Engineered ferritin with Eu^{3+} as a bright nanovector: a photoluminescence study

Luisa Affatigato[1], Alice Sciortino*[1], Giuseppe Sancataldo[1], Alessio Incocciati[2], Roberta Piacentini[2], Alessandra Bonamore*[2], Marco Cannas[1], Fabrizio Messina[1], Mariano Licciardi[3], and Valeria Militello[1]

[1]Department of Physics and Chemistry - Emilio Segrè, University of Palermo, Palermo, Italy. [2]Department of Biochemistry – A. Rossi Fanelli, Sapienza University, Rome, Italy. [3]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy.

*Corresponding author e-mail: alice.sciortino02@unipa.it (Alice Sciortino), alessandra.bonamore@uniroma1.it (Alessandra Bonamore)

25 # ABSTRACT

26 Ferritin nanoparticles play many important roles in theranostic and bioengineering applications and
27 have been successfully used as nanovectors for the targeted delivery of drugs due to their ability to
28 bind the transferrin receptor (TfR1). They can be either genetically or chemically modified for
29 encapsulating therapeutics or probes in their inner cavity. Here, we analyzed a new engineered ferritin
30 nanoparticle, made of the H chain mouse ferritin (HFt) fused with a specific lanthanide binding tag
31 (LBT). The HFt-LBT has one high affinity lanthanide binding site per each of the 24 subunits and a
32 tryptophane residue that acts as an antenna able to transfer the energy to the lanthanide ions via a
33 LRET process. In this study, among lanthanides, we selected Europium for its red emission that
34 allows to reduce overlap with tissue auto-fluorescence. Steady state emission measurements and time-
35 resolved emission spectroscopy have been employed to investigate the interaction between the HFt-
36 LBT and the Eu^{3+} ions. This allowed us to identify the Eu^{3+} energy states involved in the process and
37 to pave the way for the future use of HFt-LBT Eu^{3+} complex in theranostics.

38

INTRODUCTION

One of the major challenges for the prospective use of nanomaterials in cancer theranostics is the development of nanoplatforms capable of targeting cancer cells with high sensitivity and specificity. Ferritin H-homopolymers have been extensively studied as nanocarriers which can be applied as imaging agents (1) or as nanovectors for the targeted delivery of drugs (2, 3, 4, 5, 6) due to their ability to bind the transferrin receptor (TfR1 or CD71), highly overexpressed in most tumour cells (7, 8). TfR1 is one of the most attractive cancer targets, since tumour cells express up to 100 fold higher levels of TfR1 with respect to healthy cells, and actively internalize the H-Ferritin:TfR1 ligand-receptor complex, in order to accumulate the large amounts of iron required for unrestrained cell growth (9, 10). Recent studies demonstrate that H-ferritin nanoparticles can distinguish cancerous cells from normal cells with a sensitivity of 98% and a specificity of 95% (11).

Ferritin is a cage-like protein made of 24 subunits with an outer diameter of 12 nm and an inner diameter of 8 nm (12, 13). Ferritins are ubiquitous and well-characterized iron storage and detoxification proteins. In bacteria and plants, ferritins are homopolymers composed of H-type subunits, while in vertebrates, ferritins typically consist of 24 subunits of two different types, H and L. The H-subunit is responsible for the rapid oxidation of Fe(II) to Fe(III) and for binding the TfR1 receptor, whereas the L-subunit appears to help iron clearance from the ferroxidase centre of the H-subunit and support iron nucleation and mineralization (14, 15). The symmetrical positioning of three or four subunits in the protein shell results in the formation of the 3-fold channels and the 4-fold channels respectively. The former are hydrophilic channels connecting the inner cavity to the outside and allows the entry and exit of iron and other cations with a high selectivity (16).

