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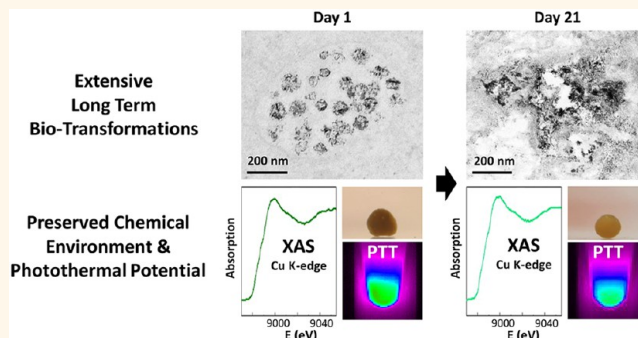
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ABSTRACT: Despite efforts in producing nanoparticles with tightly controlled designs and specific physicochemical properties, these can undergo massive nano–bio interactions and bioprocessing upon internalization into cells. These transformations can generate adverse biological outcomes and premature loss of functional efficacy. Hence, understanding the intracellular fate of nanoparticles is a necessary prerequisite for their introduction in medicine. Among nanomaterials devoted to theranostics are copper sulfide (CuS), which provides outstanding optical properties along with easy synthesis and low cost. Herein, we performed a long-term multiscale study on the bioprocessing of hollow CuS nanoparticles (CuS NPs) and rattle-like iron oxide nanoflowers@

CuS core–shell hybrids (IONF@CuS NPs) when inside stem cells and cancer cells, cultured as spheroids. In the spheroids, both CuS NPs and IONF@CuS NPs are rapidly dismantled into smaller units (day 0 to 3), and hair-like nanostructures are generated (days 9–21). This bioprocessing triggers an adaptation of the cellular metabolism to the internalized metals without impacting cell viability, differentiation, or oxidative stress response. Throughout the remodeling, a loss of IONF-derived magnetism is observed, but, surprisingly, the CuS photothermal potential is preserved, as demonstrated by a full characterization of the photothermal conversion across the bioprocessing process. The maintained photothermal efficiency correlated well with synchrotron X-ray absorption spectroscopy measurements, evidencing a similar chemical phase for Cu but not for Fe over time. These findings evidence that the intracellular bioprocessing of CuS nanoparticles can reshape them into bioengineered nanostructures without reducing the photothermal function and therapeutic potential.

KEYWORDS: biodegradation, biocompatibility, biosynthesis, CuS nanoparticles, magnetic nanoparticles, photothermia



Inorganic nanohybrids are designed with multiple capabilities, which are tailored for specific therapeutic or diagnostic purpose, and assembled all-in-one. The complex architectures of these nanomaterials imply a coexistence of multiple compositions, a control of size and shape, and a stabilization and functionalization *via* surface coating, altogether to provide original biomedical solutions.¹ Their pursued aptitudes include detection,² therapeutic efficiency with synergistic multifunction,^{3–7} and safety.^{8–10} Importantly, one major issue these engineered nanohybrids have to face is their possible modifications once in the complex biological environment, such as the formation of a protein corona inside blood circulation.^{11–14} Moreover, an increasing

number of studies demonstrated that inorganic nanoparticles (NPs) can also undergo drastic changes, such as intracellular degradation, upon endocytosis into the endosomal/lysosomal compartments.^{15–22} One of the most commonly studied NPs are the magnetic ones, based on an iron oxide core. In this case, the core degradation translates into a loss of the magnetic

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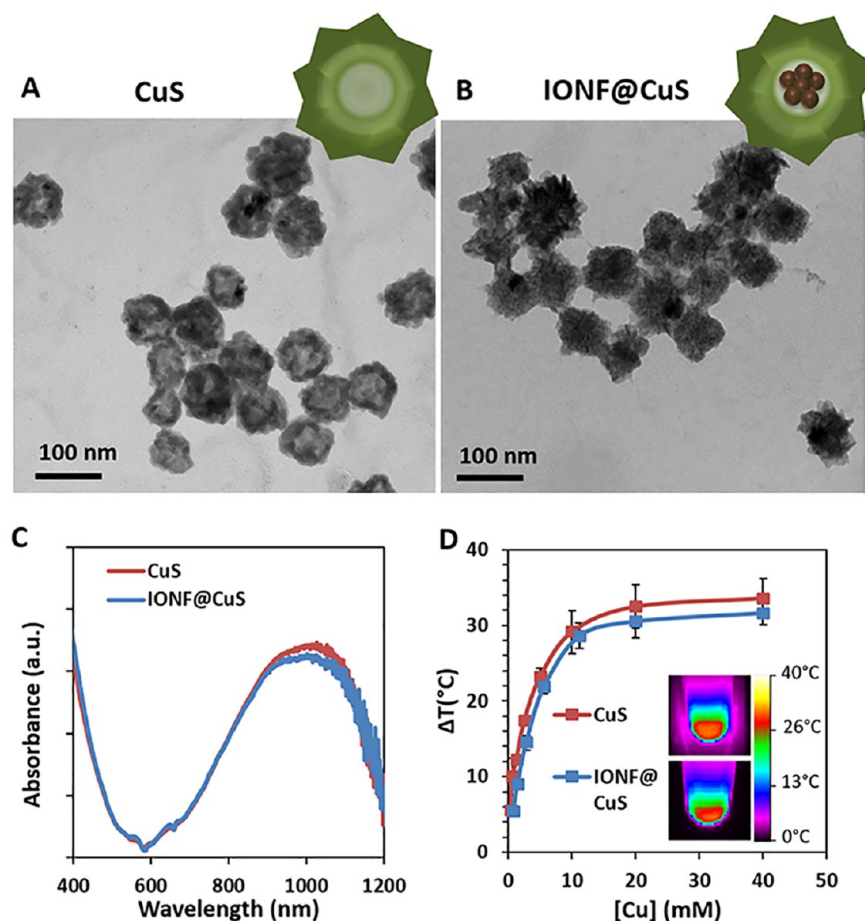


Figure 1. Copper sulfide nanoassemblies. (A, B) Transmission electron microscopy (TEM) images of hollow substoichiometric Cu_{2-x}S NPs (B) and rattle-like NPs composed of an IONF core bearing a Cu_{2-x}S assembly shell. (C) Absorbance spectra showing a similar near NIR absorption band around 1000 nm. (D) Comparative analysis of the heating of a 10 μL NP suspension at increasing concentration from $[\text{Cu}] = 0.1$ to 40 mM, upon laser exposure at 1064 nm and 0.3 W cm^{-2} laser power density, for a 5 min duration. Representative infrared (IR) thermographic camera images are shown in the inset for $[\text{Cu}] = 20$ mM.

49 properties.²³ The loss of magnetism implies a decreased
 50 efficiency for their biomedical applications²⁴ as imaging and
 51 therapeutic agents. Other types of NP composition, such as
 52 plasmonic silver, have also been reported to undergo
 53 intracellular degradation, leading to a drastic loss of the
 54 photothermal therapy (PTT) potential.²⁵ The magnetic or the
 55 silver cores covered with gold^{25,26} or polymeric^{23,27} shells can
 56 then act as a shield against degradation and favor maintenance
 57 of magnetic or plasmonic therapeutic functions. Some studies
 58 also reported the fate of NP coating *in vivo*, where a clear
 59 dismantling of the nanostructures emerged after circulation in
 60 the organism.^{28,29} Upon biodegradation, the nanoparticle core
 61 dissolution induces the release of ions (typically iron or silver),
 62 which prompts an adaptation of the cellular metabolism. The
 63 released ions were generally stored in the ferritin protein,^{25,30}
 64 but could also be used for generation of newly biomineralized
 65 magnetic nanoparticles.^{30,31}

66 Among the multifunctional NPs that are particularly
 67 promising for the biomedical field, those based on copper
 68 sulfide (CuS) are emerging as a promising class with numerous
 69 therapeutic features.^{32–34} Their light-to-heat conversion
 70 efficiencies are among the highest,^{33,35} and they provide dual
 71 laser-mediated treatments for both photothermal and photo-
 72 dynamic therapies in the most preferred second near-infrared
 73 biological window,^{33,34,36} situated between 1000 and 1700 nm,

appealing for its deep penetration, low optical absorption and
 scattering from biological substrates, and low tissue auto-
 fluorescence. One of the peculiar aspects of CuS-based
 materials resides in the origin of their optical absorption,
 which is responsible for their photothermal conversion and is
 still under debate. While some attributed such absorption to
 plasmonic effects resulting from free hole oscillations in the
 semiconductor, others assigned it to a valence band transition,
 independent from the solvent or the surrounding environment,
 as opposed to plasmonic NPs, but both mainly governed by
 the chemical composition of NPs.^{37,38}

84
 85 Hollow CuS-based nanostructures have received increasing
 86 interest in the field of nanomedicine over the last years. They
 87 can be used as cargos for drug delivery applications due to their
 88 hollow porous organization and for thermal therapy thanks to
 89 their physical plasmonic properties.^{39,40} By comparison to
 90 other CuS nanoparticles, the sacrificial templating method
 91 used for the synthesis of hollow CuS nanostructures is green
 92 (water/70 °C) and reproducible and can be easily scaled up to
 93 meet biomedical applications. Finally, the same methodology
 94 can be transposed for growing hollow copper sulfide shells of
 95 the same morphology and composition (core and surface)
 96 around any inorganic core, such as one composed of magnetic
 97 nanoparticles, what makes them even more attractive. Very
 98 recently, we have demonstrated that rattle-like magnetic iron 98

99 oxides@CuS nanohybrids are promising theranostic agents.³³
100 Considering all these advantages, it appears that the biofate
101 and bioprocessing of such nanomaterials inside cells and their
102 impact on their physical theranostic properties should be
103 investigated. To date, few studies have explored the
104 biodegradation of CuS structures. Those were performed *in*
105 *vivo*⁴⁰ or in acidic- and ionic-based environments mimicking
106 the lysosomal content⁴¹ and evidenced the bioinstability of
107 CuS-based nanomaterials. This instability has yet to be
108 explored within living cells.

