamination

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1 Zinc-substituted myoglobin is a naturally occurring  
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3 in food decontamination

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18 oxygen

19 ABSTRACT Zinc-substituted myoglobin (ZnMb) is a naturally-occurring photosensitizer that  
20 generates singlet oxygen with a high quantum yield. Using a combination of photophysical and  
21 fluorescence imaging techniques, we demonstrate the interaction of ZnMb with Gram positive  
22 *Staphylococcus aureus* and Gram negative *Escherichia coli*. An efficient antibacterial action  
23 against *S. aureus* was observed, with a reduction up to 99.9999% in the number of colony  
24 forming units, while no sizeable effect was detected against *E. coli*. Since ZnMb is known to  
25 form during the maturation of additive-free not-cooked cured ham, the use of this protein as  
26 built-in photodynamic agent may constitute a viable method for the decontamination of these  
27 food products from Gram-positive bacteria.

28

## 29 **Introduction**

30 Effective decontamination of food and food processing tools and environments is one of the  
31 major open issues in current food science. Novel methods are currently being searched with  
32 minimal effects on food quality while still warranting the complete removal of dangerous  
33 pathogens. In this context, antibacterial photosensitization-based treatments are gaining attention  
34 thanks to their unique properties. <sup>1,2</sup> Photosensitization is a light-activated reaction where a  
35 photo-excited chromophore, called a photosensitizer (PS), reacts with molecular oxygen (O<sub>2</sub>) to  
36 produce reactive oxygen species (ROS). The cytotoxicity of the ROS produced by  
37 photosensitization can be usefully exploited to inactivate microbial cells. <sup>3,4</sup> Among ROS, the  
38 non-radical and non-ionic electronically-excited state of the dioxygen molecule, called singlet  
39 oxygen (<sup>1</sup>O<sub>2</sub>), plays a crucial role in the photo-induced damage of the cells. <sup>1</sup>O<sub>2</sub> is indeed highly  
40 reactive and rapidly oxidizes substrates like membrane lipids, proteins or nucleic acids, when  
41 produced in or near a cell. <sup>5</sup> An additional strength of the photodynamic approach relies on the  
42 observation that no resurgence of photo-resistant species occurs even after multiple exposures. <sup>3,6</sup>

43 The efficacy of a PS is highly dependent on its bioavailability and biocompatibility, as well as  
44 on its affinity for the target cells. Novel materials and biomaterials have recently become  
45 available with properties that comply with these requirements and, in addition, enable their  
46 detection and monitoring. <sup>7</sup>

47 Despite PSs can be found in several classes of molecules, <sup>8</sup> only a few examples of protein-  
48 based PS have been reported. Variants of the Green Fluorescent Protein (GFP) family, <sup>9,10</sup> the  
49 protein “killer RED” <sup>11 12</sup> and the flavoprotein “mini Singlet Oxygen Generator” (miniSOG) <sup>13,14</sup>  
50 are relevant examples of genetically-encoded PS, but characterized by a low quantum yield of  
51 <sup>1</sup>O<sub>2</sub> production ( $\Phi_{\Delta}$ =0.004±0.001 for Enhanced-GFP and 0.03±0.01 for miniSOG). An

52 alternative approach is the use of protein-based carriers as they may confer water-solubility and  
53 bio-compatibility to hydrophobic PSs, along with the benefits of a nano-metric size. Widespread  
54 proteins like apo-myoglobin (apoMb), i.e. the protein portion of myoglobin (Mb), or  $\beta$ -  
55 lactoglobulin ( $\beta$ LG) have been recently proposed as carriers for the natural hydrophobic PS  
56 hypericin (Hyp).<sup>15,16</sup> The binding of Hyp to the hydrophobic pocket of these proteins leads to a  
57 remarkable increase of its quantum yields of  $^1\text{O}_2$  photosensitization ( $\Phi_{\Delta} \approx 0.1$ ) and fluorescence  
58 emission ( $\Phi_F$ ) in biological media. The latter property was recently exploited to collect  
59 STimulated Emission Depletion (STED) images of PS loaded bacteria, thus allowing a precise  
60 sub-diffraction localization of the PS in living cells.<sup>17</sup> However, the relatively weak interaction  
61 occurring between Hyp and such protein hosts limits the usefulness of these particular complexes  
62 since Hyp can translocate to e.g., serum proteins in real biological systems. In contrast,  
63 Lepeshkevich et al. recently studied the photosensitized production of  $^1\text{O}_2$  from ZnMb, where a  
64 high value for the  $\Phi_{\Delta}$  ( $0.9 \pm 0.1$ ) was obtained by substituting the iron ion in the center of the  
65 heme cofactor of Mb with a Zn(II) ion.<sup>18</sup> In this case, the zinc-protoporphyrin IX cofactor is also  
66 bound to the protein matrix by means of a coordinate bond. It is particularly interesting that zinc-  
67 protoporphyrin IX spontaneously forms as ZnMb<sup>19,20</sup> during the maturation of nitrate/nitrite-free  
68 dry cured ham.<sup>21-24</sup> Since these products are not-cooked and are additive-free, they easily suffer  
69 from bacterial contaminations. In view of the above, we hypothesized that the built-in PS ZnMb  
70 could potentially be exploited as antimicrobial agent for such hams, avoiding the introduction of  
71 exogenous products for disinfection purposes or preservative agents. In this work, we focused on  
72 the study of the interaction of ZnMb with two representative bacteria, Gram-positive  
73 *Staphylococcus aureus* and Gram-negative *Escherichia coli*, and evaluated its photoinactivation  
74 ability.