Besides their physiological function, the nanocage properties of ferritins have been investigated in several different biotechnological applications, such as drug delivery vectors (2, 16), scaffolds for vaccine development (17) and tools for bioimaging (18). Concerning these tools, many studies have been carried out on quantum dots, gold nanoparticles and fluorescent metal chelators (20, 21), whereas only a few studies have been done on ferritin-based constructs to use as smart fluorescent

65 probes. However, advanced optical imaging techniques need an expanded colour palette of bright
66 fluorescent probes for biological visualization in order to enable real-time cellular imaging with high
67 spatial resolution for close-up view into subcellular compartments, and for providing key information
68 on intracellular activities and macromolecular dynamics.
69 In this work, an engineered H-ferritin nanoparticle carrying a C-terminal lanthanide binding tag (HFt-
70 LBT) has been characterized for its interaction with europium (Eu^{3+}) ions in view of potential
71 applications in bioimaging. Fluorescent probes based on trivalent lanthanide ions are becoming
72 widespread due to their advantageous photophysical properties (22, 23): narrow band emission
73 spectra, large Stokes shift (150-300 nm), and long luminescence lifetimes from micro to milliseconds
74 (24, 25). Lanthanide f-orbitals can radiate most of the absorbed energy, but their small absorption
75 cross sections hamper their practical use (26). Lanthanides are usually not excited by direct light
76 irradiation within their absorption peak, but rather excited through small organic fluorophores, that
77 absorb in the UV region with an adequate absorption cross section and transfer the absorbed light to
78 the lanthanide atom (27, 28). Such a luminescence resonance energy transfer (LRET) strongly
79 depends on the proximity between the two fluorophores (29, 30) and can also be used as a probe of
80 the local geometry around the lanthanide site.
81 Among lanthanides, Eu^{3+} ions are of strong interest in many photonic applications (31, 32), in
82 particular in organic light emitting diodes (OLEDs), lasers, optical communications, chemical sensor
83 (33). Eu^{3+} is particularly suitable for bioimaging because its red emission allows to reduce overlap
84 with tissue auto-fluorescence and to match the biological transparency window. Besides, the fine
85 structure and the relative intensities of Eu^{3+} optical transitions depend on the local environmental
86 conditions, so that they can be used for nanosensing applications.
87 To the purposes of this work, we have used the H chain mouse ferritin fused with a lanthanide binding
88 tag (LBT) on its C-terminal end to facilitate the incorporation of Eu^{3+} ions into the ferritin
89 nanoparticle. The LBT is a stretch of 17 aminoacids (YIDTNNDGWIEGDELLA) endowed with
90 strong luminescence resonance energy transfer (LRET) sensitization properties since it has a

tryptophan (Trp) residue that can act as an antenna transferring the absorbed energy to the lanthanide ion. Furthermore, the LBT shows low nanomolar affinities for the target ions and selectively binds to lanthanides as compared to over other common metal ions (34). LBT is the most convenient option for lanthanide protein labelling in that it can be directly encoded within a recombinant protein expression construct. The tag has been designed to be located inside the inner cavity, so the lanthanide ions diffusing through the surface pores could bind to the LBT sequence (Fig. 1).

<Figure 1>

The construct would thus act both as carrier targeted to CD71 receptors and as a LRET sensitizer. Mouse ferritin was used in view of the identical sequence within the CD71 binding region as the human ferritin sequence and because of obviously more favourable immunogenic profile for forthcoming in vivo study in mouse.

Here we studied the interaction between the Eu^{3+} ions and the HFt-LBT with spectroscopic techniques, in order to investigate the interaction between the protein and the lanthanide ions and to pave the way for the future use of HFt-LBT Eu^{3+} complex in theranostics. Steady state emission measurements clearly show the interaction between them by the huge increase of Eu^{3+} emission due to an energy transfer from the Trp to the ions. Time-resolved emission spectroscopy has been employed to deeply investigate this energy transfer and allows us to discriminate the presence of two Eu^{3+} species: one bonded on the LBT and one composed by free Eu^{3+} ions in solution. Moreover, we have been able to identify the Eu^{3+} energy states involved in the process, which provides evidence to attribute the LRET process to a multipolar energy transfer ruling out the presence of FRET or Dexter-type processes.

