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# **Multifunctional temozolomide-loaded lipid superparamagnetic nanovectors: Dual targeting and disintegration of glioblastoma spheroids by synergic chemotherapy and hyperthermia treatment**

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## Abstract

Aiming at finding new solutions for fighting glioblastoma multiforme, one of most aggressive and lethal human cancer, here an *in vitro* validation of multifunctional nanovectors for drug delivery and hyperthermia therapy is proposed. Hybrid magnetic lipid nanoparticles have been fully characterized and tested on a multi-cellular complex model resembling the tumor microenvironment. Investigations of cancer therapy based on a physical approach (namely hyperthermia) and on a pharmaceutical approach (by exploiting chemotherapy drug temozolomide) have been extensively carried out, by evaluating antiproliferative and pro-apoptotic effects on 3D models of glioblastoma multiforme. A systematic study of transcytosis and endocytosis mechanisms has been moreover performed with multiple complimentary investigations, besides a detailed description of local temperature increments following hyperthermia application. Finally, an in-depth proteomic analysis corroborated the obtained findings, which can be summarized in the preparation of a versatile, multifunctional, and effective nanoplatform able to overcome the blood-brain barrier and to induce powerful anti-cancer effects on *in vitro* complex models.

## Introduction

In the current clinical practice, the golden standard therapy against glioblastoma multiforme (GBM) relies on surgical resection followed by the combination of radiotherapy and chemotherapy, usually performed with temozolomide (TMZ).<sup>[1]</sup> The poor prognosis of this pathology (12-15 months) is mainly associated to the usual occurring recurrence of GBM after these treatments. Moreover, the scarce efficacy of the treatment is related to the impossibility to completely remove GBM cells by surgery, to the inability to deliver an effective dose of TMZ to the cancer mass, and to the elevated aggressiveness of the GBM cells.<sup>[2]</sup> Moreover, GBM is the most angiogenic brain tumor,<sup>[3]</sup> and cannot be completely resected due to its indistinct margins.<sup>[4]</sup> Groups of cells that are removed by surgery develop the so-called microscopic foci; these cell populations are extremely difficult to be detected, resist to the current chemotherapy / radiotherapy approaches, and regenerate the tumor mass in a few months.<sup>[5]</sup> In this context, the current strategies dedicated to prevent the GBM recurrence require the precise targeting, at both the anatomical and cellular level, of therapeutic / theranostic agents against the microscopic foci.

The recent development of nanotechnology promises to revolutionize the delivery of chemotherapeutic agents and of other pharmacologically / biologically active compounds across the blood-brain barrier (BBB) and towards cancer cells.<sup>[6,7]</sup> Beside the passive phenomena of nanomaterial accumulation to the tumor sites due to its highly fenestrated microcapillaries, other active mechanisms for the systemic delivery of theranostic nanomaterial to brain cancer have been recently developed and validated.<sup>[8]</sup> Promising approaches include the exploitation of magnetically-responsive nanovectors for the anatomical targeting through an external magnetic guidance,<sup>[9]</sup> permeability enhancers for the transient opening of the BBB in specific brain areas,<sup>[10]</sup> and molecular "Trojan horses" for the dual targeting of BBB and GBM cells.<sup>[11]</sup> In this regard, magnetically responsive nanocarriers represent a multifunctional platform with targeting and diagnostic capabilities, adopted for the remote delivery of drugs and of magnetothermal stimuli to cancer cells.<sup>[9]</sup> Superparamagnetic nanoparticles are single-domain magnetic nanostructures characterized by excellent magnetic susceptibility; when exposed to alternating magnetic fields (AMF), they efficiently generate heat through Néel's and Brown's relaxation phenomena. Single-domain magnetic nanoparticles do not show remanence and coercivity, thus preventing their aggregation and ensuring the maintenance of their superparamagnetic behaviour.<sup>[12,13]</sup> Superparamagnetic iron oxide nanoparticles (SPIONs) are magnetic nanostructures with excellent biocompatibility, and they have been approved by the Food and Drug Administration (FDA) for the clinical treatment of anemia associated with chronic kidney disease.<sup>[14]</sup> Moreover, SPIONs have been successfully exploited in many different clinical trials for the remote hyperthermal treatment of cancer cells in response to alternated magnetic fields (AMF) and as contrast agents for magnetic resonance imaging (MRI).<sup>[15]</sup> As a supplementary function, SPIONs can be incorporated into thermosensitive nanovectors for the controlled release of specific anticancer drugs / molecules.<sup>[16]</sup> In this work, the functionalization of SPIONs- and TMZ-loaded lipid magnetic nanovectors (LMNVs) with an antibody against the transferrin receptor (TfR) for the dual targeting of the endothelial cells of the BBB and of GBM cells is reported. The targeting efficiency of the functionalized nanovectors (AbLMNVs) has been demonstrated on a multicellular organoid system in the presence of an *in vitro* BBB model. Transcytosis of functionalized nanovectors through endothelial cells and their penetration into GBM spheroids have been verified and quantified through flow cytometry analysis and several imaging techniques. Moreover, the

lipid component of the functionalized nanovectors has been modified with a lipophilic temperature sensitive fluorescent dye to monitor the intraparticle temperature in response to the AMF exposure. Chronic AMF treatments of GBM spheroids targeted with the functionalized nanovectors, either plain or loaded with TMZ, were carried out and their elevated potential to induce spheroid disintegration, cell necrosis and apoptosis was revealed. Finally, magnetothermal ability of nanovectors was successfully tested on a *post-mortem* animal brain tissue.