75

## 76 **Materials and Methods**

77 Zinc-protoporphyrin IX (ZnPP IX) and Mb from horse heart were purchased from Sigma-  
78 Aldrich and used as received.

### 79 *apoMb preparation and reconstitution with ZnPP IX*

80 The apo-form of Mb was prepared removing heme from myoglobin by the methyl ethyl  
81 ketone method.<sup>25</sup> The concentration of the apoMb stock was calculated from the absorption at  
82 280 nm ( $\epsilon=15\ 800\ \text{cm}^{-1}\ \text{M}^{-1}$ ), while residual heme concentration was estimated from the  
83 absorption at 408 nm ( $\epsilon = 179\ 000\ \text{cm}^{-1}\ \text{M}^{-1}$ ).<sup>26</sup> In all preparations, heme contamination was  
84 typically 1% of the total protein content.

85 To reconstitute ZnPP IX into apoMb, ZnPP IX was dissolved in a 10 mM NaOH solution and  
86 added dropwise to a Phosphate Buffer Saline (PBS) solution of apoMb until an equimolar  
87 concentration was reached. The whole procedure was carried out at 4 °C under dim light and the  
88 solution was kept in the dark with continuous and gentle stirring for 24 h. The sample was then  
89 centrifuged and the supernatant was dialyzed against a PBS buffer solution. The obtained ZnMb  
90 stock solution was spectroscopically checked to assess sample purity. A 1:1 stoichiometry  
91 between ZnPP IX and apoMb was regularly observed. The concentration was calculated from the  
92 absorption at 554 nm ( $\epsilon=10\ 400\ \text{cm}^{-1}\ \text{M}^{-1}$ ).<sup>18</sup>

### 93 *General spectroscopic instrumentation*

94 Absorption spectra recorded using a Jasco V-650 (Jasco Europe, Carpi, Italy). Fluorescence  
95 spectra were recorded using a Spex-Fluoromax 4 (Horiba Jobin Yvon, Edison, NJ) or a Perkin  
96 Elmer LS50 spectrofluorometers (PerkinElmer, Waltham, MA).

### 97 *Fluorescence and transmitted light microscopy*

98 Cell suspensions in sterile PBS were drop-casted on a coverslip and imaged by means of an  
99 A1r MP NIKON confocal microscope (Nikon Instruments, Tokyo, Japan). The samples were  
100 excited at 561 nm focusing a laser beam through a Plan Apo vc 100× 1.4NA oil immersion  
101 objective. The fluorescence was collected by the same lens and detected in the spectral window  
102 between 600 and 670 nm by means of a GaAsP photomultiplier tube (PMT), while the  
103 transmitted laser light was detected by a PMT through a condenser lens.

#### 104 ***Fluorescence correlation spectroscopy and time-resolved fluorescence***

105 Fluorescence Correlation Spectroscopy (FCS) and time-resolved fluorescence (TRF)  
106 experiments were performed using a Microtime 200 system from PicoQuant, based on an  
107 inverted confocal microscope (Olympus IX70) and equipped with two single photon avalanche  
108 diodes (SPADs). Excitation was achieved by a 475 nm picosecond diode laser. Fluorescence  
109 emission by ZnMb was collected through a bandpass filter (650 nm-700 nm) and split with a  
110 50/50 splitter between the two detection channels. A time-correlated single photon counting  
111 (TCSPC) operation mode was used for TRF measurements; and a cross-correlation mode was  
112 used for FCS measurements. In order to study the interaction of ZnMb with bacteria, either *E.*  
113 *coli* or *S. aureus* suspensions were added to the protein solution. Bacteria suspensions with an  
114 optical density at 600 nm corresponding to 0.4 were further diluted 100-fold. Solutions were  
115 incubated for 10 minutes before performing the experiment.

#### 116 ***Laser flash photolysis (LFP)***

117 Triplet state decays of ZnMb were monitored at 465 nm after photoexcitation with the second  
118 harmonic (532 nm) of a nanosecond Nd:YAG laser (Spectron Laser) using a previously  
119 described setup.<sup>27</sup> The effect on triplet state due to the interaction between ZnMb and bacteria  
120 was assessed by diluting ZnMb (final concentration 2 or 5 μM) in bacteria suspensions (*E. coli*

121 or *S. aureus*) with an optical density of 0.4 at 600 nm. Solutions were incubated for 10 minutes  
122 before performing the experiment.

### 123 ***Singlet oxygen measurements***

124 Time-resolved near-infrared spectroscopy (TRNIR) was used to monitor <sup>1</sup>O<sub>2</sub> phosphorescence  
125 at 1275 nm using a modified PicoQuant Fluotime 200 system. The setup uses a diode-pumped  
126 pulsed Nd:YAG laser for excitation (FTSS355-Q, Crystal Laser, Berlin, Germany; 1kHz  
127 repetition rate, λ<sub>exc</sub> = 532 nm, 1.2 μJ per pulse) and a photon counting module (H9170-45 NIR-  
128 PMT, Hamamatsu) coupled to a multichannel scaler (NanoHarp 250, PicoQuant, Germany) for  
129 detection. The time-resolved phosphorescence signals were fitted with equation (1):

$$130 \quad S = S_0 \frac{\tau_\Delta}{\tau_\Delta - \tau_T} (e^{-t/\tau_\Delta} - e^{-t/\tau_T}) + y_0 \quad (1)$$

131 where τ<sub>T</sub> and τ<sub>Δ</sub> are the lifetime of the photosensitizer triplet state and of <sup>1</sup>O<sub>2</sub> respectively, y<sub>0</sub> is  
132 an offset due to instrument dark counts and s<sub>0</sub> is an instrumental quantity proportional to Φ<sub>Δ</sub><sup>28</sup>.