109 Here, we propose a tissue model based on human
110 mesenchymal stem cells to explore in depth the long-term
111 intracellular fate of CuS, synthesized as nanoassemblies. This
112 3D cellular model, organized in a compact spheroid form,
113 allows the quantitative monitoring of NPs' functional activities
114 and structural transformations over almost a month. Hollow
115 CuS nanoassemblies and rattle-like hybrids featuring an iron
116 oxide nanoflower (IONF) core and a CuS shell were
117 investigated. The latter nanostructure allows both the analysis
118 of a combined CuS/iron oxide transformation and also the use
119 of the magnetic core as a tracer of the degradation. Cellular
120 uptake and toxicity screening including reactive oxygen species
121 (ROS) production and impact on stem cell differentiation were
122 conducted upstream. Electron microscopy was employed to
123 observe the intracellular structural evolution of the nanoma-
124 terials, highlighting an extensive structural instability. Near
125 infrared photoinduced heating (light-to-heat conversion
126 efficiency) was monitored alongside the biological processing,
127 to provide information on the evolution of the functional
128 potential and thus its time-scale range of applicability.
129 Unexpectedly, almost no loss of photothermal potential was
130 detected over the whole period of analysis (21 days). This
131 observation was confirmed in cancer cell spheroids. Con-
132 versely, an almost total loss of magnetism was revealed in the
133 case of the CuS/iron oxide nanohybrids (IONF@CuS). A final
134 proof brought by X-ray absorption spectroscopy (XAS)
135 measurements at the spheroid scale at the Fe and Cu K-edge
136 energies confirmed a strong modification of the iron chemical
137 environment, but no significant change in the copper one.
138 Thus, we demonstrate here an intracellular processing of
139 copper-based nanomaterials featuring both a massive structural
140 transformation and the preservation of its functional physical
141 properties. On a practical level, CuS nanoassemblies position
142 themselves as efficient photoresponsive nanomaterials that
143 retain their function in a biological environment in the long-
144 term and despite their biological processing.

145 RESULTS AND DISCUSSION

146 **Photothermal-Responsive Copper Sulfide Assem-**
147 **blies.** Substoichiometric hollow Cu_{2-x}S nanoassemblies,
148 hereafter named copper sulfide (CuS), and a rattle-like iron
149 oxide nanoflower core surrounded by copper sulfide assemblies
150 (IONF@CuS) were prepared by a two-step water-based
151 template sacrificial synthesis followed by their surface
152 modification with PEG (Figure 1 A and B, respectively), as
153 described in the **Materials and Methods section**.³³ The CuS
154 and IONF@CuS nanoparticles featured an average diameter of
155 62 ± 8 nm and 68 ± 9 nm, respectively, measured on
156 transmission electron microscopy (TEM) images. In the case
157 of IONF@CuS, the diameter of the nanoflower-like magnetic
158 core was 24 ± 5 nm (typical TEM image shown in Figure S1).
159 Both nanostructures exhibit a similar hydrodynamic size with
160 an average of about 90 nm, stable in cellular media (DLS,

Figure S2), and a similar broad near-infrared (NIR) absorption
161 band centered around 1000 nm (Figure 1C). Their exposure
162 to a 1064 nm laser at low power density (0.3 W/cm^2) for 5
163 min induced a high temperature elevation (Figure 1D), with,
164 for [Cu] over 10 mM, a 30°C temperature increase ΔT
165 reached for both nanoassembly formulations. Figure S3 also
166 provides the calculation of the light-to-heat conversion
167 efficiency, with an average value of $(42 \pm 6)\%$.
168

Internalization and Photothermia in Cells. Hollow
169 CuS and rattle-like IONF@CuS NPs were internalized in
170 primary human mesenchymal stem cells (hMSCs), selected as
171 a resourceful and versatile model that provides fine
172 biocompatibility information on long-term culture. The CuS
173 and IONF@CuS NPs were incubated for 4 h with hMSCs at
174 Cu concentrations ranging from 0.1 to 1.6 mM. TEM images
175 show the two stages of internalization. In Figure 2A and B,
176 respectively CuS and IONF@CuS NPs can be seen close to
177 the outer cell membrane, just before their internalization,
178 which is obtained on the 2D culture in a nonspecific way.
179 Figure 2C–F show both NPs accumulated within the
180 endosomes, with their structural architecture still intact.
181 Additional images are presented in Figure S4 and Figure S5.
182 In the case of IONF@CuS NPs, the iron oxide core is easily
183 recognizable with its nanoflower-like shape and 24 nm size
184 compared to the surrounding CuS, which appears bigger,
185 forming a hollow shell, as can be seen from the thin
186 longitudinal sectioning of the samples (40 nm slices; Figure
187 2B and F). As emerged from elemental analysis (Figure S6),
188 NP internalization increases with the incubation concentration
189 for both nanoformulations. Once the nanoassemblies were
190 internalized, high-density collected cells (250 000 cells in 10
191 μL , at different intracellular doses) were exposed to photo-
192 thermia, in the same configuration (volume and laser power
193 density) as previously achieved for aqueous dispersions (Figure
194 1). Heating efficiency in the cell environment was found to be
195 similar for both CuS and IONF@CuS NPs (Figure 2G and H)
196 and in the same range as obtained in water (Figure 1D). The
197 photothermal potential of CuS-based NPs is thus similar in an
198 aqueous dispersion or within cells.
199

200 Biological Responses Using High-Content Imaging.

201 Results of cytotoxicity analyses of cellular interaction with the
202 different NPs for 72 h investigating cell viability, mitochondrial
203 reactive oxygen species (MitoROS), and the size of the
204 mitochondrial network are presented in Figure 3. Representa-
205 tive images of the InCell analysis experiment for viability and
206 MitoROS are presented in Figure 3D and Figure S7.
207 Altogether, these results indicate that CuS NPs induce toxic
208 effects only at the highest concentrations (Figure 3A). The
209 level of observed cell death was accompanied by only a
210 marginal increase in MitoROS (Figure 3B), while mitochon-
211 drial area (an indicator for mitochondrial stress) displayed a
212 significant reduction in size of the cellular mitochondrial
213 network (Figure 3C). The latter indicates a clear increase in
214 mitochondrial stress, at high concentrations, but this does not
215 seem to be linked to the generation of ROS.

Long-Term Impact on Stem Cell Spheroids. In order to
216 perform longer-term toxicity assessment, stem cells labeled
217 with CuS or IONF@CuS NPs (incubated at extracellular [Cu]
218 = 0.4 mM) were cultured as spheroids for 21 days (Figure 4A).
219 This 3D model is commonly used for the chondrogenic
220 differentiation of stem cells, which is initiated by both the 3D
221 organization of the cells and the addition of specific culture
222 conditions. It results in an important production of 223

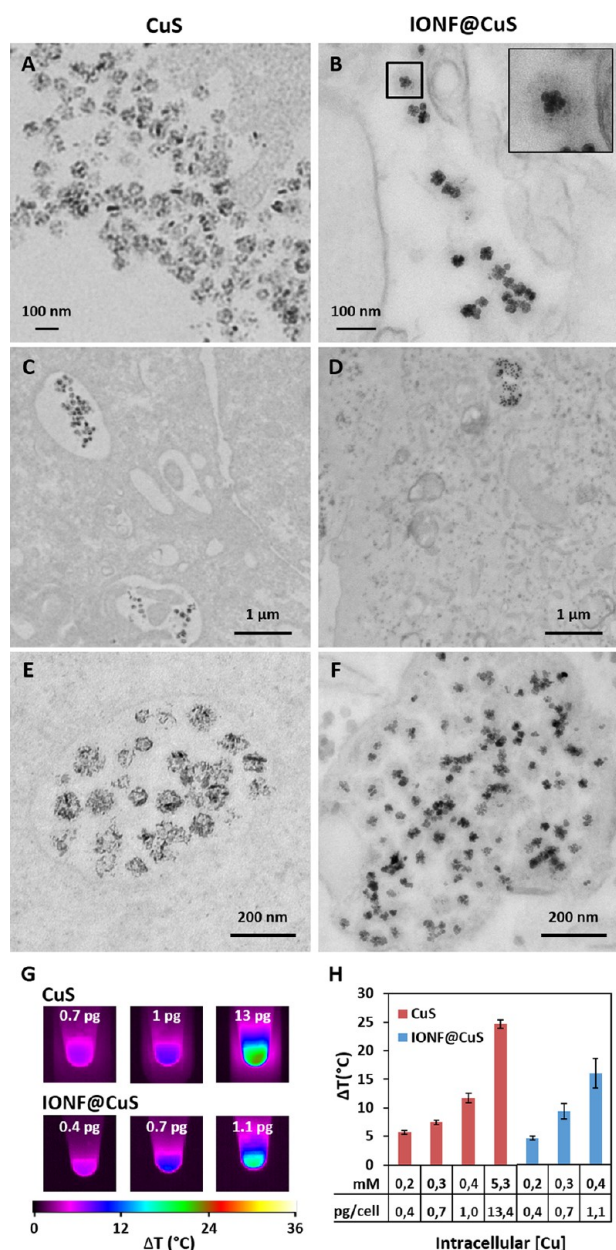


Figure 2. NP internalization in primary stem cells. (A–F) TEM micrographs showing CuS (A, C, E) and IONF@CuS (B, D, F) nanoassemblies upon 4 h incubation with hMSCs at a Cu concentration of 0.5 mM. Some nanoassemblies are still visible at the cell outer membrane before endocytosis (A, B). Most of the nanoassemblies were however already found inside endosomes, where they appear still intact and highly accumulated (C, D, E, F). (G) Representative IR thermographic camera images of a nanoassembly-containing cell suspension (250 000 cells in 10 μL) after 5 min of 1064 nm laser exposure, at different doses of Cu. For the CuS (top), the three conditions correspond to extracellular incubation at [Cu] = 0.2, 0.4, and 1.6 mM, resulting in intracellular Cu amounts of 0.7, 1, and 13.4 pg Cu per cell, respectively. For the IONF@CuS (bottom), they correspond to [Cu] = 0.1, 0.2, and 0.4 mM, resulting in intracellular Cu amounts of 0.4, 0.7, and 1.1 pg Cu per cell, respectively. (H) Average heating of CuS and IONF@CuS treated hMSCs upon 4 h of incubation, plotted against their intracellular mass of Cu in pg per cell (*x*-axis, bottom) or the resulting molar concentration of Cu in the 10 μL cell dispersion (*x*-axis, top).

extracellular matrix (e.g., collagen II, aggrecan). Importantly, under this setup, cells can remain viable in culture for extensive time frames (even months) without dividing, allowing one to assess the long-term biological impact in a quantitative manner.