## Results

### AbLMNV characterization

Lipid magnetic nanovectors (LMNVs) loaded with superparamagnetic iron oxide nanoparticles (SPIONs) were functionalized with antibody against the transferrin receptor (anti-TfR Ab) in order to obtain a dual targeting of the endothelial cells of the blood-brain barrier (BBB) and of the GBM cells, as both these types of cells highly express the TfR.<sup>[17,18]</sup> The scheme of the nanovector functionalization, the TEM imaging, and the analysis of the Ab functionalization efficiency are shown in **Figure 1**. **Figure 1a** depicts the surface modification, which has been obtained by exploiting a biotin-streptavidin interaction between biotin-functionalized 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-conjugated methoxypoly(ethylene glycol) (biotin-mPEG-DSPE) and streptavidin-conjugated Ab. TEM imaging of the Ab-functionalized nanovectors (AbLMNVs) is reported in **Figures 1b-c**. AbLMNVs are characterized by a spheroidal morphology and a diameter of  $36 \pm 25$  nm; the presence of the SPIONs can be appreciated owing their higher electron-density with respect to the lipid matrix where they are embedded. The average hydrodynamic diameter ( $D$ ) and the polydispersity index (PDI) of the AbLMNVs resulted, respectively,  $D = 101.3 \pm 1.1$  nm and  $PDI = 0.19 \pm 0.01$  in water, and  $D = 94.7 \pm 1.0$  nm and  $PDI = 0.48 \pm 0.01$  in complete medium. Further details concerning the physicochemical and magnetic characterization of the nanovectors can be found in a previous work of our group.<sup>[16]</sup>

The nanovector functionalization was confirmed by SDS-PAGE (**Figure 1d**). After the molecular weight indicator, the first three lanes represent the bands of the free Ab (5, 1 and 0  $\mu$ g of Ab are loaded from left to right, respectively); the fourth lane is instead loaded with AbLMNV (30  $\mu$ l of a 5 mg ml<sup>-1</sup> dispersion). The characteristic bands of the anti-TfR Ab can be found in the AbLMNVs sample, therefore confirming the successful functionalization of the

particles. The last two lanes consist of two negative controls (biotin-LMNVs and LMNVs, respectively, from left to right), that do not present any band, as expected. The correlation between the intensity of the band attributed to the Ab light chain (MW ~ 25 kDa) and the amount of free Ab loaded in the gel is shown as Supplementary Information (**Figure S1**); owing to this calibration curve, an amount of 1.9  $\mu\text{g}$  of Ab was estimated in 30  $\mu\text{l}$  of a 5  $\text{mg ml}^{-1}$  AbLMNV dispersion. This result indicated that the 42% of the Ab used in the functionalization reaction successfully associated to the nanovectors.

### **Dual targeting and magnetic guidance of functionalized AbLMNVs**

The dual targeting of AbLMNVs was investigated by using a multicellular model of the blood-brain barrier (BBB, with brain endothelial cells and astrocytes) separating a luminal from an abluminal compartment, the latter of which contains cancer spheroids (**Figure 2**). The scheme of the multicellular organoid system used for studying the BBB crossing and the following GBM targeting of the nanovectors is shown in **Figure 2a**. Astrocytes and endothelial cells of the BBB are respectively seeded on the abluminal and luminal side of the membrane separating the two compartments. Endothelial cells of the BBB model developed a dense monolayer and highly expressed *zonula occludens-1* (ZO-1), a specific marker of the tight junctions; trans-endothelial electrical resistance (TEER) between luminal and abluminal compartment was  $99.0 \pm 6.6 \Omega \cdot \text{cm}^2$  after 4 days of culture. The complete formation of the endothelial layer on the luminal side of the BBB can be appreciated by the confocal laser scanning microscopy imaging of ZO-1 provided in Supplementary Information (**Figure S2**). Plain or functionalized nanovectors (LMNVs or AbLMNVs), fluorescently labeled with DiO, were incubated in the luminal compartment and their crossing through the BBB in the abluminal chamber was investigated with or without the presence of a static magnetic field (SMF), at different time points (24, 48 and 72 h; **Figure 2b**). In this study, we observed that the presence of SMF and the functionalization with anti-TfR Ab were able to independently and synergistically promote the BBB crossing. Regarding the samples non-treated with SMF, the concentration of LMNVs in the abluminal compartments at 48 h ( $3.0 \pm 1.3 \mu\text{g}$ ) and 72 h ( $7.1 \pm 1.3 \mu\text{g}$ ) resulted significantly lower with respect to that one of AbLMNVs measured at 48 h ( $7.1 \pm 0.7 \mu\text{g}$ ;  $p < 0.05$ ) and at 72 h ( $13.3 \pm 1.3 \mu\text{g}$ ;  $p < 0.05$ ). No significant differences in BBB crossing were detected for LMNVs and AbLMNVs at 24 h ( $p > 0.05$  %). The presence of SMF induced a remarkable increase of BBB crossing of both LMNVs ( $8.4 \pm 6.9 \mu\text{g}$  at 24 h,  $22.2 \pm 5.9 \mu\text{g}$  at 48 h,  $42.4 \pm 13.8$  at 72 h), and AbLMNVs ( $9.5 \pm 3.9 \mu\text{g}$  at 24 h,  $41.0 \pm 2.2 \mu\text{g}$  at 48

h,  $99.9 \pm 10.2 \mu\text{g}$  at 72 h) with respect to the corresponding plain or functionalized nanovectors incubated without SMF (for LMNVs  $2.9 \pm 2.2 \mu\text{g}$  at 24 h;  $2.0 \pm 0.3 \mu\text{g}$  at 48 h,  $p < 0.05$ ;  $5.9 \pm 0.2 \mu\text{g}$  at 72 h,  $p < 0.05$ ; for AbLMNVs  $0.3 \pm 4.1 \mu\text{g}$  at 24 h,  $p < 0.05$ ;  $7.1 \pm 0.6 \mu\text{g}$  at 48 h,  $p < 0.05$ ;  $13.3 \pm 1.3 \mu\text{g}$  at 72 h,  $p < 0.05$ ). It is important to highlight as both the investigated factors (the SMF and the functionalization with the anti-TfR Ab) independently enhanced the BBB crossing, and that the best performances were observed by synergistically exploiting AbLMNVs+SMF for 72 h ( $99.9 \pm 10.2 \mu\text{g}$  of crossing nanovectors).