### 133 ***Microbial strains and growth conditions***

134 *S. aureus* CECT 239 and *E. coli* CECT 101, obtained from the Spanish Type Culture  
135 Collection (CECT), were grown overnight in sterile Tryptic Soy Broth (TSB) or in Luria Bertani  
136 medium (LB) at 37°C. Stock inoculum suspensions were prepared in sterile PBS and adjusted to  
137 an optical density of 0.4 at 600 nm.

### 138 ***Photodynamic inactivation of S. aureus and E. coli***

139 Cell suspensions in sterile PBS were incubated for 30 min in the dark at room temperature  
140 with the PS. The final concentration of the PS in the cell suspensions ranged between 0 and 50  
141 μM. Then, 0.3 mL of the suspensions were placed in 96-well plates. The plates were illuminated  
142 from the top with green light (LED, 520 nm) for 15 or 30 min (18 and 37 J cm<sup>-2</sup>, respectively,  
143 measured with a calibrated power meter), serially diluted, seeded on tryptic soy agar, and



144 incubated in the dark for 24 h at 37 °C. Colony-forming units (CFUs) were counted in order to  
145 calculate the survival fraction. Experiments were carried out in duplicate for each condition,  
146 including cell controls without the addition of PS and dark controls.

147

## 148 **Results and discussion**

### 149 **Photophysical properties of ZnMb in PBS solution**

150 The absorption and fluorescence emission spectra of ZnMb in PBS buffer at 20 °C closely  
151 match those reported in the literature.<sup>18</sup> The absorption spectrum of the protein is characterized  
152 by an intense Soret band centered at 428 nm and two Q-bands, about 16-times weaker, centered  
153 at 554 and 595 nm (Figure 1). The fluorescence emission spectrum shows an intense narrow  
154 band with a maximum at 597 nm and a broader, less intense emission band around 650 nm  
155 (Figure 1).

156 Fluorescence decay occurs with a single exponential decay with lifetime  $\tau_F = 2.0 \pm 0.1$  ns (Table  
157 1). The triplet-state lifetime ( $\tau_T$ ), measured by monitoring the transient triplet-triplet absorption  
158 at ~465 nm, ranges from  $26 \pm 1$   $\mu$ s in air-equilibrated PBS to  $13 \pm 1$  ms in deaerated solutions  
159 (Table 1), in agreement with previous determinations.<sup>18,29</sup> Compared to  $\tau_T$  of typical PSs in air-  
160 saturated aqueous solutions (2-3  $\mu$ s),<sup>30</sup>  $\tau_T$  is substantially longer, which indicates that ZnMb is  
161 somewhat more shielded from oxygen, in agreement with the localization of the porphyrin within  
162 the protein cavity.  $\tau_T$  values determined by TRNIR (eq. 1) were in good agreement, with small  
163 differences being due to minor differences in experimental conditions. In turn, the singlet oxygen  
164 decay lifetimes were also consistent with literature values for similar systems.<sup>15,16</sup> All the above  
165 photophysical parameters are likewise consistent with the values previously reported for ZnMb  
166 in 50 mM citrate-phosphate buffer pH=7.4.<sup>18</sup>

167

## 168 **Interaction between ZnMb and bacterial cells**

169 The remarkable  $^1\text{O}_2$  photosensitizing efficiency of ZnMb suggests that this compound could be  
170 exploited to obtain efficient bacterial photoinactivation.

171 Direct demonstration of the interaction between ZnMb and bacterial cells was obtained by  
172 exploiting the fluorescence emission of the compound to collect images through a confocal  
173 microscope. *S. aureus* and *E. coli* suspensions were incubated with 1  $\mu\text{M}$  ZnMb for 10 minutes  
174 prior to image collection. Comparison of the confocal images with those obtained in transmitted  
175 light mode clearly shows accumulation of the fluorescent ZnMb both on *S. aureus* (Figure 2,  
176 panels A-C) and *E. coli* (Figure 3, panels A-C). Washed samples show a substantial reduction of  
177 the protein's fluorescence from both *S. aureus* (Figure 2, panels D-F) and *E. coli* (Figure 3,  
178 panels D-F), which becomes barely distinguishable from the background. This fact indicates that  
179 the interaction between the protein and the bacterial cell wall is rather weak, ZnMb  
180 internalization being negligible.

181 Incidentally, we noticed that attempts to exploit the inherent fluorescence emission by ZnMb  
182 to reach subdiffraction resolution using a STED microscope failed, possibly because of the low  
183 fluorescence yield and transient absorption properties of the compound.