Representative TEM images taken at day 21 show that the cells labeled with CuS-based NPs have produced an organized extracellular matrix (Figure 4B), a clear sign of their advanced stage of differentiation. Histology images also confirm an abundance of proteoglycans within these CuS-incorporated spheroids similar to control spheroids, as indicated by positive toluidine blue staining (Figure 4C). Gene expression of cartilage-specific constituents (aggrecan and collagen II) was assessed by qPCR for the spheroids labeled with IONF@CuS NPs and shows an increased expression over time, similar to the control at day 21, confirming the chondrogenic differentiation of the cells (Figure S8). Overall, the intracellular presence of CuS-based NPs appears not to hinder the typical biological maturation of the stem cell spheroids.

Long-Term Expression of Oxidative Stress and Metal Ion-Related Genes. Biological outcomes of the internalization of CuS and IONF@CuS materials in stem cells cultured as spheroids in the long-term (up to 21 days) were assessed by measuring the expression of genes involved in the oxidative stress response and in copper and iron metabolisms (Figure 5). First, Figure 5A shows the expression of GLRX and SOD1 (encoding for glutaredoxin and superoxide dismutase 1, respectively), both involved in the antioxidant defense system, and of NOX2- α (encoding for NADPH oxidase 2), involved in ROS production. The expression of these oxidative stress related genes is not impacted by the presence of the CuS-based NPs. This supports the previous findings (Figure 3B) that no significant mitochondrial ROS were produced. Figure 5B evidences next that most of the copper- and iron-related genes were not upregulated, except for CP, encoding for ceruloplasmin, one of the most important copper carriers also involved in iron metabolism. By contrast, three genes encoding for metallothioneins (involved in the homeostasis of heavy metals, such as copper) are upregulated in a time-dependent manner after exposure to CuS and IONF@CuS (Figure 5C, D, and E). For the IONF@CuS, upregulation is maximal at day 3 and progressively returns to control levels over time, for all the isoforms analyzed (MT1E, MT1X, and MT2A). For CuS NPs, upregulation is less important at day 3, but remains sustained for longer duration. Hence, metallothionein gene expression is induced after exposure to both formulations of CuS-based nanomaterials.

Overall, this analysis provides insights into Cu and Fe metabolic pathways. First it suggests a role of the metallothioneins in the cellular response to Cu-based materials. The upregulation of CP for both CuS and IONF@CuS is consistent with its role not only as Cu transporter but also for Fe.⁴² The particular case of ATP7A is also relevant, with an upregulation only for IONF@CuS. ATP7A encodes for the copper-transporting ATPase 1 that has for a main function the transport of Cu, and potentially Fe, across the cell membrane, and is thus related to cellular ion levels.⁴³

Structural Transformations Observed at the Nano-scale by Electron Microscopy. TEM observations were undertaken throughout the 21 days of spheroid maturation. They evidenced that the integrity of the nanoassemblies is rapidly affected by the biological environment, with structural modifications already starting at the third day of spheroid

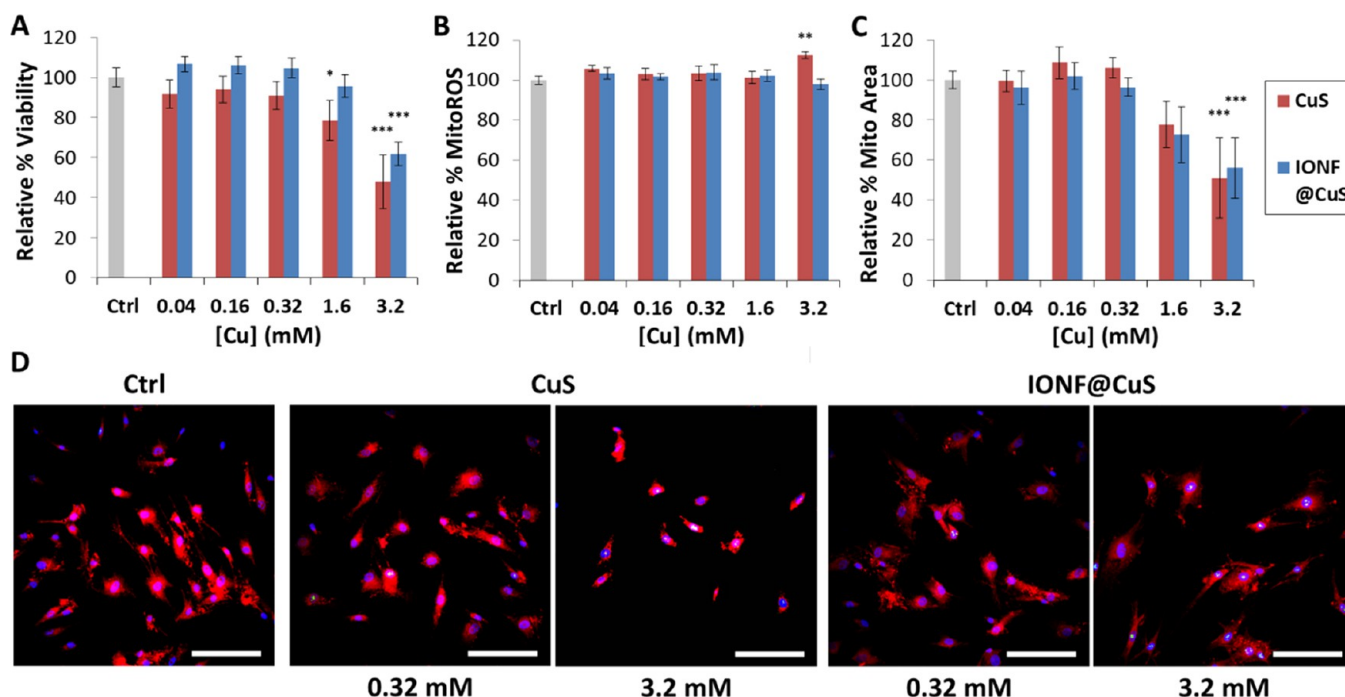


Figure 3. Nanotoxicity screening results of hMSC exposed to various concentrations of CuS and IONF@CuS NPs for 72 h. (A) Biocompatibility analysis, (B) mitochondrial ROS, and (C) size of the mitochondrial network of hMSCs treated for 72 h with CuS and IONF@CuS at concentrations ranging from 0.04 to 3.2 mM of Cu and relative to untreated control cells (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). (D) Representative merged images of the hMSCs treated with CuS and IONF@CuS nanoassemblies at 0.32 and 3.2 mM of Cu. Nuclei are stained in red (Hoechst), and dead cells' nuclei are counter-stained in green (as light green spots). For the 3.2 mM conditions, many nuclei appear green labeled. Mitochondrial network stained in red (MitoTracker Red CMXRos) (Scale bar = 200 μm).

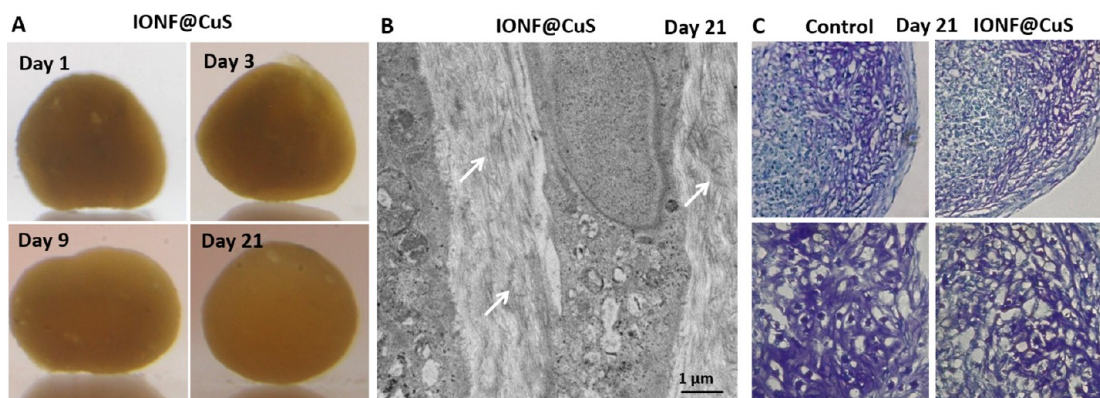


Figure 4. Formation of spheroids from stem cells loaded with CuS-based NPs for long-term culture. (A) Representative photographs of spheroids made of stem cells loaded with IONF@CuS NPs (4 h of incubation at day 0, with 0.4 mM Cu). The spheroids become more cohesive and spherical during the 21-day culture period. (B) TEM image of a spheroid harvested and sectioned at day 21 showing the presence of a dense extracellular matrix in between the cells (white arrows). (C) Histological images of spheroids fixed and sectioned at day 21 and stained with toluidine blue. A similar staining is obtained for the control (left) and the IONF@CuS condition (right), indicating the abundance of proteoglycans in both conditions.