MATERIAL AND METHODS

116 **Protein expression and purification:** Mouse H ferritin fused with a lanthanide binding tag (HFt-
117 LBT) was expressed in *Escherichia coli* BL21 upon induction with 1 mM IPTG (Isopropyl-β-D-1-
118 thiogalactopyranoside) at $OD_{600} = 0.6$ for 16 hours. Bacterial paste from 2 L culture was resuspended
119 and sonicated in 100 mL of 20 mM HEPES buffer, pH 7.5, containing 200 mM NaCl, 1 mM TCEP
120 (tris(2-carboxiethyl)phosphine), and protease inhibitors. The supernatant was heated at 78 °C for 10
121 minutes and the denatured proteins were removed by centrifugation. The soluble fraction was treated
122 with 50% and 70% $(NH_4)_2SO_4$ and both the pellets were resuspended in 20 mM sodium phosphate
123 buffer pH 7.2 containing 20 mM $MgCl_2$, extensively dialyzed versus the same buffer, and then
124 digested with 5 mg of deoxyribonuclease I for 1h at 37°C. After digestion, the protein sample was
125 dialyzed versus 20 mM HEPES buffer pH 7.4 containing 150 mM NaCl and loaded onto a HiLoad
126 26/600 Superdex 200 pg column previously equilibrated in the same buffer, using an ÄKTA-Pure
127 apparatus (Cytiva). The eluted ferritin was concentrated using Amicon Ultra-15 centrifugal filter
128 devices (100 kDa cut-off), sterile filtered, and stored at 4 °C. Protein concentration was calculated by
129 measuring the UV spectrum using an extinction coefficient of 32400 $M^{-1}cm^{-1}$ and protein purity was
130 checked by SDS-PAGE. Protein yield was about 50 mg per 1 L culture.

131 **Photoluminescence of HFt-LBT Eu^{3+}:** Intrinsic luminescence emission spectra were detected
132 using a Jasco FP-6500 equipped with a Jasco peltier thermostat; samples were positioned in a quartz
133 cuvette of 1 cm and all emission spectra were recorded at 0.5 nm wavelength intervals with excitation
134 and emission bandwidth of 10 nm, scan speed of 100 nm/min and integration time of 1 s upon
135 excitation at 280 nm at 25°C. Luminescence static spectra were performed using 10 μM HFt-LBT in
136 3 mL HEPES 20 mM and NaCl 150 mM buffer solution pH 6.4. Luminescence spectra of the protein
137 Eu^{3+} complexes were recorded after 30 min incubation and after addition of incremental amount of
138 $EuCl_3$ in buffer solution in order to saturate all possible Eu^{3+} binding sites in HFt-LBT. Before
139 recording spectra, protein solutions were exchanged with buffer (Europium free) by doing dialysis
140 using a molecular porous membrane tubing MWCO: 3.5 kD (Spectral/Por Dialysis Membrane
141 Standard RC Tubing) in order to remove unbound and weakly bound metal ions (4 exchanges steps).

142 Protein concentration was measured again and adjusted to the final concentration with buffer.
143 Luminescence titrations were carried out by adding in the sample incremental amount (5 μL or
144 multiples) of a 3 mM europium stock solution under stirring. Emission spectra were recorded 30
145 minutes after addition of $EuCl_3$ solution aliquots. Luminescence intensity of Hft-LBT Eu^{3+} complex
146 as a function of the Eu^{3+}/HFt-LBT ratio has been reported. Luminescence intensity was recorded at
147 615 nm and 329 nm corrected for the dilution factor.