184 Further evidence for the existence of spontaneous interactions between ZnMb and bacterial  
185 cells was provided by FCS measurements. The fluorescence intensity time-traces from *E. coli*  
186 and *S. aureus* suspensions incubated with ZnMb ( $\sim 1 \mu\text{M}$ ) were characterized by several spikes,  
187 corresponding to very slow diffusing species at low ( $\sim \text{nM}$ ) concentration, that are reasonably  
188 identified with bacteria, decorated with several copies of ZnMb. An analysis of the cross-  
189 correlation curves calculated on the spikes led to an estimate of the diffusion coefficient  $D$  for

190 these species:  $(0.10 \pm 0.05) \mu\text{m}^2/\text{s}$  and  $(0.3 \pm 0.1) \mu\text{m}^2/\text{s}$  for ZnMb incubated with *E. coli* and *S.*  
191 *aureus*, respectively (Table 1). According to the Stokes-Einstein equation for spherical particles,  
192 these values correspond to diffusing species of radius  $\sim 2$  and  $\sim 0.7 \mu\text{m}$ , respectively, roughly in  
193 keeping with the expected size of the investigated bacterial cells. The radius for *E. coli* must be  
194 considered as a rough estimate, since the bacteria are rod shaped and not spherical.

195 No substantial changes in the photophysical properties of ZnMb could be observed when the  
196 compound was bound to the cells (Table 1), except for a slight increase of the triplet lifetime  $\tau_T$   
197 in the presence of *S. aureus* (from  $26 \pm 1 \mu\text{s}$  to  $34 \pm 3 \mu\text{s}$ ). The larger value of  $\tau_T$  may result from a  
198 reduced accessibility of  $\text{O}_2$  to the protein pocket, possibly caused by an interaction with the cell  
199 wall. The value of  $\tau_T$  for ZnMb incubated with *E. coli* is  $29 \pm 3 \mu\text{s}$ , larger but still consistent with  
200 the one obtained in the absence of bacteria. The assignment of the transient absorption to the  
201 triplet state was confirmed by the increase of its lifetime upon removal of oxygen from samples  
202 (Table 1). ). It should be noted that a related porphyrin photosensitiser, Photofrin, showed a  
203 shorter triplet lifetime ( $6 \mu\text{s}$ ) when bound to *S. aureus*, which suggests that the ZnPPIX  
204 chromophore remains bound to myoglobin.<sup>31</sup>

205 Figure 4 compares the fluorescence emission spectra and the  $^1\text{O}_2$  phosphorescence kinetics  
206 obtained for ZnMb in solution and incubated with bacterial cells. The results are similar for both  
207 *S. aureus* (panels A - B) and *E. coli* (panels C - D): after the incubation with the cells (blue  
208 curves), both fluorescence emission and  $^1\text{O}_2$  phosphorescence kinetics are substantially  
209 unchanged with respect to ZnMb in PBS (black curves). Thus, the protein's ability to  
210 photosensitize the production of  $^1\text{O}_2$  appears to be unaltered by the presence of the cells.

211 Additional experiments were carried out washing the sample after the incubation period, by  
212 means of centrifugation and re-suspension of the pellet in fresh PBS buffer. No sizeable ZnMb

213 fluorescence nor  $^1\text{O}_2$  phosphorescence emission was detected for the washed samples (red  
214 curves), confirming the conclusions of the imaging data, namely that the interaction between  
215 ZnMb and the bacteria is relatively weak.

216 Accordingly, fluorescence emission from the supernatant (green curves) is almost  
217 indistinguishable from that observed before centrifugation, an indication that ZnMb dissociated  
218 from the bacterial wall upon centrifugation.

219

## 220 **Photodynamic inactivation**

221 Photodynamic inactivation of bacteria by green-light irradiation of ZnMb was tested at  
222 different protein concentrations and at different light fluences for *S. aureus* (Figure 5 panel A  
223 and B) and *E. coli* (Figure 5 panels C and D). A substantial difference in the efficacy of the  
224 photo-inactivation on the two strains can be immediately recognized: while *S. aureus* were  
225 efficiently inactivated, with a reduction in the number of bacterial colony forming units (CFU)  
226 up to 6 logarithmic units, *E. coli* was not affected by the photodynamic treatment. The reason for  
227 this opposite response of the two strains is very likely related to the different structure of the  
228 corresponding bacterial cell walls.

229

230 The Gram-negative *E. coli* are characterized by a more complex cell wall in comparison to the  
231 Gram-positive *S. aureus*, with an outer impermeable membrane that counteracts the action of the  
232 photosensitizer, by preventing the diffusion to the sensitive inner membrane<sup>32</sup>. Given the short  
233 diffusion of  $^1\text{O}_2$  in the cellular environment, limited to at most ~200 nm from the site of  
234 photosensitization,<sup>33</sup>  $^1\text{O}_2$  that is produced on the surface of *E. coli* cells is unable to induce a

235 lethal damage, probably because the outer membrane is more protective against the produced  
236  $^1\text{O}_2$ .

237 Despite the observed accumulation of ZnMb on both cells types, the PS is able to  
238 photoinactivate only the Gram positive *S. aureus*, as reported for other protein based PSs, like the  
239 complexes between Hyp and apoMb,<sup>16 17</sup> or  $\beta$ -lactoglobulin.<sup>15</sup> Unlike the developed protein  
240 based PSs, there are several examples that proved effective against Gram negative bacteria,  
241 including, e.g. cationic PSs like methylene blue and toluidine blue, derivatives of  
242 phthalocyanines, chlorins, porphyrins, chlorophyll, functionalized fullerenes and nanoparticles.  
243<sup>32</sup>

244 As discussed above, this result is consistent with the presence of a weak interaction between  
245 ZnMb and the cells leading to a localization of the protein only at the cells surface. Since the  
246 isoelectric point of myoglobin is 7.2, the net charge on the protein is essentially zero (-0.5).  
247 Thus, ionic interactions between the protein and the bacteria wall surface are not expected to  
248 influence substantially the binding process.