The targeting of nanovectors to the GBM spheroids, after the BBB model crossing, was investigated by confocal laser scanning microscopy (CLSM) imaging and flow cytometry at 72 h of treatment. **Figure 2c** shows the 3D rendering obtained from CLSM imaging of representative spheroids at 72 h of incubation with LMNVs / AbLMNVs in the luminal compartment, with or without the presence of the SMF. The percentage of the spheroid volume occupied by nanovectors is reported in **Figure 2d**; the anti-TfR Ab was able to significantly promote the spheroid targeting both in the presence of SMF ( $7.2 \pm 1.2\%$  for LMNVs+SMF and  $62.3 \pm 16.5\%$  for AbLMNVs+SMF,  $p < 0.05$ ), and without the SMF ( $0.8 \pm 1.1\%$  for LMNVs and  $2.2 \pm 1.3\%$  for AbLMNV,  $p < 0.05$ ). Similarly to data collected concerning the BBB crossing, the best results in terms of spheroid targeting were observed by synergistically combining the Ab functionalization with the SMF application ( $p < 0.05$ ). Obtained results were then confirmed through flow cytometry by analyzing the fluorescence of the cells dissociated from spheroids (**Figure 2e**). The higher percentage of nanovector-positive (nanovector<sup>+</sup>) cells were observed in AbLMNVs+SMF (72.4%), followed by LMNVs+SMF (39.8%), AbLMNVs (26.2%), and LMNVs (11.8%).

In this experimental configuration, the GBM spheroids targeting efficiency of the nanovectors might be affected by the different levels of their BBB crossing. For this reason, subsequent tests have been performed by directly incubating the spheroids with nanovectors at those concentrations that have been found in the abluminal compartment upon BBB crossing. In these conditions, analysis of nanovector targeting / penetration in GBM spheroids has been carried out (**Figure 3**). After 24 and 48 h of LMNVs / AbLMNVs incubation ( $167 \mu\text{g ml}^{-1}$ , corresponding to the highest concentration found in the abluminal compartment after 72 h of BBB crossing), CLSM imaging was performed (**Figure 3a**); the percentage of the spheroid volume occupied by nanovectors is reported in **Figure 3b**. Interestingly, results indicated that AbLMNVs associated to and internalized in spheroids with improved efficiency with respect

to plain LMNVs. Specifically, the percentage of spheroid volume occupied by AbLMNVs ( $1.5 \pm 0.7\%$  at 24 h and  $40.5 \pm 2.9\%$  at 48 h), was remarkably higher with respect to that one observed for LMNVs ( $0.8 \pm 0.7\%$  at 24 h and  $8.1 \pm 0.5\%$  at 48 h;  $p < 0.05$ ). The preferential targeting of AbLMNVs was then confirmed by focused ion beam (FIB) milling combined with scanning electron microscopy (SEM; **Figure 3c**). Owing to this approach, it has been possible to observe an increased amount of highly electron-dense nanoparticles internalized in the cells inside spheroids when incubated with AbLMNVs with respect to LMNVs. Moreover, the different phases of the nanovector internalization were observed: the nanovector-plasma membrane contact, the cell membrane invagination, and the nanovector internalization in intracellular vesicles (**Figure 3d**).

### **Analysis of the nanovector internalization pathway**

We hypothesized that the increased BBB crossing of the functionalized nanovectors with respect to the plain ones could be associated to a different internalization pathway; to test this hypothesis, the analysis of the mechanisms involved in the uptake of plain / functionalized nanovectors by the endothelial cells of the BBB was carried out (**Figure 4**). Three different markers expressed in caveosomes (caveolin-1), clathrin-coated vesicles (clathrin) and transcytosis vesicles (rab11) were investigated by CLSM in endothelial cells of the BBB incubated with LMNVs or AbLMNVs (**Figure 4a**). The CLSM imaging revealed higher intracellular levels of AbLMNVs compared to the LMNVs; the enhanced uptake of the functionalized particles by the endothelial cells can be associated with the observed increased level of BBB crossing. Moreover, a relatively high co-localization of the AbLMNV signal with clathrin and rab11 markers can be appreciated. The quantitative analysis of the Pearson's correlation coefficients is reported in **Figure 4b**. A limited internalization of both LMNVs and AbLMNVs in caveosomes was also observed (Pearson's correlation coefficient of  $0.03 \pm 0.03$  for LMNVs and  $0.03 \pm 0.05$  for AbLMNVs). Increased internalization of AbLMNVs in clathrin<sup>+</sup> (Pearson's correlation coefficient of  $0.32 \pm 0.08$ ) and rab11<sup>+</sup> (Pearson's correlation coefficient of  $0.54 \pm 0.19$ ) vesicles was observed with respect to the non-functionalized LMNVs (Pearson's correlation coefficient  $0.12 \pm 0.03$  for clathrin,  $p < 0.05$ ; Pearson's correlation coefficient of  $0.28 \pm 0.03$  for rab11,  $p < 0.05$ ). These results indicate as the presence of Ab on the nanovector surface promotes a clathrin-mediated endocytosis and the transcytosis through rab11<sup>+</sup> vesicles. **Figure 4c** reports a SEM imaging after FIB transverse sectioning of endothelial cells during the uptake of LMNVs (top) and AbLMNVs (bottom).