287 maturation (day 3), both in the presence and in the absence of
 288 the IONF core (Figure 6, panels A–D). At this stage, some
 289 lysosomes still contain intact NPs, while others hold
 290 nanostructures resembling the initial nanoassemblies but
 291 disassembled into smaller units, with the IONF core also
 292 dismantled in the case of IONF@CuS (Figure 6C and D).
 293 Additional TEM images are shown in Figure S9 and Figure
 294 S10. After 21 days (Figure 6, panels E–L) absolutely no intact
 295 assemblies can be detected within the cells anymore. By
 296 contrast, neo-formed nanostructures can be clearly seen in
 297 lysosomes, generally leaned against the internal side of the
 298 membrane, in a “hair-like” manner. Similar additional images

(day 9 and day 21) are shown in Figure S11 and Figure S12. 299
 Importantly, energy-dispersive X-ray spectroscopy (XEDS) 300
 elemental analysis of the transformed nanostructures at day 21 301
 confirmed the presence of copper along the membrane of 302
 lysosomes (Figure S13). Interestingly, only for the IONF@ 303
 CuS condition could we also detect small 5–7 nm NPs, that 304
 we identify as iron deposits in 5–7 nm ferritins previously 305
 observed after degradation of iron oxide nanoparticles. 306
 In summary, both CuS and IONF@CuS nanoassemblies 307
 undergo a profound morphological reshaping inside the 308
 lysosomes, which occurs in close association to the inner 309
 side of the lysosomal membrane, for the Cu processing. 310

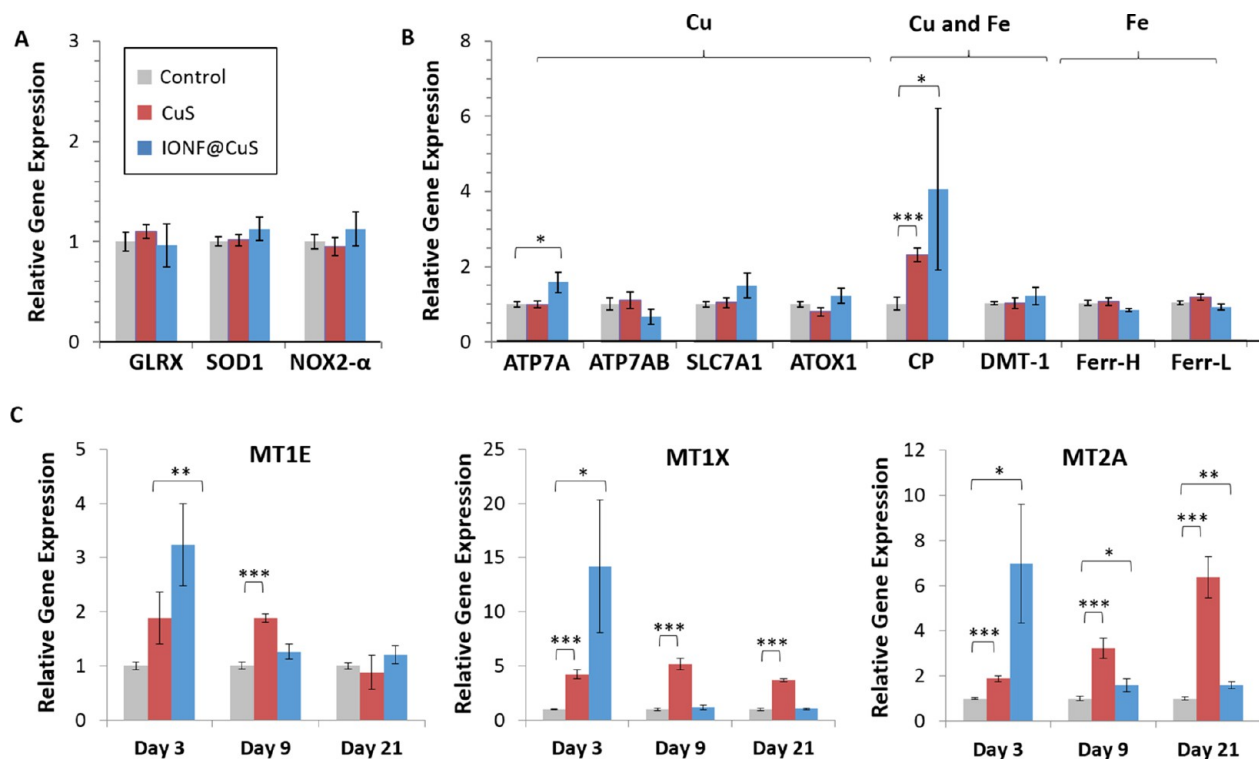


Figure 5. Impact of the internalization of CuS or IONF@CuS on the expression of genes involved in (A) oxidative stress (day 9), (B) copper and iron metabolism (day 9), and (C–E) metallothioneins (MT1E, C; MT1X, D; MT2A, E). Gene expression was normalized to RPLP0 mRNA and expressed relative to the average values of nonlabeled spheroids harvested the same day (control). Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

311 Finally, it is important to emphasize that the *in vivo*
 312 complexity is only partially reproduced at the cell culture level.
 313 We therefore included an *in vivo* exploration of the IONF@
 314 CuS NPs fate at the organism level by injecting them
 315 intravenously in mice, harvesting the liver 1 day after the
 316 injection or 22 days later and postprocessing it for TEM
 317 imaging. The corresponding images, shown in Figure S14,
 318 evidence very similar structures to the ones observed at the
 319 cellular level.

320 Photothermal Potential of the Biotransformed NPs.

321 The spheroids containing intact NPs (day 1) and processed
 322 ones (from day 3 to day 21) were exposed to a NIR laser
 323 (Figure 7). Overall, seven independent incubation conditions
 324 were tested, corresponding to three and four different
 325 extracellular doses for IONF@CuS and CuS NPs, respectively.
 326 Each time point corresponds to spheroids ($n = 6$) that have
 327 been fixed after 1, 3, 9, or 21 days of maturation. Unexpectedly,
 328 the photothermal heating efficiency was globally kept constant
 329 during the course of spheroid maturation for all the tested
 330 doses. Figure 7A and D show representative IR images of the
 331 heating at the first (day 1) and last (day 21) time point. A
 332 complete IR panel including also images at day 3 and day 9 can
 333 be found in Figure S15. Figure 7B and E present the average
 334 temperature increases ΔT over time. All data point out the
 335 stability of the photothermal potential of the CuS-based
 336 nanoassemblies. For all conditions, the quantity of copper (and
 337 iron) within the spheroids remains unchanged over time
 338 (Figure 7C and F), direct proof that the spheroids generally do
 339 not lose labeled cells nor eject nanomaterials or copper ions.
 340 We also tested the photothermal potential of CuS NPs
 341 dispersed in culture medium at 37 °C, without cells, for 7 days.
 342 Results are shown in Figure S16 for different concentrations,

clearly demonstrating a significant reduction of the photo-
 thermal efficiency, over 30%, in 1 week of incubation.

Overall, it appears that despite the massive intracellular
 structural modification of the CuS and IONF@CuS NPs over
 weeks in stem cells, their heating potential is exceptionally
 conserved throughout the analysis period, in a consistent
 manner at all doses tested.

Magnetometry and X-ray Absorption Spectroscopy at the Spheroid Level Reveal Degradation of the Magnetic Core but No Change in Cu Chemical State.

Magnetometry was performed on the IONF@CuS spheroids,
 to quantitatively detect the magnetic signature of the IONF
 magnetic core inside the cells over time, as an indicator of its
 integrity. As shown in Figure 8A (single spheroid magnet-
 ization curves at room temperature) and Figure 8B (average of
 spheroid saturation magnetization), a sharp reduction of the
 magnetization is already experienced 1 day after IONF@CuS
 internalization. The magnetic signal then remains very low
 from day 3 to day 21, demonstrating that the magnetic core is
 totally degraded.

X-ray absorption spectroscopy (XAS) of the spheroids at the
 Fe K-edge in the X-ray absorption near-edge structure regime
 (XANES) (Figure 8C) confirmed the degradation of the IONF
 magnetic core upon cellular uptake. Right after internalization
 in cells, IONFs were identified as maghemite by spectrum
 comparison with iron oxide references. Besides, the spectrum
 matches perfectly the one obtained before internalization, for
 IONFs dispersed in water (see also Figure S17A). By contrast,
 after 21 days, although the Fe ions remained in the +3
 oxidation state, the spectra obtained evolve to a ferrihydrite-
 like structure and could be modeled with a high content of
 ferrihydrite (80%) over maghemite (Figure S17B). It confirms