249 *S. aureus* disinfection (3-logs CFU reduction, 99.9%) could be achieved with a ZnMb  
250 concentration of 3  $\mu\text{M}$  and a light fluence of 18  $\text{J}/\text{cm}^2$ . The most efficient conditions for *S.*  
251 *aureus* photo-inactivation were obtained by increasing the protein concentration to 20  $\mu\text{M}$  and  
252 the light fluence to 37  $\text{J}/\text{cm}^2$ , which allowed to decrease the *S. aureus* population by 99.9999% (6  
253 logs), without appreciable dark toxicity. The administration of an even higher concentration of  
254 ZnMb (50  $\mu\text{M}$ ) induced some dark toxicity and resulted less efficient in the photo-inactivation  
255 than the lower concentrations, particularly for the lower light dose. This effect was attributed to a  
256 screening (inner filter) effect due to the major absorption of light by the protein in solution, as  
257 already observed for other systems with high PS concentration.<sup>34</sup>

258 Importantly, already at the lowest ZnMb concentration employed (3  $\mu\text{M}$ ) the reduction in CFU  
259 amounts to 4-log units at 18  $\text{J}/\text{cm}^2$ , and reaches 5-log units at 37  $\text{J}/\text{cm}^2$ . This concentration is  
260 comparable to the one estimated in ham, ranging between 1.6 and 2.8  $\mu\text{M}$ .<sup>22</sup>

261 The above results can be compared with those we have obtained with other protein-based  
262 photosensitizers. The same protein scaffold (apomyoglobin, i.e. the protein portion of myoglobin  
263 where the heme was removed) was used to host the naturally occurring photosensitizer hypericin  
264 in the heme cavity.<sup>16</sup> This complex proved effective against *S. aureus* and was able to induce a  
265 5-log reduction. Similarly, the complex between hypericin and  $\beta$ -lactoglobulin was found to be  
266 effective on *S. aureus*, leading to a 5-log decrease upon exposure to a light dose of 20 J.<sup>15</sup>

267 The photodynamic action of ZnMb on *S. aureus* can be compared to the effect reported for the  
268 cofactor ZnPP IX in the absence of a carrier by Ishikawa *et al.*<sup>35</sup> Under experimental conditions  
269 that are similar to ours, the survival of *S. aureus* appears to be a bit higher, possibly because of  
270 the tendency of ZnPP IX towards aggregation and the presence of two negative charges on its  
271 structure, that preclude cell-wall binding due to electrostatic repulsion. Thus, we conclude that  
272 the use of the protein carrier leads to an improvement in the efficiency of ZnPP IX alone.

273 The above results demonstrate that ZnMb is a promising natural photosensitizing agent that is  
274 found in cured ham. It is endowed with a very large capacity to photosensitize the production of  
275  $^1\text{O}_2$  upon exposure to harmless visible light. It spontaneously accumulates on bacterial cells of *S.*  
276 *aureus* and *E. coli* species and is an effective PDT agent capable of reducing the population of  
277 Gram-positive *S. aureus* up to 6 log CFU with no appreciable dark toxicity. Conversely, the  
278 Gram-negative *E. coli* was unaffected by the PDT treatment with ZnMb in all the conditions  
279 tested. This study demonstrates the possibility to employ ZnMb as a bio-compatible PS for the  
280 inactivation of Gram-positive bacteria. This nano-metric size, bio-compatible protein structure is

281 particularly interesting for its potential application in the decontamination of non-cooked,  
282 additive-free cured ham because it is spontaneously produced during the maturation.  
283 Experiments with contaminated food (hams) and food handling materials will assess the  
284 feasibility of the approach in industrial applications.

285

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### 291 **Author Contributions**

292 The manuscript was written through contributions of all authors. All authors have given approval  
293 to the final version of the manuscript.

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403

404 **Figure Captions**

405 **Figure 1.** Absorption (blue) and fluorescence emission (black) spectra for ZnMb (4  $\mu$ M) in PBS  
406 buffer. T=20°C.

407

408 **Figure 2.** Comparison of selected images acquired using a confocal microscope: transmitted  
409 light (A, D); confocal fluorescence (B, E) exploiting the emission of ZnMb under excitation at  
410 561 nm and detection at 600-670 nm; transmitted light and fluorescence overlay (C, F) of the  
411 portions marked in A and D. Cell contours are marked with a dashed line. Pixel size 62x62nm;  
412 pixel dwell time 42  $\mu$ s. A – B: *S. aureus* cells incubated with 1 $\mu$ M ZnMb; C – D: *S. aureus* cells  
413 incubated with 1  $\mu$ M ZnMb and washed by means of centrifugation. The color bars are in  
414 arbitrary units. The scale bars are 5  $\mu$ m.