### **AbLMNVs-assisted magnetothermal stimulation affects GBM cell membrane functionality**

The ability of AbLMNVs-assisted magnetothermal stimulation to affect plasma membrane functionality was firstly investigated on 2D living cultures of GBM cells (**Figure 5**). For these experiments, GBM cultures were incubated with  $167 \mu\text{g ml}^{-1}$  of AbLMNVs, which corresponds to nanovector concentration that was detected in the abluminal compartment after 72 h of BBB crossing. Firstly, the analysis of intracellular temperature during AMF stimulation was monitored at different time points by exploiting the ER-thermo yellow temperature sensor (**Figure 5a-b**). ER-thermo yellow is a thermosensitive fluorescent dye, the fluorescence emission of which proportionally decreases in response to the temperature increment.<sup>[19-21]</sup> GBM cultures non-incubated with AbLMNVs that underwent AMF were considered as negative controls. A significant decrease of the fluorescence intensity in GBM cells incubated with AbLMNVs was observed (**Figure 5a**). The fluorescence ( $F/F_0$ ) and temperature ( $T$ ) time courses are shown in **Figure 5b** (graphs on the left and on the right, respectively). The temperature levels of the samples stimulated with AbLMNVs+AMF rose from  $25^\circ\text{C}$  until  $\sim 41^\circ\text{C}$ , while an increase of just  $2.5^\circ\text{C}$  was detected in non-incubated negative controls (AMF). The AMF-mediated AbLMNVs-assisted magnetothermal stimulation was carried out on GBM cells in the presence of propidium iodide (PI) in the extracellular medium, as an indicator of membrane integrity. In **Figure 5c** the fluorescence and transmission light images by CLSM are reported for two different time points ( $t = 0$  min and  $t = 70$  min; AMF stimulus started at  $t = 12$  min). Three different experimental conditions were considered: AbLMNVs-incubated cells non-stimulated with AMF (AbLMNVs), cultures non-incubated with AbLMNVs that underwent AMF stimulation (AMF) and cells incubated with AbLMNVs and stimulated with AMF (AbLMNVs+AMF). It is possible to observe cells internalizing the PI during the magnetothermal stimulation generated by the combined AbLMNVs+AMF treatment (white arrows); the PI internalization indicates the loss of cell membrane integrity and this is attributable to the temperature increase.<sup>[22]</sup> Instead, AMF stimulation or the AbLMNV incubation did not singularly affect the membrane functionality, highlighting the safeness of this remote stimulation approach. The fluorescence time-lapses of the three experimental conditions are available as Supplementary Information (Video S1, S2, and S3, respectively). The fluorescence ( $F/F_0$ ) time courses relative to PI uptake are reported in **Figure 5d** (the black arrow indicate the starting of the AMF stimulus), while the CLSM imaging of the whole samples at the end of the experiment is shown in **Figure 5e**. A remarkable increase of  $\text{PI}^+$

cells was observed in response to the magnetothermal AbLMNVs+AMF treatment with respect to the all the other experimental classes.

### **Temperature imaging in AbLMNVs in response to AMF**

The monitoring of the intraparticle temperature during exposure to AMF was performed by exploiting the temperature-sensitivity of the fluorescence emission spectra of the Dil lipophilic dye (**Figure 6**). In **Figure 6a**, the emission spectra of Dil in DMSO solution during two temperature cycles are shown ( $\lambda_{ex} = 560$  nm). The amplitude of the fluorescence emission peak ( $\lambda_{em} = 583$  nm) linearly decreases in response to the temperature increment; the phenomenon is reversible and the Dil fluorescence emission returns to its original state after subsequent temperature cycles (**Figure 6b**). Fluorescence imaging of the Dil-stained AbLMNVs during heating ( $\Delta T_1 = 0.0$  °C;  $\Delta T_2 = 3.5$  °C;  $\Delta T_3 = 7.8$  °C;  $\Delta T_4 = 10.9$  °C;  $\Delta T_5 = 12.3$  °C;  $\Delta T_5 = 14.3$  °C) induced by an infrared (IR) laser source are reported in **Figure 6c**. The measurement of the temperature increments generated by different laser powers (LP) of the IR source is reported as Supplementary Information (**Figure S3**). The fluorescence intensity ( $F/F_0$ ) of the Dil-labeled AbLMNVs decreases during heating (**Figure 6d**) following a linear relation with the temperature increase ( $\Delta F/F_0 = -0.0224 \cdot \Delta T$ ;  $R^2 = 0.99$ ; **Figure 6e**). In **Figure 6f**, the temperature imaging of the Dil-AbLMNVs internalized into a GBM spheroid before, during, and after the AMF stimulation is shown (the complete time-lapse is available as Supplementary Information, Video S4). The graph reporting the fluorescence ( $F/F_0$ ) and the temperature ( $T$ ) time courses of the Dil-labeled AbLMNVs in GBM spheroid during exposure to AMF is shown in **Figure 6g**; after 17 min of AMF stimulation, the intraparticle temperature reached and stabilized at  $\sim 43.5$  °C.