148 **Time-resolved photoluminescence of HFt-LBT Eu^{3+}:** Luminescence decay was recorded
149 using 10 μM HFt-LBT in 3 mL HEPES 20 mM and NaCl 150 mM buffer solution pH 6.4 with
150 different concentrations of Eu^{3+}. The emission spectra have been recorded by exciting the samples by
151 5 ns laser pulses of 0.1−0.2 mJ energy derived from a tunable laser (410-700 nm) consisting of an
152 OPO (optical parametric oscillator) pumped by the third harmonic of a pulsed Q-switched Nd:YAG
153 laser and finally duplicated by an UV-module in order to obtain 280 nm nanosecond beam. The
154 spectra and the kinetics have been detected by an intensified CCD camera which acquires the
155 emission spectra within a temporal window of variable duration, with controlled delays with respect
156 to the laser pulse. To obtain an equivalent of a steady state measurement, the temporal window is
157 fixed at 1 ms. To record the decay kinetics, the temporal window varies from 0.5 ns to 100 μs. The
158 Eu^{3+} emission decay profiles were least-squares fitted to a multi-exponential fitting function $I(t) =$
159 $A_1 \exp\left\{-\frac{t}{\tau_1}\right\} + A_2 \exp\left\{-\frac{t}{\tau_2}\right\} +..$

160

161 # RESULTS

162 **Photoluminescence of HFt-LBT Eu^{3+}:**

163 The steady-state emission properties of HFt-LBT Eu^{3+} were investigated in order to find evidence of
164 the interaction between HFt-LBT and Eu^{3+} ions, by comparison with the optical properties of free
165 Eu^{3+} in solution. Steady state Eu^{3+} emission spectra at different concentrations were thus recorded for
166 comparison, by photoexciting at 280 nm a solution of the lanthanides dissolved in the same buffer

167 solution (Fig. 2A). As expected, the emission efficiency collected from bare lanthanides is very low,
168 as demonstrated by the signal-to-noise ratio in Figure 2A.
169
170 <Figure 2>
171
172 Despite the low signal, it is possible to recognize several sharp spectral lines as the electronic
173 transitions typical of Europium ions. Under the same experimental conditions, the luminescence of
174 HFt-LBT Eu^{3+} complexes, at the same lanthanides concentrations, has been recorded (Fig. 2B).
175 Before each measurement, the samples were dialyzed with buffer (Europium free) to remove unbound
176 and weakly bound metal ions. It is evident that Eu^{3+} emission efficiency is at least two orders of
177 magnitude more intense with respect to that observed with free Eu^{3+} ions. The emission enhancement
178 is ascribed to HFt-LBT Eu^{3+}complex formation in which Eu^{3+} is excited via LRET. A second relevant
179 modification is the variation of the ratio between the intensity of different electronic transitions. The
180 $^5D_0 \rightarrow ^7F_2$ emission line peaking at 615 nm is much more intense than the other transitions. The electric
181 dipole (ED) $^5D_0 \rightarrow ^7F_2$ transition is called "hypersensitive transition", which means that its intensity is
182 more sensitive to the coordination environment around the Eu^{3+} ion, and to its geometry and
183 symmetry, with respect to the other electronic transitions (35, 36). This transition is dominant in the
184 emission spectrum of the HFt-LBT Eu^{3+}complex, and thus primarily responsible for its high emission
185 efficiency, probably due to the stable chemical environment around the Eu^{3+} ion (37). The variation
186 of the ratio between 615 nm peak and the others in the HFt-LBT Eu^{3+} implies the successful
187 incorporation of the ion with a consequent perturbation of the electronic transitions of the ion.
188 The huge increase of Eu^{3+}emission suggests that, when the complexes are formed, the UV excitation
189 is absorbed by the protein which acts as an antenna, and then releases the energy exciting to the
190 Europium ions. The Trp are the residues involved in the protein emission (as shown in Figure 2C),
191 and, in particular after the photoexcitation the Trp of the tag which should be tightly bound to the
192 lanthanide is expected to transfer the energy to the Europium, thus increasing its emission. As a matter