415

416 **Figure 3.** Comparison of selected images acquired using a transmitted light (A, D) and a  
417 confocal fluorescence (B, E) microscope exploiting the emission of ZnMb under excitation at  
418 561 nm and detection at 600-670 nm; transmitted light and fluorescence overlay (C, F) of the  
419 portions marked in A and D. Cell contours are marked with a dashed line. Pixel size 62x62nm;  
420 pixel dwell time 42  $\mu$ s. A – B: *E. coli* cells incubated with 1  $\mu$ M ZnMb; C – D: *E. coli* cells  
421 incubated with 1 $\mu$ M ZnMb and washed by means of centrifugation. The colors bar are in  
422 arbitrary unit. The scale bars are 5  $\mu$ m.

423

424 **Figure 4:** Comparison of fluorescence emission spectra (panels A and C) and time-resolved  
425 singlet oxygen phosphorescence traces (panels B and D) for ZnMb in PBS buffer (black), ZnMb  
426 incubated with *S. aureus* (blue, panels A and B) or *E. coli* (blue, panels C and D) and ZnMb  
427 incubated with bacteria and washed (red). A fluorescence spectrum for the supernatant is  
428 reported in green in panel A. Fit curves for phosphorescence kinetics are reported for ZnMb in  
429 PBS (yellow) and ZnMb incubated with bacteria (orange). All samples are air-equilibrated at  
430 room temperature with  $[ZnMb] = 10 \mu M$ . Fluorescence spectra are collected under excitation at  
431 552 nm. Phosphorescence kinetics are collected under excitation at 532 nm, detection at 1270  
432 nm, with 256 ns resolution and 10 min acquisition at 1 kHz repetition rate.

433

434 **Figure 5.** Light fluence (at 520 nm) effect on *S. aureus* (A) and *E. coli* (C) incubated with ZnMb  
435 3  $\mu M$  (red), 10  $\mu M$  (green), 20  $\mu M$  (blue), 50  $\mu M$  (magenta). Control experiments are performed  
436 without addition of ZnMb (black). ZnMb concentration effect on *S. aureus* (B) and *E. coli* (D) at  
437 18  $J/cm^2$  (orange) and 37  $J/cm^2$  (violet) light fluences. Control experiments are performed in the  
438 dark (black).

439

440 **Table 1.** Photophysical parameters for ZnMb in PBS buffer and ZnMb incubated with *E. coli*  
 441 and *S. aureus*.

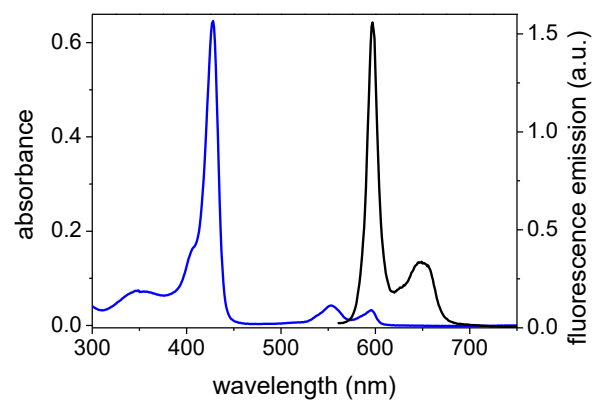
Sample	$\tau_F$ (ns)	D ( $\mu\text{m}^2/\text{s}$ )	$\tau_T$ ( $\mu\text{s}$ )	$\tau_T$ (ms)	$\tau_\Delta$ ( $\mu\text{s}$ )
			air equilibrated	nitrogen saturated	
ZnMb	$2.0 \pm 0.1^{\S}$		$26 \pm 1^*$	$13 \pm 1^*$	$2.6 \pm 0.3^{\xi}$
			$19 \pm 2^{\xi}$		
ZnMb with <i>E. coli</i>	$2.1 \pm 0.1^{\S}$	$0.12 \pm 0.02^{\S}$	$29 \pm 3^*$	$11 \pm 1^*$	$2.6 \pm 0.3^{\xi}$
			$20 \pm 2^{\xi}$		
ZnMb with <i>S. aureus</i>	$2.1 \pm 0.1^{\S}$	$0.31 \pm 0.05^{\S}$	$34 \pm 3^*$	$13 \pm 1^*$	$2.6 \pm 0.3^{\xi}$
			$22 \pm 2^{\xi}$		

442  $^{\S}$ : FCS / TCSPC  $\lambda_{\text{exc}}=475$  nm,  $\lambda_{\text{det}}=650-700$  nm, 20 MHz,  $[\text{ZnMb}] \approx 1$   $\mu\text{M}$  ;  $^*$ : LFP  $\lambda_{\text{exc}}=532$  nm,  
 443  $\lambda_{\text{det}}=465$  nm,  $[\text{ZnMb}]=2$  or  $5$   $\mu\text{M}$  ;  $^{\xi}$ : TRP  $\lambda_{\text{exc}}=532$  nm,  $\lambda_{\text{det}}=1275$  nm, 1 KHz rep. rate, 10 min  
 444 acquisition time,  $[\text{ZnMb}]=10$   $\mu\text{M}$ ; deoxygenation: 1 hour flux pure nitrogen.