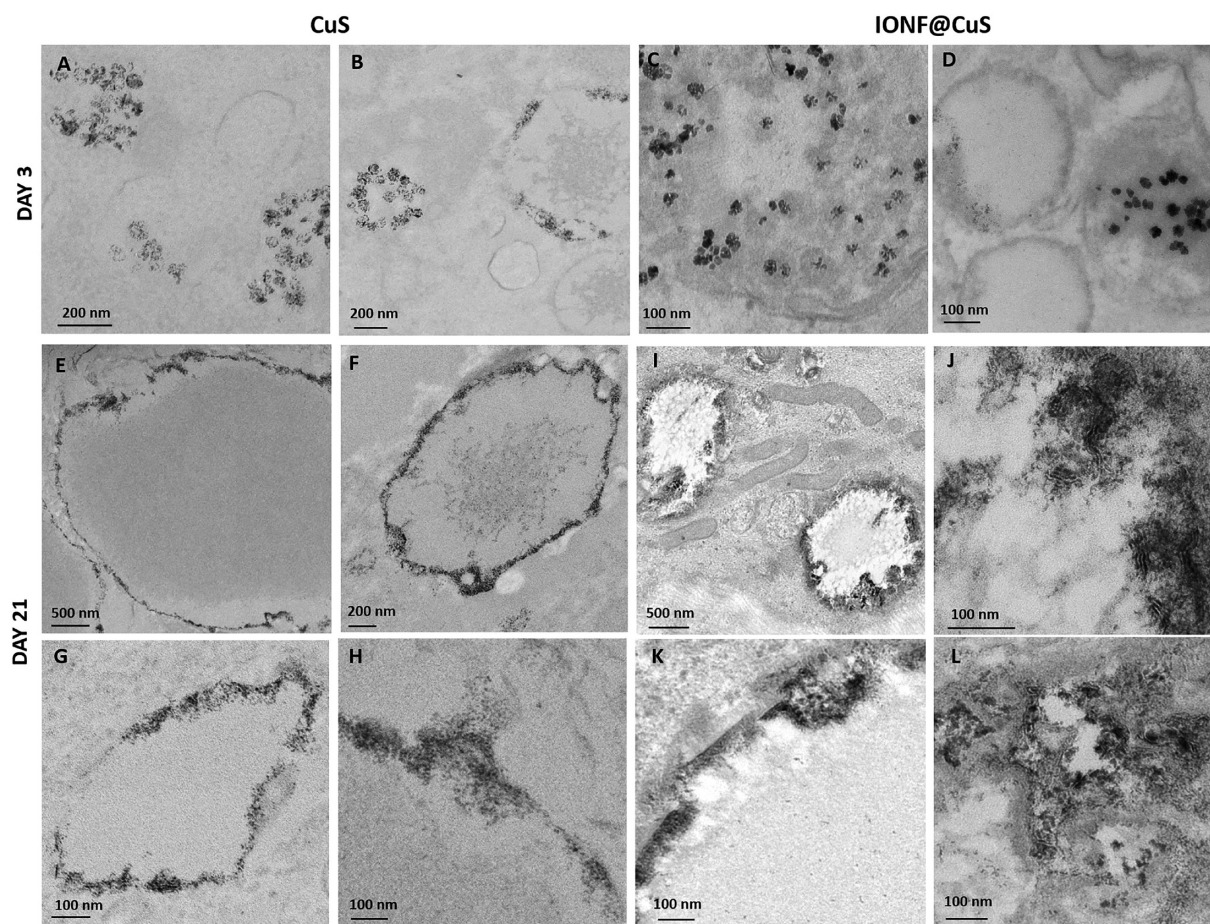


Figure 6. TEM study of the intracellular structural transformation of nanoassemblies over time. Representative TEM images at day 3 for CuS (A, B) and IONF@CuS assemblies (C, D) show the presence of both still intact nanoassemblies and their much smaller degradation products. At day 21 of spheroid maturation, no original nanoassemblies are visible for both CuS (E–H) and IONF@CuS (I–L), while the lysosome inner membrane appears decorated with nanomaterial deposits, reshaped in smaller “hair-like” structures.

375 that the degradation products issued from the magnetic core
 376 are stored within the ferritin protein as ferrihydrite, as already
 377 reported for the degradation of magnetic NPs alone.^{30,31} By
 378 contrast, XANES analysis of the spheroids at the Cu K-edge
 379 (Figure 8D) demonstrates no change for the chemical phase
 380 and environment of Cu over time. Moreover, the XANES
 381 spectrum of IONF@CuS NPs dispersed in water displays
 382 identical edge positions and spectral features to those obtained
 383 in cell spheroids at all maturation durations. A fit using Cu-
 384 based reference compounds yielded a composition of mainly
 385 CuS phase (Figure S17C and D). This is in total agreement
 386 with the preservation of the photothermal potential and
 387 provides the unequivocal evidence of the massive intracellular
 388 transformation of the nanoassemblies into CuS biologically
 389 reshaped materials with the same structural chemistry and,
 390 thus, physical photothermal properties.

391 **Maintenance of the Therapeutic Programmed Tasks**
 392 **in a Cancer Model.** The stem cell spheroids, made of primary
 393 human mesenchymal stem cells, were used as a model to
 394 enable a long-term study of the intracellular remodeling of the
 395 CuS NPs, as well as to evaluate the biological impact of the
 396 NPs on healthy cells. Nevertheless, the photothermal property
 397 of CuS is usually used to treat diseases, for example, to kill
 398 tumor cells. Within the spheroids, the stem cells stop dividing
 399 and create a rich extracellular matrix, which allows them to be
 400 kept viable over months; by contrast, highly proliferating

401 cancer cells are more challenging to culture in 3D culture in
 402 the long-term. Yet, we managed to transfer the stem cell
 403 spheroid model to glioblastoma cancer cells, which successfully
 404 formed 3D spheroids of similar size and number of cells and
 405 were maintained viable over 9 days (Figure 9A). Two doses of
 406 CuS NPs were investigated, corresponding to 0.5 and 0.9 pg of
 407 Cu per cell, in the same range as what was obtained in stem
 408 cells. The intracellular heating performances of the CuS NPs
 409 were maintained over the 9 days, with heat measurements
 410 performed at days 1, 3, 6, and 9. This is evidenced in Figure
 411 9B, C, and D, showing typical IR images of single spheroid
 412 heating, typical temperature elevation curves for each dose and
 413 each measurement day, and a temperature increase (at plateau)
 414 averaged over five independent spheroids, as a function of
 415 time. Importantly, these series of measures were achieved
 416 starting from 37 °C, with laser exposure at low power density
 417 (0.3 W/cm²), totally approved for clinical use, and for 10 min,
 418 a duration typically used for photothermal cancer therapy.
 419 With these settings, the preservation of the heat generation
 420 over time perfectly translated into a conserved therapeutic
 421 efficacy, as spheroid viability after laser exposure at days 1, 3, 6,
 422 and 9 was systematically decreased to 60% and 30% compared
 423 to the nonexposed control spheroids, for 0.5 and 0.8 pg of Cu
 424 per cell, respectively.

425 **Hypothetical Mechanism.** The data presented here
 426 provide insights into the landscape of intracellular reshaping

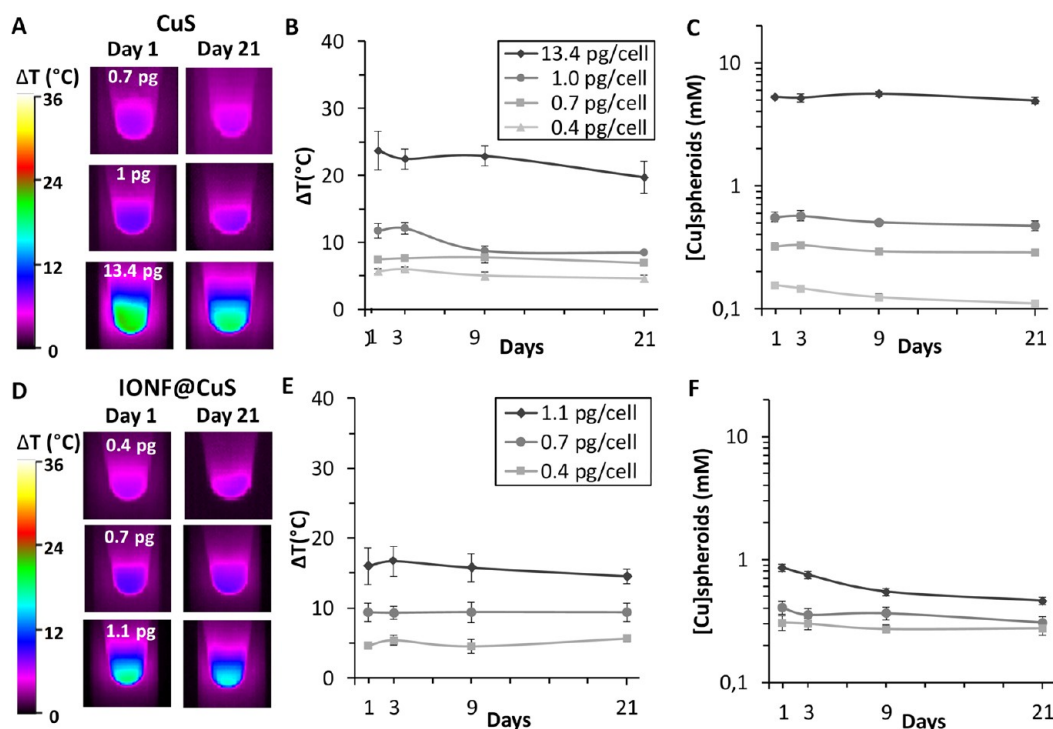


Figure 7. Laser-induced photometric study on CuS and IONF@CuS NP-containing spheroids over time. A and D show typical IR images of three concentrations of CuS- and IONF@CuS-treated spheroids, respectively, at day 1 and 21 of spheroid maturation. Images were taken after 5 min of 1064 nm laser irradiation at 0.3 W cm^{-2} (heating plateau achieved at 1–2 min of laser application). B and E present the average time plots of the heating efficiencies for all incorporated doses for spheroids treated with CuS (B) and IONF@CuS (E) at days 1, 3, 9, and 21 of spheroid maturation. C and F present [Cu] measurements obtained by ICP of the $10 \mu\text{L}$ solution containing the individual CuS- (C) and IONF@CuS-containing (F) spheroids.