193 of fact, in order to better analyse the variations in the emission spectra of the Trp and the Eu^{3+}, and
194 the interplay between the two, we carried out a titration by adding free Eu^{3+} ions to the HFt-LBT
195 solution monitoring both the Europium and Trp emission (Fig. 2D). The results demonstrate a
196 progressive increase of the intensity at 615 nm for Europium and an anticorrelated quenching of the
197 Trp luminescence at 329 nm, when the Eu^{3+}/HFt-LBT concentration ratio increases. The titration
198 endpoint, that is the Eu^{3+}/HFt-LBT ratio at which saturation is reached, was observed at 2 equivalent
199 amounts of Eu^{3+} per subunit instead of the predicted 1 equivalent based on the presence of one LBT
200 moiety per subunit. This suggests that the Trp residue of the LBT acts as an antenna not only for the
201 very close Eu^{3+} ion, but also for a few extra Eu^{3+} atoms bound to the natural ferritin binding sites. As
202 reported in a previous study on HFt-LBT with Terbium, the HFt-LBT construct is capable of: 1) high
203 affinity binding of 24 Ln^{3+} atoms, one per each lanthanide binding tag; 2) intermediate affinity
204 binding of 24 Ln^{3+} atoms at the ferroxidase binding site; and 3) lower affinity binding of 8 Ln^{3+} atoms
205 at the entrance of the 3-fold channels (1). Hence the HFt-LBT is able to bind a total of 58 Ln^{3+} with
206 different affinities, leading to an overall stoichiometry that approaches 2.3 Ln^{3+} atoms per subunit.
207 In addition to the protein emission quenching, comparison of the emission spectrum of the HFt−LBT
208 Eu^{3+} with that of bare HFt-LBT (Fig. 2C) shows a blue shift of the peak in the presence of ions due
209 to the energy changes in the electronic transitions (38).

210 **Time-resolved photoluminescence of HFt-LBT Eu^{3+}:**

211 Time resolved measurements were carried out by recording both the emission band of the Eu^{3+} and
212 of the Trp in order to investigate the dynamics of the energy transfer in HFt-LBT Eu^{3+}. The singlet-
213 singlet Trp emission at 340 nm of the bare protein, excited at 280 nm, decays with a time constant of
214 $\tau_0 \sim 3.4$ ns as obtained by a fitting procedure from the kinetics in Figure 3. We compared this value
215 with the lifetime of the Trp in the HFt-LBT Eu^{3+}complex (Fig. 3B). As shown in Figure 3B, the
216 lifetime of the complexes shortens reaching a value of $\tau_{Eu} \sim 3.0$ ns indicating an interaction between
217 Europium ions and H-Ferritin which is consistent with an energy transfer from the S_1 state of the Trp
218 to the electronic states of Eu^{3+} (39). More precisely, from the variation of the lifetime induced by the

219 presence of Europium it is possible to directly estimate the energy transfer rate k as $\frac{1}{\tau_{Eu}} - \frac{1}{\tau_0} = 0{,}04\ ns^{-1}$.

220

221 <Figure 3>

222

223 We also analysed the luminescence decay kinetics of the bare Eu^{3+} ions (Fig. S1) and of the ions
224 forming complexes with the HFt-LBT protein (Figure 4). The kinetics of the $^5D_0 \rightarrow {}^7F_2$ emission at
225 615 nm of bare Eu^{3+} can be described as a double exponential decay with two characteristic
226 timescales: τ_1= (175 ± 10) μs and τ_2= (530 ± 20) μs. The presence of two timescales is probably
227 related to different geometrical configurations around the solvated ions in the buffer solution (40).
228 When europium ions bind to HFt-LBT, the recorded kinetics change and, in addition, they depend on
229 ions concentration (Figure 4B). The decay timescales of the 615 nm emission recorded after adding
230 15 μM of Eu^{3+} to the HFt-LBT solution were observed to be τ_1= (100 ± 10) μs and τ_2= (250 ± 20) μs,
231 both values being very different from the ones observed for free Eu^{3+} in solution, thus demonstrating
232 a binding interaction with HFt-LBT. Then, increasing the amount of europium from 15 μM to 24 μM
233 modifies the kinetics as shown in Figure 4B. A fitting procedure on these data gives back the same
234 two timescales (100 μs and 250 μs) but with different weights (Table 1). Adding even more Eu^{3+} does
235 not modify anymore the kinetics (Fig. 4B). The results of the fitting procedures on the three decay
236 curves are reported in Table 1.
237 The double-exponential nature of these decay curves indicates the presence of two different protein
238 environments possibly corresponding to two distinct binding sites for Eu^{3+} ions. Interestingly, the
239 amplitude of the slower kinetic component, τ_2= (250 ± 20) μs, is always higher than the first one,
240 especially at the lowest concentration of added Eu^{3+}. Considering the high binding affinity between
241 the ions and the LBT tag, it is thus reasonable to associate such a timescale to the electronic transition
242 of Europium ions which are coordinated to LBT. By contrast, the shorter phase, characterized by τ_1=
243 (100 ± 10) μs is possibly related to Eu^{3+} ions in the 3-fold channels with lower binding affinity. As a