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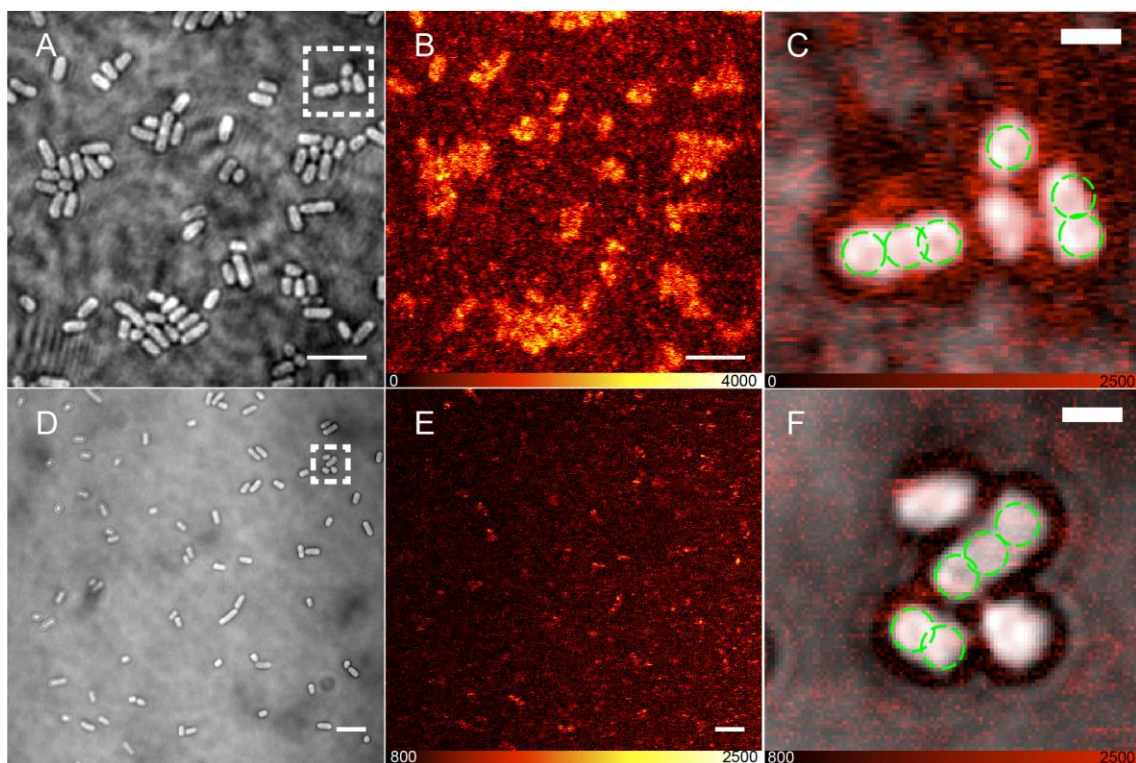


448

449 **Figure 1.**

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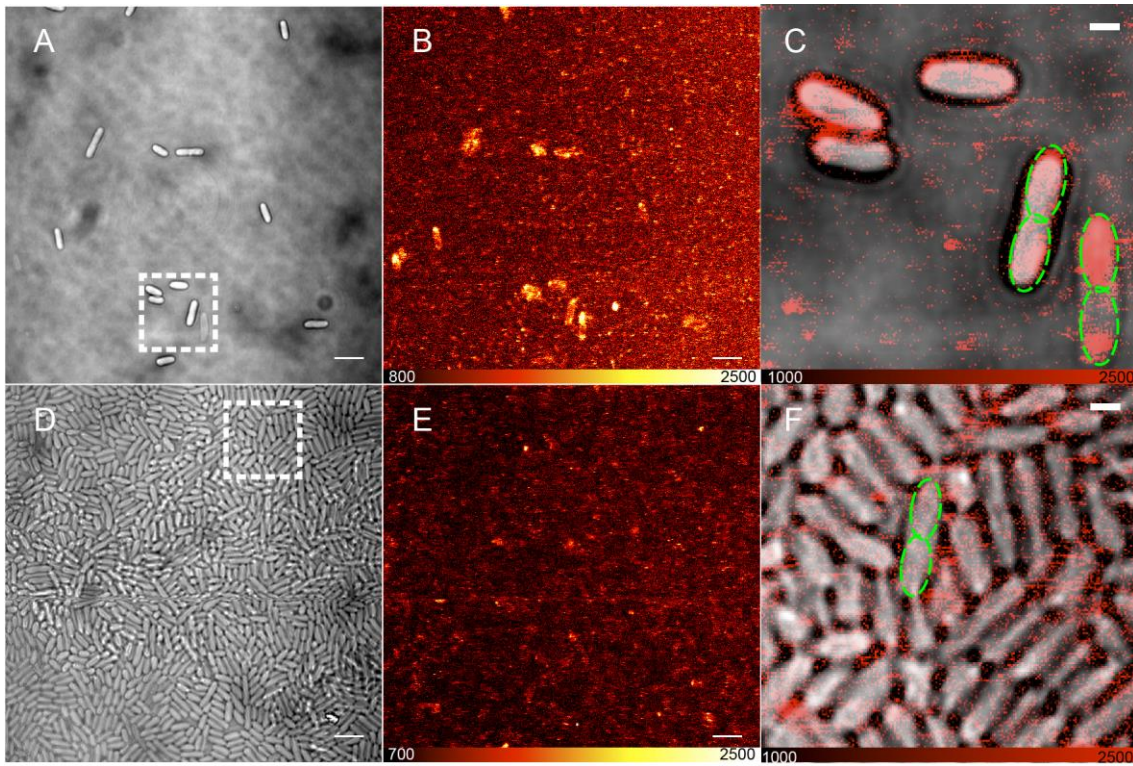




