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Massive Intracellular Remodeling of CuS Nanomaterials Produces Nontoxic Bioengineered Structures with Preserved Photothermal Potential

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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 $[\text{Cu}] = 20 \text{ mM}$.

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

Among the multifunctional NPs that are particularly promising for the biomedical field, those based on copper sulfide (CuS) are emerging as a promising class with numerous therapeutic features.^{32–34} Their light-to-heat conversion efficiencies are among the highest,^{33,35} and they provide dual laser-mediated treatments for both photothermal and photodynamic therapies in the most preferred second near-infrared 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 autofluorescence. 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}

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

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

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

RESULTS AND DISCUSSION

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

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

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

Biological Responses Using High-Content Imaging. Results of cytotoxicity analyses of cellular interaction with the different NPs for 72 h investigating cell viability, mitochondrial reactive oxygen species (MitoROS), and the size of the mitochondrial network are presented in Figure 3. Representative images of the InCell analysis experiment for viability and MitoROS are presented in Figure 3D and Figure S7. Altogether, these results indicate that CuS NPs induce toxic effects only at the highest concentrations (Figure 3A). The level of observed cell death was accompanied by only a marginal increase in MitoROS (Figure 3B), while mitochondrial area (an indicator for mitochondrial stress) displayed a significant reduction in size of the cellular mitochondrial network (Figure 3C). The latter indicates a clear increase in mitochondrial stress, at high concentrations, but this does not seem to be linked to the generation of ROS.

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

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 μ L) 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 μ L 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.

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

(day 9 and day 21) are shown in Figure S11 and Figure S12. Importantly, energy-dispersive X-ray spectroscopy (XEDS) elemental analysis of the transformed nanostructures at day 21 confirmed the presence of copper along the membrane of lysosomes (Figure S13). Interestingly, only for the IONF@CuS condition could we also detect small 5–7 nm NPs, that we identify as iron deposits in 5–7 nm ferritins previously observed after degradation of iron oxide nanoparticles.^{23,30,31} In summary, both CuS and IONF@CuS nanoassemblies undergo a profound morphological reshaping inside the lysosomes, which occurs in close association to the inner side of the lysosomal membrane, for the Cu processing.

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 *** $p < 0.001$.

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

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

clearly demonstrating a significant reduction of the photothermal 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 magnetization 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.

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

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

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

Hypothetical Mechanism. The data presented here 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.

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

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

Moreover, TEM observation of these CuS confirmed their porous and polycrystalline architecture, made of tiny particles 7–11 nm in diameter. Such tiny crystals are organized in a hollow porous morphology resulting from the Kirkendall effect in the presence of polyvinylpyrrolidone (PVP). The degradation of similar hollow CuS NPs in different physiological buffers including PBS, RMPI, and DMEM was investigated by Ortiz de la Solorzano *et al.*⁴¹ at 37°C , who showed their dissolution into water-soluble sulfate-based copper species. This dissolution led to the decrease of the NIR absorption peak with almost a total disappearance after 13 days. Consequently, it is expected that the disappearance of the NIR absorption should lead to a loss of the photothermal properties, which was not observed in our investigation for both types of nanoparticles in cells. These results are in good agreement with XAS data and indicate that the degradation of CuS in the endosomes by dissolution of water-soluble copper species is not the predominant mechanism, at least on the scale of 29 days. At day 3, in addition to intact hollow structures, smaller CuS units are also observed by TEM, and these units decorate the internal membrane of the endosomes. Interestingly, 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 ($\gamma\text{-Fe}_2\text{O}_3$ (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.

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

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

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

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

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).

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

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

CONCLUSION

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

stored within the ferritin protein, as generally observed for magnetic NPs alone. This magnetic degradation reflects the disassembling of the hybrid IONF@CuS structure, making it possible for the lysosomal harsh environment to access the core and degrade it. However, the loss of magnetism was not associated with a comparable loss of the photothermal potential of the spheroids over time, provided by the CuS physicochemical structure of the metal chalcogenide semiconductor. The demonstration by XAS that the copper chemical phase remained the same during the whole 3 weeks of the spheroid maturation period further confirmed their functional stability. The copper sulfide nanoassemblies were therefore reshaped by the cells, triggering a process of nontoxic metabolization in which the cellular environment managed to transform the materials into smaller and stable structures with the same high photothermal potential. Since these nanoparticles are devoted to therapeutic applications, the photothermal follow-up was also performed on spheroids made of cancer cells. The photothermal potential and its related therapeutic function were maintained all through the cancer spheroid maturation (over 9 days). In order to implement efficient theranostic applications, it appears pivotal to study the balance between biocompatibility, degradation, and bioprocessing while maintaining their physical properties. Herein, we demonstrate that a theranostics nanomaterial can undergo a severe clear cell-mediated breakdown but still maintain its 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 (Macheney-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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Notes

The authors declare no competing financial interest.

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