244 control, we performed time resolved measurements on a HFt Eu^{3+} complexes without the LBT. The
245 recorded time courses are reported in Figure S1C in comparison with the tagged protein. From Figure
246 S1C, it is evident that the kinetics are markedly different, indicating that the presence of the tag
247 dramatically modifies the photon decay dynamics. The fitting procedure on HFt Eu^{3+} kinetics returns
248 two rate constants τ_1= (110 ± 10) µs and τ_2= (330 ± 20) µs (Table S1). The shorter time is compatible
249 with the shorter time obtained in presence of tag. This indicates, again, that this timescale does not
250 depend on the tag suggesting the binding of Eu^{3+} at a site of the 3-fold channel with lower binding
251 affinity. The longer timescale, instead, is different from that one obtained in presence of the tag, thus
252 indicating the direct involvement of the tag in the energy transfer transition.
253
254 <Figure 4>
255 <Table 1>
256
257 In order to understand which europium transition is involved in the energy transfer, we compare the
258 absorption spectrum of the ion with the fluorescence spectrum of Trp (Fig. S2). The comparison
259 shows that the $S_1 \rightarrow S_0$ transition of Trp spectrally overlaps with several europium electronic
260 transitions especially with the $^7F_0 \rightarrow {}^5H_5$ which most likely plays the role of the acceptor transition.
261 However, a classical FRET mechanism can be ruled out as being the pathway of energy transfer. In
262 fact, from the known rate of energy transfer of 0.04 ns^{-1} which was estimated from the changes of
263 Trp lifetime (Figure 3), we tried to estimate the r_0 (Förster distance) (41) through the known relation
264 $k_T(r)=\frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$. The obtained value of r = 0,13 Å, is so low to rule out a classical FRET mechanism.
265 Besides, FRET, which is an electric dipole driven transition, is also inconsistent with the selection
266 rules with this donor acceptor pair because the acceptor transition ($^7F_0 \rightarrow {}^5H_5$) implies a change of
267 angular momentum higher than 1 ($\Delta J>1$). Therefore, based on the literature, we propose that this
268 energy transfer is a multipolar interaction between the Trp and the Eu^{3+} (42). The energy transfer is

269 followed by a fast internal conversion to $^5D_0$ energy state which decays to $^7F_2$ emitting photons in the

270 microsecond range. A simple model of the interaction is shown in Figure 5.

271

272 <Figure 5>

273

274 ## CONCLUSIONS

275 A ferritin nanocage has been designed in order to guide the allocation of metal sites inside the cavity
276 and display the best geometry for an efficient energy transfer. Thereby, mouse ferritin endowed with
277 a specific metal binding tag, able to act as an antenna system for Europium has been obtained. Energy
278 transfer dynamics in HFt-LBT Eu^{3+} complex after photoexcitation of the antenna moiety using
279 photoluminescence has been observed for the first time, demonstrating the successful design of a
280 complex capable of amplifying the emission intensity of Eu^{3+} through multipolar energy transfer from
281 Trp to the $^7F_0 \rightarrow {}^5H_5$ accepting transition of the lanthanide ions The detailed study of the luminescent
282 properties of the complex revealed two distinct lanthanide binding environments, one related to the
283 specific coordination at the binding tag, and a secondary site with lower binding affinity associated
284 to Eu^{3+} ions in the threefold channels on the protein surface. In this framework, the key physical
285 properties of the tag are thus coupled to the CD71 receptor recognition properties of the ferritin in
286 order to produce a unique luminescent nanovector capable of a strong emission at the typical Eu^{3+}
287 peak at 615 nm upon Trp excitation at 280 nm. The results are very promising in view of the use of
288 these nanomaterials in prospective theranostic applications.