451

452 **Figure 2.**

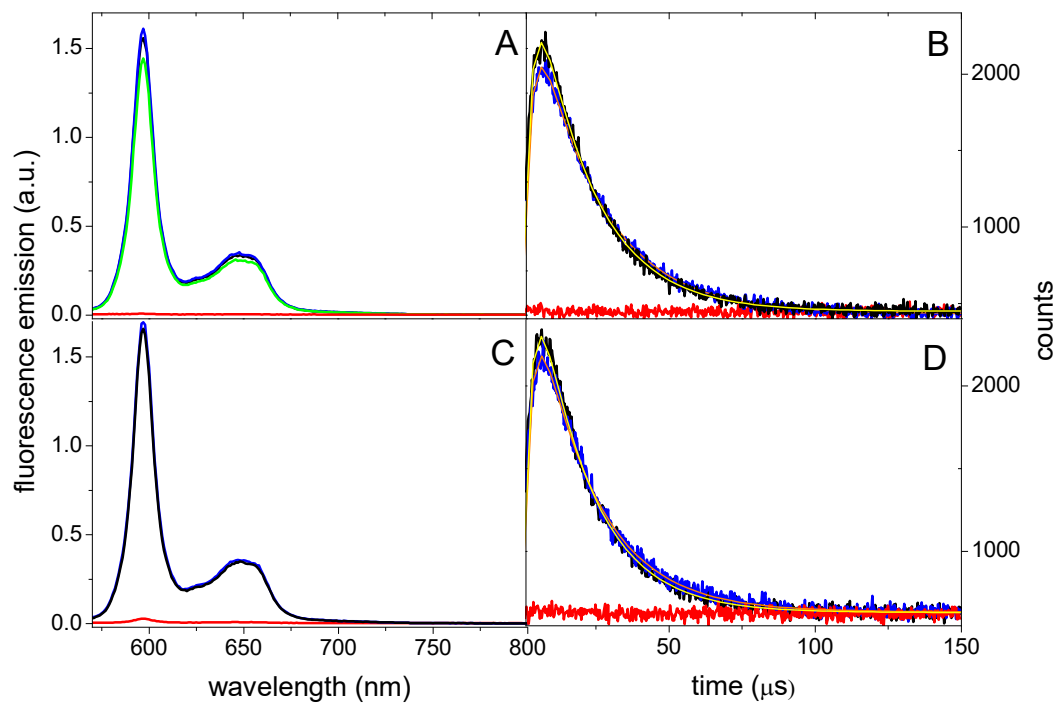
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454

455 **Figure 3.**

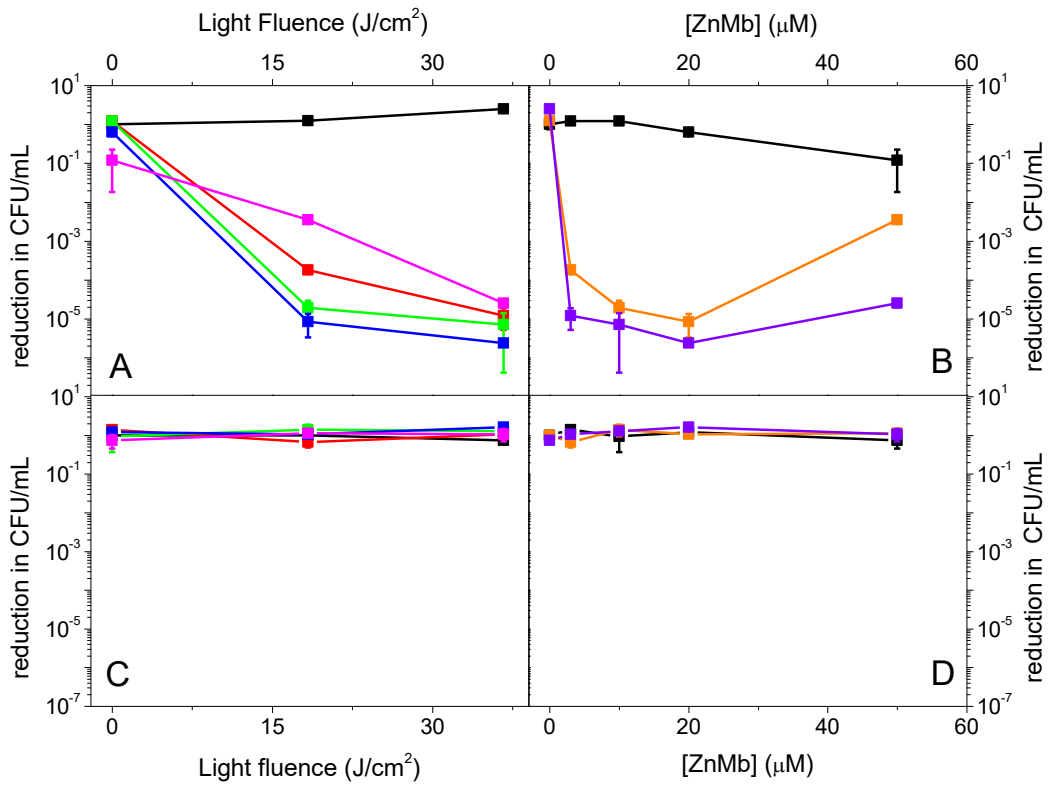
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459 **Figure 4.**

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462

463 **Figure 5.**

464