427 of functional nanomaterials, with the evidence that a
 428 nanoparticle can be significantly bioprocessed and transformed
 429 into different shapes and sizes while maintaining its photo-
 430 thermal function. This behavior was observed only for copper
 431 sulfide material, while magnetic cores were already processed
 432 to nonmagnetic after a few days as demonstrated by magnetic
 433 measurements and XAS. Such differences in cell bioprocessing
 434 of both materials may have different origins including the low
 435 solubility of metal sulfides compared to metal oxides, the
 436 differences in size and shape, but also the differences in cellular
 437 management of both metal ions, where Fe is typically
 438 metabolized rapidly after cellular uptake.⁴⁴ Naturally, Fe is
 439 transported from the outside of the cell to the inside by
 440 transferrin, which, after binding to the transferrin receptor,
 441 ends up in endosomes/lysosomes compartments. The receptor
 442 can recycle back to the cell membrane, where the Fe ion can
 443 bind small molecules such as citrate that will aid in its
 444 solubilization and transport it out of the endosomes into the
 445 labile iron pool of the cell.⁴⁵ For Fe brought inside the cells *via*
 446 NPs, it is generally the same pathway, where the NPs
 447 sequestered in endosomes are gradually degraded and free
 448 Fe ions are transported out of the endosomes into the
 449 cytoplasm.⁴⁶ For copper, the process is different and less
 450 understood. Naturally, Cu enters the cells mainly *via* the
 451 copper transporter 1 (CTR1) and is immediately linked to one
 452 of its chaperones, such as copper chaperone for SOD (CCS) or
 453 Sco1, an enzyme involved in the synthesis of cytochrome *c*
 454 oxidase.⁴⁷ Cu can be stored at low levels in metallothioneins,
 455 but overall, transport of Cu ions from endo- and lysosomes
 456 toward the cytoplasm is very limited, as it does not occur
 457 naturally. For Cu-containing NPs, this poses an entirely

458 different scenario, in which the cells are not equipped to deal
 459 with excess levels of Cu ions in their endosomal compartments.
 460 CuO NPs, which typically dissolve quite quickly, result in free
 461 Cu ions already present in the cell medium and cellular uptake
 462 of excess Cu ions that can lead to cell death.⁴⁸ Alternatively,
 463 the low solubility of CuS and the small contact time during
 464 incubation allowed the internalization of the CuS-based
 465 nanostructures, still intact, in the endosomes of the cells, on
 466 the first day.

467 Moreover, TEM observation of these CuS confirmed their
 468 porous and polycrystalline architecture, made of tiny particles
 469 7–11 nm in diameter. Such tiny crystals are organized in a
 470 hollow porous morphology resulting from the Kirkendall effect
 471 in the presence of polyvinylpyrrolidone (PVP). The degrada-
 472 tion of similar hollow CuS NPs in different physiological
 473 buffers including PBS, RMPI, and DMEM was investigated by
 474 Ortiz de la Solorzano *et al.*⁴¹ at $37 \text{ }^\circ\text{C}$, who showed their
 475 dissolution into water-soluble sulfate-based copper species.
 476 This dissolution led to the decrease of the NIR absorption
 477 peak with almost a total disappearance after 13 days.
 478 Consequently, it is expected that the disappearance of the
 479 NIR absorption should lead to a loss of the photothermal
 480 properties, which was not observed in our investigation for
 481 both types of nanoparticles in cells. These results are in good
 482 agreement with XAS data and indicate that the degradation of
 483 CuS in the endosomes by dissolution of water-soluble copper
 484 species is not the predominant mechanism, at least on the scale
 485 of 29 days. At day 3, in addition to intact hollow structures,
 486 smaller CuS units are also observed by TEM, and these units
 487 decorate the internal membrane of the endosomes. Interest-
 488 ingly, despite the presence of such dissociated structures, no

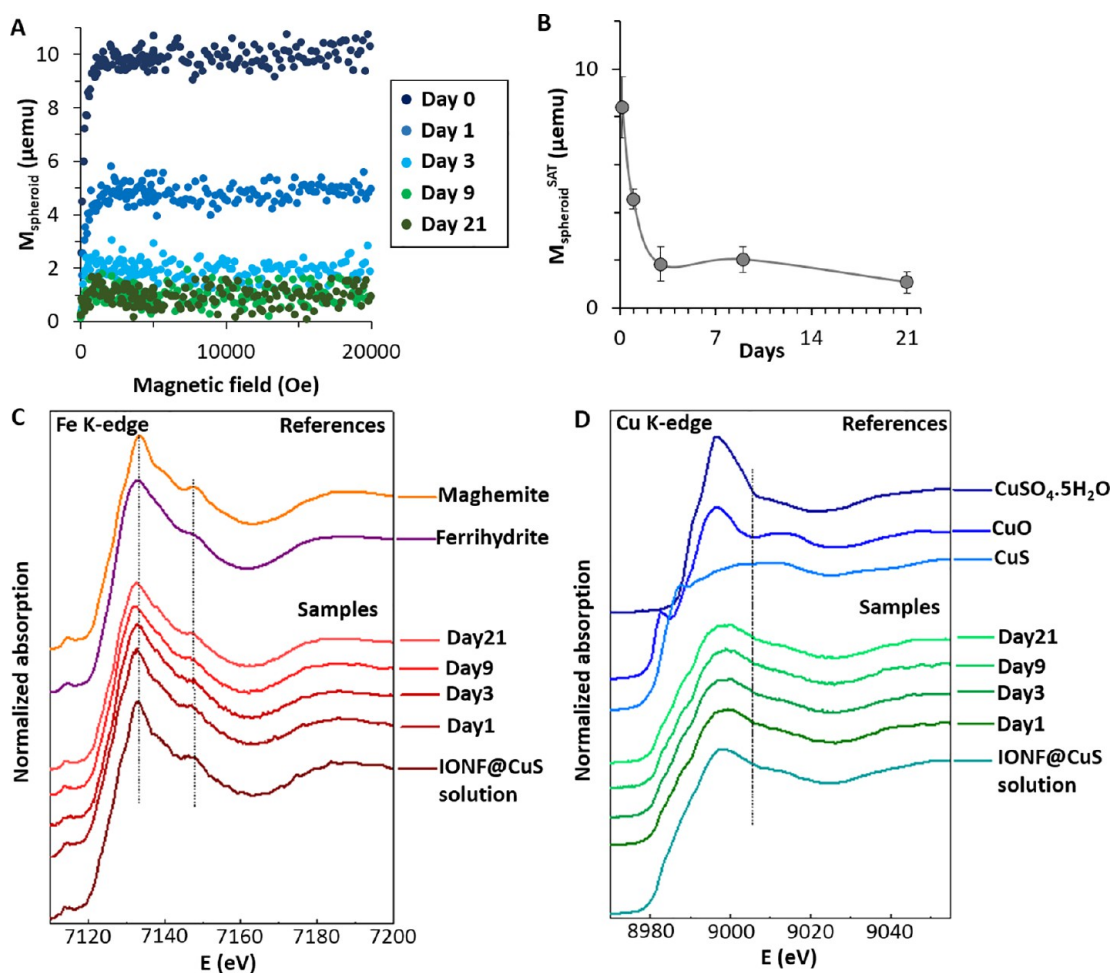


Figure 8. Structural characterization of IONF@CuS NPs processed by the spheroids from incubation day (day 0) to 3 weeks of maturation (day 21), taking advantage of the magnetic core as an additional integrity tracer. (A) Typical magnetometry curves for single spheroids at room temperature over time. (B) Magnetization at saturation averaged over four independent spheroids at each time point (total of 20 spheroids analyzed). (C) XANES spectra at the Fe K-edge of iron oxide references (γ - Fe_2O_3 (maghemite) and $\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$ (ferrihydrite)), IONF@CuS NPs incubated from day 1 to day 21, and IONF@CuS NPs dispersed in water. (D) XANES spectra at the Cu K-edge of copper-based compound references ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, CuO, CuS), IONF@CuS nanoassemblies incubated from day 1 to day 21, and IONF@CuS NPs dispersed in water.

489 change in photothermia or chemical phase was observed.
 490 Consequently, we attributed the formation of such free units to
 491 the disassembly (by dissolution of PVP) of the tiny
 492 nanoparticles from the shell of CuS. After coating with
 493 positively charged endosomal proteins they undergo inter-
 494 action and accumulation at the negatively charged lysosomal
 495 inner membrane. A comparable disintegration mechanism has
 496 been observed *in vivo* in blood plasma for similar hollow shells
 497 by Guo *et al.*⁴⁰ At day 21, TEM showed that no intact CuS
 498 nanostructures remained for both types of nanostructures,
 499 while the lysosome inner membrane appeared decorated with
 500 4–8 nm nanoparticles organized in a hair-like structure. Such
 501 structures are copper-based, as confirmed by XEDS analysis.
 502 Therefore, we may hypothesize that these structures were
 503 generated by continuous disintegration of the CuS over time
 504 and reorganization of the tiny subunits released.

505 At high concentrations, the nanohybrids induced mitochon-
 506 drial stress but no ROS generation, while Cu-based nano-
 507 particles are typically associated with the induction of
 508 ROS.^{36,49} The lack of mitochondrial ROS also reflects a low
 509 level of dissolution of CuS. However, the observed
 510 mitochondrial stress and the increasing activity of genes

related to copper suggest that free copper species are released 511
 during the bioprocessing of the nanostructures. Indeed, XAS 512
 analysis shows that the CuS shell is composed of a mixture of 513
 CuS and CuO phases and free $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Given the higher 514
 solubility of CuO compared to CuS also established in cells, we 515
 may hypothesize that, upon the nanomaterial bioprocessing, 516
 free CuO species are released upon the nanomaterial 517
 bioprocessing and that they are stored in the copper-related 518
 proteins. 519

Concerning the stability of photothermal potential upon the 520
 degradation of the CuS-based NPs into small structures, it is 521
 difficult to establish a clear reason, as the origins of absorption 522
 in copper sulfide remain poorly understood and are still a 523
 subject of debate. While some associate this NIR absorption to 524
 d–d transition of Cu^{2+} ions, which is not affected by the 525
 solvent or the surrounding environment, others attribute such 526
 a behavior to a plasmonic effect generated by free hole 527
 oscillations.^{37,38} In this case the effect of morphology on the 528
 localized surface plasmon resonance is not clearly understood, 529
 and it seems that the morphology has less influence on their 530
 plasmon oscillation mode with respect to the composition.³⁸ In 531
 all cases we can attribute the conservation of the photothermal 532