### **AbLMNV-assisted chronic magnetothermal stimulation induces necrosis, apoptosis and tumor spheroids disintegration**

Prior to carry out chronic magnetothermal treatments, spheroids at day 4 of culture in non-adherent conditions were imaged by CLSM (imaging is available as Supplementary Information in **Figure S4**) and their equivalent diameter resulted of  $315 \pm 91$   $\mu\text{m}$ . The anticancer effects of AMF stimulation on GBM spheroids were investigated by pre-incubating spheroids with  $167 \mu\text{g ml}^{-1}$  of AbLMNVs (**Figure 7**). The temperature of the medium in the proximity of spheroids, pre-incubated or non-incubated with AbLMNVs, was monitored during AMF stimulation by an optical sensor. In **Figure 7a** and **7b**, the graphs respectively report representative temperature curves and the average temperatures reached at the

equilibrium. The samples treated with AbLMNVs+AMF underwent a significant increase of temperature, which stabilized at  $41.3 \pm 0.4^\circ\text{C}$ . A scarce increase of temperature ( $\Delta T \sim 2.5^\circ\text{C}$ ) was instead recorded for samples stimulated with AMF without the pre-incubation with AbLMNV.

Chronic AMF stimulations (2 h *per* day for 4 days) were carried out on GBM spheroids non-incubated with AbLMNVs (Control+AMF), treated with the TMZ (TMZ+AMF), treated with AbLMNVs (AbLMNVs+AMF) or treated with TMZ-loaded AbLMNVs (TMZ-AbLMNVs+AMF). These experimental classes were then compared to the corresponding samples non-exposed to AMF (Control, TMZ, AbLMNVs and TMZ-AbLMNVs). Concerning the TMZ and TMZ+AMF experimental classes, free drug concentration was  $6.8 \mu\text{g ml}^{-1}$ , the amount of TMZ loaded in  $167 \mu\text{g ml}^{-1}$  AbLMNVs (being the TMZ  $4.1 \pm 0.5\%$  w/w).<sup>[16]</sup> **Figure 7c** and **7d** respectively show the imaging and the size analysis of spheroids after 4 days of treatment. Spheroids that underwent chronic magnetothermal stimulation (both AbLMNVs+AMF and TMZ-AbLMNVs+AMF samples) resulted disaggregated, and only few small groups of cells could be detected; these samples mostly consisted of necrotic debris. Equivalent diameters of spheroids that did not undergo magnetothermal stimulation ( $540 \pm 37 \mu\text{m}$  for Control,  $464 \pm 83 \mu\text{m}$  for TMZ,  $560 \pm 104 \mu\text{m}$  for AbLMNVs and  $433 \pm 157 \mu\text{m}$  for TMZ-AbLMNVs) resulted higher with respect to the average size before starting the treatment ( $315 \pm 91 \mu\text{m}$ ), indicating as the non-stimulated GBM spheroids were growing during the 4 days of experiment, even when treated with TMZ. No significant differences among the spheroid sizes in these experimental classes (Control, TMZ, AbLMNVs, and TMZ-AbLMNVs) was found ( $p > 0.05\%$ ). Instead, the equivalent diameter detected after the magnetothermal treatment ( $192 \pm 92 \mu\text{m}$  for AbLMNVs+AMF and  $167 \pm 86 \mu\text{m}$  for TMZ-AbLMNVs+AMF) was significantly smaller not only with respect to the other experimental classes ( $p < 0.05$ ), but even compared to the pre-treatment ( $p < 0.05$ ). In other words, the magnetothermal therapy induced the disaggregation of the GBM spheroids, being the groups of cells after the treatment significantly smaller, in terms of equivalent diameter, with respect to the spheroids at the starting of the experiment.

The viability of the cells in the spheroids after magnetothermal treatment was moreover investigated. Cells dissociated from GBM spheroids were stained with PI / FITC-annexin V and, subsequently, the percentage of healthy, apoptotic, and necrotic cells was analyzed by flow cytometry (**Figure 7e**). The plot highlights that the best anticancer results were obtained

with in the TMZ-AbLMNVs+AMF group (7.7% of healthy cells, 87.5% of necrotic cells, 1.6% of early apoptotic cells, and 3.2% of late apoptotic cells), followed by the AbLMNVs+AMF group (49.6% of healthy cells, 38.8% of necrotic cells, 4.9% of apoptotic cells, and 6.7% of late apoptotic cells). Concerning the other experimental classes, no remarkable apoptotic / necrotic effects were observed, with a percentage of healthy cells  $\geq 96$  % in all the experimental groups. Scatter plots of a representative experiment for each experimental class is shown as Supplementary Information (**Figure S5**).

### **Proteomic analysis of synergic magnetothermal and chemotherapy treatment**

Proteomic analysis on the combined effect of magnetothermal and chemotherapy treatment has been performed (number of protein analyzed = 4208); results are shown in **Figure 8**. In these experiments, non-functionalized LMNVs have been used in order not to affect the proteomic profile in the experimental classes treated with nanovectors, due to the presence of anti-TfR Ab. In terms of number of differentially represented proteins (DRPs) among experimental classes, the co-action of AMF, TMZ and LMNVs results into the highest number of DRPs, closely followed by the association of AMF with LMNVs. Almost without exceptions, down-regulated proteins outnumber up-regulated ones. Concerning the effects generated by the single experimental valuable, LMNVs elicit the broadest response when taken as a single experimental variable (it should be noted that TMZ-LMNVs were considered a combination of two factors). In comparison, both AMF and TMZ seem to produce wide effects on the proteome just when combined with other treatments. Results are summarized in Table 1. Four different comparisons can be used to study AMF, TMZ or LMNVs separately; we intersected them to produce four-way Venn diagrams. These highlight a comparatively high number of DRPs shared by at least some comparisons of the LMNVs diagram, with 52 proteins found at the intersection of all four parental sets. For AMF, the Venn diagram shows a relatively pronounced impact mainly when LMNVs were provided. TMZ promoted important variations in the member of DRPs only when combined with both AMF and LMNVs. Experimental variables can also be investigated as if they were compound factors: we adopted two-way Venn diagrams to study AMF + TMZ, AMF + LMNVs, or TMZ-LMNVs. **Figure 8a** shows Venn diagrams.