289

290 **ACKNOWLEDGMENTS**: The authors acknowledge the ‘molecular biophysics and
291 nanotechnologies’ group and in particular Prof. Maurizio Leone for helpful suggestions and
292 discussion.

293

294 ## AUTHOR CONTRIBUTIONS

295 **Conceptualization:** Valeria Militello, Alessandra Bonamore, Fabrizio Messina, Luisa Affatigato,
296 Giuseppe Sancataldo, Alice Sciortino

297 **Data Curation:** Luisa Affatigato, Alessio Incocciati, Giuseppe Sancataldo, Alice Sciortino

298 **Formal analysis:** Luisa Affatigato, Roberta Piacentini, Giuseppe Sancataldo, Alice Sciortino

299 **Funding Acquisition:** Alessandra Bonamore, Valeria Militello, Mariano Licciardi, Marco Cannas

300 **Methodology:** Luisa Affatigato, Alessio Incocciati, Alice Sciortino

301 **Project Administration:** Valeria Militello

302 **Supervision:** Alessandra Bonamore, Fabrizio Messina, Valeria Militello

303 **Writing – Original draft:** Luisa Affatigato, Alice Sciortino

304 **Writing – review & editing:** Alessandra Bonamore, Mariano Licciardi, Marco Cannas, Fabrizio
305 Messina, Valeria Militello

306

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409 **FIGURE CAPTIONS**

410 **Figure 1.** Structural model of HFt-LBT. On the left, the structure of the protein cage in which the
411 LBT, pointing inside the cavity, is highlighted in grey and represented as spheres. On the right, detail
412 of the monomer.

413 **Figure 2.** (A) Steady state emission spectra in the spectral range of Europium emission, as excited
414 at 280 nm of Eu^{3+} in buffer solution. (B) Steady state emission spectra in the spectral range of
415 Europium emission, as excited at 280 nm of the HFt-LBT Eu^{3+} complex, as obtained at four different
416 concentrations of Eu^{3+}. (C) Normalized emission spectra in the near-UV of HFt-LBT and HFt−LBT-
417 Eu^{3+} complexes excited at 280 nm. (D) Luminescence intensity of HFt−LBT Eu^{3+} complex as a
418 function of the Eu^{3+}/HFt-LBT ratio per subunit. Luminescence intensity shown in the graph was
419 recorded at 615 nm (blue points) for the Europium emission and at 329 nm (orange points) for protein
420 emission.

421 **Figure 3.** (A) 2D Fluorescence decay map of the HFt-LBT excited at 280 nm. (B) Kinetics decays
422 at 340 nm of bare protein (red curve) and of HFt-LBT-Eu^{3+} complexes (blue curve) both excited at
423 280 nm.

424 **Figure 4.** 2D Fluorescence decay map of Eu^{3+}-HFt-LBT excited at 280 nm and recorded in the
425 visible range. (B) Decay kinetics at 615 nm of HFt-LBT-Eu^{3+} complexes at three different Eu^{3+}
426 concentrations.

427 **Figure 5.** Schematic energy transfer process from the Trp residue of the tag to the Eu^{3+} ion in the
428 complex HFt-LBT Eu^{3+}.

429 **Table 1.** Decay times τ_1 and τ_2 (and their respective weights) of the Eu^{3+}, added at three different
430 concentrations to a solution of HFt-LBT.

	HFt-LBT Eu 15 µM	**HFt-LBT Eu 24 µM**	**HFt-LBT Eu 36 µM**
$W\tau_1$	26%	44%	46%
τ_1	100 ± 10 µs	100 ± 10 µs	100 ± 10 µs
$W\tau_2$	66%	53%	52%
τ_2	250 ± 20 µs	250 ± 20 µs	250 ± 20 µs