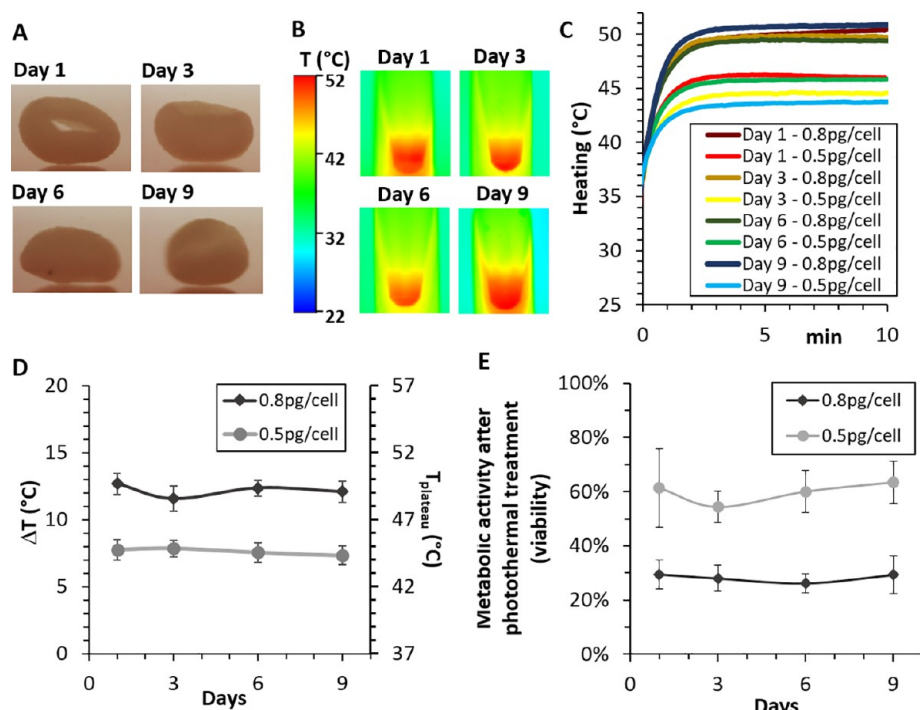


Figure 9. Preservation of photothermia and subsequent therapeutic potential in a glioblastoma spheroid model. (A) Representative photographs of single spheroids made of 2.5×10^5 cancer cells containing CuS NPs at a dose of 0.8 pg of Cu per cell, at days 1, 3, 6, and 9. (B) Typical IR images of cancer spheroids (loaded with CuS NPs at 0.8 pg of Cu per cell) in a 0.5 mL tube (10 μ L volume), at days 1, 3, 6, and 9 of spheroid maturation, upon 10 min of laser irradiation at 0.3 W cm^{-2} . (C) Typical heating curve for single spheroids, at each day (1, 3, 6, and 9) and for the two doses (0.5 and 0.8 pg of Cu per cell). (D) Plateau temperature, expressed as temperature elevation (left vertical axis) and absolute temperature (right secondary axis), averaged over five independent spheroids, as a function of time. (E) Metabolic activity (Alamar blue assay) of the spheroids ($n = 5$ per day and per dose), measured 24 h after photothermal treatment, expressed in % compared to nontreated control spheroids ($n = 5$ per day and per dose).

533 potential to the preservation of the chemical composition, as
 534 confirmed by XAS performed over days on the units formed
 535 after disintegration of the original CuS nanomaterials.

536 These results show clearly that multifunctional materials
 537 with predefined properties can be bioprocessed by cells into
 538 new reshaped biological forms, which may present a high
 539 biocompatibility while maintaining their functionalities and
 540 their programmed therapeutic tasks.

541 CONCLUSION

542 Herein, we studied the effect that a long intracellular exposure
 543 of CuS-based nanomaterials could have on both their own
 544 physical functionalities and on the biology of the stem cells
 545 exposed. CuS were produced as nanoassemblies, without
 546 (CuS) and with a magnetic core (IONF@CuS), internalized in
 547 stem cells further assembled in spheroids to allow long-term
 548 3D culture. Overall, the CuS and IONF@CuS NPs did not
 549 trigger an adverse biological response either immediately (over
 550 the cell viability, oxidative stress), or in the longer-term (over
 551 stem cell differentiation, oxidative stress, iron and copper
 552 metabolism). However, the nanoassemblies were massively
 553 transformed inside the endosomes of the cells, with only few
 554 intact structures observed already after 3 days of cellular
 555 processing. These transformations were quantitatively con-
 556 firmed by magnetometry by the disappearance of the spheroid
 557 magnetism initially provided by the IONF core and confirmed
 558 by XAS synchrotron measurements, revealing the trans-
 559 formation of maghemite into ferrihydrite over time. The
 560 magnetic core was, thus, totally degraded by the cells and

561 stored within the ferritin protein, as generally observed for
 562 magnetic NPs alone. This magnetic degradation reflects the
 563 disassembling of the hybrid IONF@CuS structure, making it
 564 possible for the lysosomal harsh environment to access the
 565 core and degrade it. However, the loss of magnetism was not
 566 associated with a comparable loss of the photothermal
 567 potential of the spheroids over time, provided by the CuS
 568 physicochemical structure of the metal chalcogenide semi-
 569 conductor. The demonstration by XAS that the copper
 570 chemical phase remained the same during the whole 3 weeks
 571 of the spheroid maturation period further confirmed their
 572 functional stability. The copper sulfide nanoassemblies were
 573 therefore reshaped by the cells, triggering a process of nontoxic
 574 metabolization in which the cellular environment managed to
 575 transform the materials into smaller and stable structures with
 576 the same high photothermal potential. Since these nano-
 577 particles are devoted to therapeutic applications, the photo-
 578 thermal follow-up was also performed on spheroids made of
 579 cancer cells. The photothermal potential and its related
 580 therapeutic function were maintained all through the cancer
 581 spheroid maturation (over 9 days). In order to implement
 582 efficient theranostic applications, it appears pivotal to study the
 583 balance between biocompatibility, degradation, and biopro-
 584 cessing while maintaining their physical properties. Herein, we
 585 demonstrate that a theranostics nanomaterial can undergo a
 586 severe clear cell-mediated breakdown but still maintain its
 587 programmed tasks after intracellular processing.

588 MATERIALS AND METHODS

589 **Materials.** All reagents were of analytical purity and used without
590 further purification. Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$,
591 99%), sodium hydroxide (NaOH, 99.99%), diethylene glycol (DEG,
592 99%), *N*-methyl-diethanolamine (NMDEA, 99%), nitric acid (HNO_3 ,
593 70%), copper(II) nitrate hemi(pentahydrate) ($\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$,
594 $\geq 99.99\%$), polyvinylpyrrolidone (PVP, M_w 55 kDa), poly(ethylene
595 glycol) methyl ether thiol (PEG-SH, M_w 2 kDa) hydrazine hydrate
596 (55%), ammonium sulfide solution ($(\text{NH}_4)_2\text{S}$, 20%), sodium
597 cacodylate trihydrate ($\geq 98\%$), glutaraldehyde solution (25% in
598 H_2O), and formalin solution (10%) were purchased from Sigma-
599 Aldrich (France). Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 99%)
600 and ethanol were obtained from VWR (France). Live–Dead Green
601 dead cell and MitoTracker Red CMXRos were purchased from Life
602 Technologies (Thermo Fisher, Belgium).

603 **Iron Oxide Nanoflower Core.** IONFs were synthesized using a
604 modified polyol synthesis as previously described.⁵⁰ In brief, the iron
605 precursors were solubilized in a DEG and NMDEA mixture (1:1 v/v)
606 and heated to 220 °C for 2.5 h to obtain the alkaline hydrolysis. The
607 obtained magnetic nanoflowers were cleaned with ethanol and ethyl
608 acetate and treated with 10% nitric acid to complete the oxidation.
609 They were then redispersed in water and mixed with 0.3% PVP (55
610 kDa) prior to the following step.

611 **Copper Sulfide Assembly.** The synthesis of the substoichiometric
612 copper sulfide Cu_{2-x}S assembly has been carried out in the
613 presence or in the absence of an IONF core using a two-step reaction
614 through a template sacrificial synthesis method, modified from ref 33.
615 In the first step, 10 mg of cupric nitrate, $\text{Cu}(\text{NO}_3)_2$, was dissolved in
616 30 mL of Milli-Q H_2O and mixed with 0.3 g of PVP (55 kDa) and
617 IONF (if present) at $[\text{Fe}] = 0.3$ mM. After 15 min of shaking at room
618 temperature 100 μL of hydrazine 5.5% was added rapidly in the
619 mixture to induce the formation of Cu_2O NPs. The obtained product
620 was cleaned by centrifugation at 9000g for 45 min and resuspended in
621 30 mL of Milli-Q H_2O . The second step of the reaction consisted in
622 the sulfidation of the Cu_2O shell previously synthesized using 0.1 M
623 sodium sulfide followed by heating at 50 °C for 2 h. After several
624 washings by centrifugation and resuspension in Milli-Q H_2O , the
625 surface of the nanoassemblies was PEGylated by shaking the sample
626 overnight in the presence of PEG-SH (final concentration 10 mg/
627 mL) at 4 °C and subsequent washing by centrifugation. These
628 reactions resulted in the production of copper sulfide nanoassemblies
629 (hollow) or of iron oxide nanoflower-like cores surrounded by copper
630 sulfide nanoassemblies (IONF@CuS) when achieved in the presence
631 of IONF (rattle-like).

632 **Morphological and Optical Characterization.** TEM images
633 were obtained using a Hitachi HT 7700 TEM operated at 80 kV
634 (Elexience, France), and images were acquired with a charge-coupled
635 device camera (AMT). UV–vis–NIR characterization was performed
636 with a real-time Avaspec-USB2 spectrometer. Cu and Fe concentration
637 was determined by elemental analysis using ICP-AES (iCAP
638 6500, Thermo Scientific).

639 **Laser-Induced Thermometric Measurements.** Heating profiles
640 of aqueous solutions were obtained by placing 10 μL of CuS or
641 IONF@CuS dispersions at concentrations ranging from 0.5 to 40 mM
642 of Cu in a 0.5 mL tube at a 4 cm distance from the laser source. The
643 samples were irradiated with a 1064 nm laser at a power density of 0.3
644 W cm^{-2} until equilibrium temperature was reached (typically in 1–2
645 min, measurements were performed over 5 min to be sure to measure
646 the plateau temperature). The increase in temperature was measured
647 using an FLIR SC7000 infrared thermal camera. The spheroids were
648 analyzed in the same configuration. All values are reported as means
649 of at least three separate experiments.