Concerning the analysis of coherent proteins and second-order intersections, we defined coherent a DRP if it is systematically either up- or down-regulated at a given intersection. We observed that most of DRP shared between different comparisons are coherent. For selected

subsets in our Venn diagrams, we reported the number of coherent proteins (**Figure 8a**). With AMF + TMZ-loaded LMNVs and AMF + LMNVs being the most relevant compound variables, we also intersected coherent genes yielded by the AMF + LMNVs Venn diagram with comparison 8 vs. 1, which studies TMZ-LMNVs + AMF; results are shown in **Figure 8a**. Concerning the gene ontology terms associated to nanopharmacological treatment, coherent DRPs for our second-order intersection between TMZ-LMNVs + AMF and AMF + LMNVs were used to understand which biological dynamics might have taken place in spheroids following the synergic treatments. To do this, we adopted a gene ontology (GO) strategy. GO terms significantly associated to the combined effects of TMZ-LMNVs + AMF were identified for biological processes, functions and components (**Figure 8b**). Relevant phenomena are examined in detail in the “Discussion” section).

### **Magnetothermal stimulation of post-mortem mammal brain tissue**

As a proof of concept, AbLMNVs were injected and exposed to an AMF in a bovine *post-mortem* brain tissue, and temperature was recorded during the experiment (**Figure 9**). In details, 300  $\mu\text{l}$  of a 7  $\text{mg ml}^{-1}$  AbLMNV dispersion was injected in the brain tissue (**Figure 9a**), transferred into a plastic tube (**Figure 9b**), and exposed for 2 h to AMF stimulation. The graph in **Figure 9c** shows the temperature levels at the injection site, which reached and stabilized at  $T = 42.5^\circ\text{C}$ .

## **Discussion**

In this work, we report for the first time the preparation of lipid magnetic nanovectors functionalized with anti-TfR Ab (AbLMNVs) for the dual targeting of BBB and GBM cells, and for the combined delivery of magnetothermal stimulation and chemotherapeutic agents (*i.e.*, TMZ). The thermosensitive properties of the DiI lipophilic tracer were investigated and exploited to measure the temperature increase of the lipid matrix of the AbLMNVs in GBM spheroids, which was able to reach  $43.5^\circ\text{C}$ . The disaggregation of TMZ-AbLMNV-targeted GBM spheroids has been obtained by chronic exposures to AMF with remarkable anticancer results (92.3% of dead cells, 87.5% of which resulted necrotic and 4.8% apoptotic). The enhanced BBB crossing and GBM spheroid targeting obtained with the anti-TfR Ab functionalization of nanovectors was demonstrated through flow cytometry, 3D confocal laser scanning imaging, spectrofluorimeter analysis, and FIB-SEM imaging, by exploiting the high accessibility of the developed multicellular organoid system. The TfR Ab-mediated dual

targeting of nanoparticles to endothelial cells of BBB and to cancer cells has been previously proposed in the literature by independent groups.<sup>[23-26]</sup> TfR is indeed highly expressed in both endothelial cells of brain vessels and in glioma;<sup>[17,18]</sup> TfR is necessary for glioma tumorigenesis, and its expression is indicative of the increased tumorigenicity (2-fold higher TfR expression in grade IV gliomas with respect to lower grade tumors).<sup>[18]</sup> Both Tf ligand and anti-TfR Ab has been used to target the TfR; however, considering the quite high concentration of Tf in the blood (1.7-3.7 mg ml<sup>-1</sup> for humans), the targeting with anti-TfR Ab is preferable because the Ab does not compete with the endogenous Tf to bind its receptor.<sup>[27]</sup> Johnsen *et al.* reported as Ab-functionalized gold nanoparticles actively accumulate in brain capillaries and cross the BBB before entering into the brain parenchyma.<sup>[27]</sup> Furthermore, in another work, Johnsen *et al.* observed that anti-TfR Ab-functionalized immunoliposomes were more efficiently transported to the brain with respect to non-functionalized ones.<sup>28</sup> Here, the brain targeting resulted successful by using a density of antibodies for nanoparticle surface of  $\sim 8.1 \cdot 10^2$  Ab  $\mu\text{m}^{-2}$  (considering 50 Ab/immunoliposome and an immunoliposome size of 140 nm), an order of magnitude similar to that one of our nanosystem ( $\sim 14.8 \cdot 10^2$  Ab  $\mu\text{m}^{-2}$ ).

The increased endothelial cell targeting and uptake of the functionalized AbLMNVs was associated to a significantly higher level of endocytosis in clathrin-coated vesicles of these nanovectors with respect to the non-functionalized LMNVs. This result is in line with different evidences found in the literature, where the clathrin-mediated endocytosis resulted the preeminent internalization pathway for both iron-complexed Tf and TfR-targeting nanoparticles.<sup>[29-31]</sup> The internalization of both functionalized and non-functionalized nanovectors in caveosomes did not result significant, probably due to the relatively large size of these nanovectors.<sup>[32]</sup> Furthermore, a higher amount of AbLMNVs was found in rab11<sup>+</sup> vesicles with respect to the LMNVs. Rab11 is a small GTPase that prevents the transfer of the cargo to the lysosome compartments, and mediates the transendothelial transport across the BBB.<sup>[33,34]</sup> Moreover, rab11 regulates the vesicle exocytosis, and the rab11 depletion inhibits tethering and fusion of the TfR<sup>+</sup> vesicles to the plasma membrane.<sup>[35]</sup> Besides TfR targeting, which promotes the increase of nanoparticle crossing through the BBB by transcellular pathway, other approaches have been proposed to transiently and safely increase the BBB permeability and the consequent paracellular transport of nanoparticles/compounds to the brain. The most relevant examples include the injection of

recombinant human vascular endothelial growth factor,<sup>[36]</sup> the use of focused ultrasound,<sup>[37]</sup> and the magnetothermal stimulation.<sup>[38]</sup> In this context, AbLMNV represents a multifunctional nanoplatform, that can be locally accumulated with a static magnetic field, it is able to promote dual targeting towards both the endothelial cells of the BBB and the GBM cells, and, finally, it induces significant temperature increment when exposed to an AMF; in principle, the nanoparticle heating could be exploited, transiently, for promoting the BBB crossing, and, chronically, for GBM treatment.

In this work, we have also reported for the first time the temperature sensitivity of the Dil lipophilic dye. The good thermosensitivity of Dil was then exploited to measure the temperature reached in the lipid matrix of the AbLMNVs in response to an AMF. The temperature inside the particles reached 43.5°C during the stimulation, slightly higher than that one measured in the medium (~41°C.). Obviously, the equilibrium of the temperature inside the particles and the one in solution depends on the average distance of the nanovectors encapsulating the SPIONs as well as on the localization of the SPIONs inside the nanovectors. A similar finding was also reported by Dong and Zink,<sup>[39]</sup> which developed mesoporous silica nanoparticles encapsulating SPIONs and NaYF<sub>4</sub>:Yb<sup>3+</sup>, Er<sup>3+</sup> thermosensitive nanocrystals. At the end of the exposure to AMF (frequency of 375 kHz; induction power of 5 kW), the temperature increment of the nanoparticles was two-times higher than that one observed in the solution. Beside the reported application of the Dil as intraparticle thermometer, its good temperature sensitivity and its great affinity with the cell membrane makes this fluorescent probe an interesting tool for monitoring the temperature of the plasma membrane in living cells.

Nanotechnology-based strategies for the temperature-dependent remote control of drug release include the use of near infrared (NIR)-absorbing plasmonic nanomaterials for photothermal conversion, superparamagnetic nanoparticles for magnetothermal effect, and the ultrasound-induced heating of thermosensitive nanoparticles.<sup>[40]</sup> Particularly noteworthy in this regard is the recent work of Goodman *et al.*,<sup>[41]</sup> which demonstrated the NIR-mediated release of a human epidermal growth factor receptor (HER2)-targeted breast cancer drug, lapatinib, from a nanoshell-based human serum albumin protein host complex. Thanks to a femtosecond pulsed laser, the lapatinib was released and induced selective toxicity in HER2<sup>+</sup> breast cancer cells without significantly affecting control macrophages. However, the penetration capacity of NIR radiation in biological tissues, although elevated compared to the

visible light, is not sufficient to safely treat deep tumors in humans (the incident radiation is reduced to 1/10 at about 2 cm and to 1/100 at 4 cm depth).<sup>[42]</sup> On the contrary, alternating magnetic fields can be efficiently and safely delivered in scarcely accessible deep tissues, like the human brain.<sup>[43]</sup> As an example, clinical hyperthermia-based treatments against brain tumors with plain SPIONs have been carried out by the group of Andreas Jordan by exploiting MFH<sup>®</sup>300F technology (MagForce Nanotechnologies) with a variable field strength of 0-18 kA m<sup>-1</sup> and a frequency of 100 kHz for the brain delivery of AMF.<sup>[44]</sup> In our work, the localized heat of nanovectors was exploited for both remotely triggering the TMZ release from nanovectors and for inducing hyperthermia in GMB spheroids. The release profile of TMZ from LMNVs was previously investigated during exposures to AMF;<sup>[16]</sup> this study was conducted both in lysosome-mimicking conditions (pH 4.5 and 50 μM H<sub>2</sub>O<sub>2</sub>) and in control conditions (pH 7.4 and 0 μM H<sub>2</sub>O<sub>2</sub>); results were compared to samples non-exposed to AMF. In this previous work we highlighted that, after 3 days of treatment, the lysosome-mimicking conditions slightly affected the TMZ release (4.9 ± 0.1% of cumulative release) with respect to the control (1.0 ± 0.3% of cumulative release), while the chronic AMF stimulation (2 h *per* day) induced an elevated release of TMZ (65.1 ± 1.9%); the complete release was instead reached after 7 days of chronic magnetothermal stimulation at pH 4.5 and 50 μM H<sub>2</sub>O<sub>2</sub>. The gradual and remotely controlled drug release from nanovectors can be therefore obtained after repeated applications of AMF.