650 **Cell Culture and Nanoparticle Uptake.** Human mesenchymal
651 stem cells were purchased from Lonza and were cultured in hMSC-
652 basal medium at 37 °C, 5% CO_2 , and 95% relative humidity. Human
653 glioblastoma cells U87 were purchased from ATCC and cultured in
654 DMEM medium supplemented with 1% (w/v) streptomycin, 1% (w/
655 v) penicillin, and 10% (w/v) FBS. At 90% confluence, cells were
656 incubated with the nanoassemblies diluted in serum-free RPMI

medium. The copper concentration in the medium ranged from 0.1 to 657
1 mM. After 4 h of incubation, the medium was removed and the cells 658
were rinsed and incubated further for 2 h in complete hMSC-basal 659
medium to remove any noninternalized copper nanoassemblies. Then, 660
cells were detached by trypsinization, counted, and immediately 661
analyzed or further processed. To assess the intracellular NP content, 662
 2.5×10^5 cells were digested in pure nitric acid for 48 h until total 663
dissolution, diluted up to 2% HNO_3 in ultrapure H_2O , and analyzed 664
by elemental analysis. 665

Nanotoxicity Study. For the biocompatibility assays, 1.25×10^3 666
or 2.5×10^3 cells per well were seeded in 96-multiwell culture plates 667
at 100 μL total volume and incubated overnight. IONF@CuS or CuS 668
nanoassemblies at the concentrations of 0.04, 0.16, 0.32, 1.6, and 3.2 669
mM of Cu were dispersed in cell culture media and incubated with 670
hMSC cells for 24 h (2.5×10^3 cells) or 72 h (1.25×10^3 cells). At 671
the end of the incubation, the media was removed and fresh media 672
was provided containing Live–Dead Green dead cell and 673
MitoTracker Red CMXRos (Molecular Probes, Life Technologies 674
Europe, BV, Belgium), after which the cells were further incubated for 675
30 min in a humidified atmosphere at 37 °C and 5% CO_2 . Next, the 676
cells were washed three times with PBS, fixed with 4% 677
paraformaldehyde (PFA), and counterstained with Hoechst 33342 678
nuclear stain solution (Life Technologies, Belgium). Next, the plates 679
were analyzed using the INCell Analyzer 2000 (GE Healthcare Life 680
Sciences, Belgium), while 2000 cells per condition were acquired in 681
triplicates using a 20 \times objective lens for the DAPI/DAPI (Hoechst), 682
FITC/FITC (Live–Dead Green), and TexasRed/TexasRed (Mito- 683
Tracker Red CMXRos) channels. The acquired images were 684
processed using the InCell Investigator software (GE Healthcare 685
Life Sciences, Belgium). Cell viability was calculated by segmenting 686
cell nuclei and dead cells (signal crossing the threshold in the green 687
channel overlapping with the nuclei) using the InCell Developer 688
software (GE Healthcare Life Sciences, Belgium). The number of live 689
cells per each condition was calculated as the total number of nuclei 690
counted minus the number of dead cells. The values were then 691
normalized by the control conditions (= 100%). Finally, for 692
mitochondrial stress, the total area of cellular mitochondria was 693
used, while for mitochondrial ROS, the intensity of the mitochondrial 694
stain was determined. The respective channel was segmented using 695
the Hoechst images as seed, and the total size and intensity of the 696
mitochondrial network were determined for each individual cell. 697
These values were then normalized by the respective control 698
conditions (= 100%). Results represent quantitative data for the 699
analysis of a minimum of 2000 cells per condition. Values are 700
presented as mean + SEM ($n = 3$). 701

Stem Cell Spheroid Formation and Characterization. A total 702
of 2.5×10^5 stem cells were centrifuged (1200 rpm for 5 min) in 15 703
mL tubes to form a pellet and cultured in order to induce cell 704
differentiation (chondrogenesis). The cells then spontaneously 705
formed a spheroid, which could be kept in culture for months 706
(here up to 3 weeks of spheroid maturation). The differentiation 707
medium was composed of high-glucose DMEM supplemented with 708
1% penicillin–streptomycin, 0.1 μM dexamethasone, 1 mM sodium 709
pyruvate, 50 μM L-ascorbic acid 2-phosphate, 0.35 mM L-proline 710
(Sigma), 1% ITS-Premix (Corning), and 10 ng/mL TGF- β 3 711
(Interchim) and was changed twice a week. For histological analysis, 712
spheroids harvested after 21 days of maturation were fixed overnight 713
in 10% formalin, before paraffin inclusion and cutting. Slices that were 714
4 μm thick were treated with toluidine blue 0.04% for collagen 715
staining and then analyzed by optical microscopy. 716

At days 1, 3, 9, and 21, the spheroids were fixed with 4% PFA for 2 717
h at room temperature and transferred in PBS for photothermal 718
analysis, magnetic characterization, and elemental characterization. 719
For the electron microscopy analysis, other spheroids at the same time 720
points were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer 721
for 2 h, contrasted with oolong tea extract (OTE) 0.5% in 0.1 M Na 722
cacodylate buffer, postfixed with 1% osmium tetroxide containing 723
1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30% to 724
100%), and gradually embedded in epoxy resins. Ultrathin slices (70 725

726 nm) were collected onto 200 mesh copper grids and counterstained
727 with lead citrate prior to being observed by TEM.

728 **Gene Expression Quantification by qPCR.** Total RNA was
729 extracted from spheroids at different maturation times using a
730 NucleoSpin RNA II kit (Macherey-Nagel). Reverse transcription into
731 cDNA was achieved using SuperScript II reverse transcriptase
732 (Invitrogen) with random hexamers as primers according to the
733 manufacturer's instructions. qPCR was performed with StepOnePlus
734 (Applied Biosystems) using the SYBR Green reagent (Applied
735 Biosystems). The expression of reference gene RPLP0 was used as a
736 housekeeping transcript gene. Each value is obtained by the average of
737 at least two wells gathering a minimum of three independent
738 repetitions. The sequences of primers used are listed in Table S1.

739 **Magnetometry of the Internalized IONF Core by VSM.** Cell
740 magnetization right after IONF@CuS nanoassembly incubation and
741 in samples fixed at different spheroid maturation times was measured
742 by magnetometry using a PPMS device equipped with a vibrating
743 sample magnetometer (VSM) option (Quantum Design). The
744 analysis was performed at 300 K, between 0 and 20 000 Oe, and
745 provides the saturation magnetization of the sample (in emu).

746 **XAS/Synchrotron Measurements.** XAS measurements were
747 performed on the spheroids pooled in groups of 4 or 5 for each
748 maturation time to increase the signal-to-noise ratio. Measurements
749 were achieved in the XANES regime at the CRG beamline BM25-
750 SpLine of the European Synchrotron Radiation Facilities (ESRF) in
751 Grenoble (France). The spectra were acquired at the Fe K-edge
752 (7112 eV) and Cu K-edge (8980 eV) at room temperature and
753 atmospheric pressure in transmission and fluorescence modes. Metal
754 foils of Fe and Cu elements were measured as energy calibration
755 references. Iron oxides (as maghemite and ferrihydrite) and several
756 copper-based materials (Cu-S-O compounds as copper sulfides,
757 copper oxides, and copper sulfates) were chosen as standards. For
758 each condition (at days 1, 3, 9, and 21 of maturation), a group of 3 or
759 4 multicellular spheroids was measured to improve the signal-to-noise
760 ratio. XANES spectra of IONF, IONF@CuS, and CuS initial solution
761 samples were also evaluated. Data normalization, energy calibration,
762 and analysis of the XAS data were carried out using the Demeter
763 software package (Athena program).⁵¹

764 **Cancer Cell Spheroid Formation and Photothermal
765 Therapy.** In the exact same way as for the stem cells, 2.5×10^5
766 U87 cancer cells were pelleted and kept in complete culture medium
767 for 9 days. At days 1, 3, 6, and 9, the spheroids (5 per day) were
768 collected and transferred to 0.5 mL tubes (one spheroid per tube) in
769 10 μ L of culture medium. The tubes were placed in a thermostatic
770 device so that the sample is maintained at a temperature of 37 °C
771 before exposure to the laser. Heating was achieved with the spheroids
772 placed 4 cm away from the laser source (1064 nm), corresponding to
773 a laser power density of 0.3 W cm⁻².

774 The samples were irradiated for 10 min. The increase in
775 temperature was measured using an FLIR SC7000 infrared thermal
776 camera. After laser treatment, the spheroids were transferred to a 48-
777 multiwell plate, and each single spheroid's metabolic activity was
778 measured 24 h later, by the Alamar blue assay, and renormalized by
779 nontreated control spheroid values (5 control spheroids for each
780 measurement day).

781 **Statistical Analysis.** All values are reported as means and
782 standard error of the mean. Significant differences were determined
783 using Tukey's test in one-way analysis of variance (ANOVA). *
784 denotes a *p*-value < 0.05 (significant result), ** a *p*-value < 0.01 (very
785 significant), and *** a *p*-value < 0.001 (highly significant).

786 ASSOCIATED CONTENT

787 **SI** Supporting Information

788 The Supporting Information is available free of charge at
789 <https://pubs.acs.org/doi/10.1021/acsnano.1c00567>.

790 Additional nanoparticle and *in vivo* TEM images, DLS
791 analysis, light-to-heat conversion coefficient, elemental
792 analysis, InCell analyzer microscopy images, gene
793 expression, STEM-HAADF and XEDS characterization,

supplementary IR images, *in vitro* photothermal analysis, 794
XANES synchrotron spectra, and the qPCR primers list 795
(PDF) 796

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