Concerning the chronic magnetothermal and chemotherapy treatment (TMZ-AbLMNVs+AMF), the biological material resulting after 4 days of stimulation mainly consisted of cell debris, while the small group of cells collected consisted for the 92.3% of death cells (87.5% of necrotic cells and 4.8% of apoptotic cells). The magnetothermal treatment without TMZ (AbLMNVs+AMF) was able to disintegrate the GBM spheroids with a similar efficacy compared to the TMZ-AbLMNVs+AMF; however the groups of cells found after the AbLMNVs+AMF stimulation were characterized by a higher amount of healthy cells with respect to the TMZ-AbLMNVs+AMF. The anticancer effects of TMZ during magnetothermal stimulation (41.9% of increased cell death) were remarkably higher with respect to those induced by TMZ alone. Indeed, treatments of GBM spheroids with free TMZ at the same concentrations loaded in the nanovectors induced a low cell death with respect to the controls (+2.8%), while no significant effects in GBM spheroid size were observed with respect to non-treated controls.

Concerning proteomic analysis, AMF alone seems to have little effect on proteins and to be well tolerated by spheroids (only 19 DRPs of 4208 total proteins were found in AMF vs. negative controls). In our experimental conditions, TMZ also displayed scarce effects in proteome (only 32 DRPs in TMZ vs. negative controls). TMZ appears to elicit a significant response, within our experimental framework, only when delivered *via* LMNVs and just when AMF is also present (1272 DRPs were found in TMZ-LMNVs + AMF vs. negative controls). In principle, it is desirable to observe noxious effects from chemotherapeutic agents only in combination with other components of a complex treatment. Hence, we believe that the modest rise in DRPs observed when TMZ is added to LMNVs + AMF is a promising hint of enhanced drug delivery or lethality. A higher number of proteome alterations were instead found in LMNVs with respect to the other single experimental variables (362 DRPs of 4208 total proteins were found in LMNVs vs. negative controls). Despite no apoptotic/necrotic and size effects were observed in spheroids treated with plain nanovectors, the higher number of DRPs is compatible with biological effects of these nanovectors *per se*. However, it should be taken into account that a precise tuning of dosages is beyond the goals of the current study, and remains highly dependent on experimental setups. Similarly, it is worth stressing that our study does not focus on selective toxicity, which continues to be an open question for future investigations. Moreover, synergic effects between AMF and LMNVs, that is compatible with LMNVs heating up upon selective magnetic stimulation, was found (846 DRPs were found in LMNVs + AMF vs. negative controls). Sets of DRPs among different experimental classes showed a remarkable degree of coherence. The 535 factors selected through our second-order Venn diagram are all coherent, confirming the general soundness of our approach. When searching for biological phenomena associated to such factors, we enriched in semantic spaces that, overall, are in line with an activation of catabolic paths preparing spheroids to death. Examples of specific GO terms for biological processes, functions or components are “stimulatory C-type lectin receptor signaling pathway”, “threonine-type peptidase activity”, and “proteasome core complex”. All these are suggestive of a stress or even apoptotic state.<sup>[45,46]</sup> The pervasive protein down-regulation observable in our dataset may be a further indication of cell suffering, which could possibly be due to increased transcriptional repression and/or protein catalysis.

Coming back to translational issues, promising clinical outcomes have been obtained by applying the magnetothermal therapy through direct injection of superparamagnetic

nanoparticles in GBM tumors. In the context of a first phase I trial, aminosilane-coated SPIONs were stereotaxically injected at a concentration of  $112 \text{ mg ml}^{-1}$  into the tumor of 14 patients diagnosed with primary or recurrent GBM. Patients underwent 6 sessions of AMF (variable field strength of  $2.5\text{-}18.0 \text{ kA m}^{-1}$  and frequency of  $100 \text{ kHz}$ ), each performed for 60 min, 2 times *per* week. An average intratumoral temperature of  $44.6^\circ\text{C}$  was observed during AMF applications.<sup>[47]</sup> In a subsequent phase II clinical trial involving 59 patients with recurrent GBM, a significantly prolonged overall survival (13.4 months) was observed thanks to this treatment.<sup>[48]</sup> In this context, the magnetothermal stimulation of the *post-mortem* brain tissue directly injected with AbLMNVs was carried out as a proof of concept. Interestingly, mild hyperthermia ( $42.5^\circ\text{C}$ ) was reached by injecting  $0.3 \text{ ml}$  of  $7 \text{ mg ml}^{-1}$  AbLMNVs, a 15-times lower concentration and an 11-times lower volume than the plain SPIONs injected in the GBM of patients during the previously mentioned clinical trials. In this regard, it is important to highlight as the lipid matrix where the SPIONs are embedded in AbLMNVs, not only has the function of encapsulating the drug for a temperature-dependent controlled release, yet also prevent the aggregation / precipitation of SPIONs, therefore guaranteeing the stability of their superparamagnetic behavior and of their optimal magnetothermal performances.<sup>[49]</sup>

## Conclusions

The nanoplatform proposed in this work represents an efficient tool for the synergic magnetothermal hyperthermia and chemotherapy treatment of sub-millimetric glioblastoma spheroids: cell populations that, in patients, cannot be surgically resected, resist to the current chemotherapy / radiotherapy approaches, and are the main cause of cancer recurrence. The remote activation of lipid magnetic nanovectors by alternating magnetic fields induces the heating of the nanovectors, the chemotherapy drug release, and the hyperthermia-dependent loss of plasma membrane integrity. The single and combined effects of the alternating magnetic fields, lipid superparamagnetic nanovectors, and temozolomide drug on glioblastoma spheroids were deeply investigated. Magnetothermal stimulation without chemotherapy (defined as the combined effect of alternating magnetic fields and lipid superparamagnetic nanovectors) was able to efficiently disintegrate the GBM spheroids, however, just when magnetothermal hyperthermia was combined with the temozolomide treatment (present in the nanovectors at subtoxic doses) the GBM spheroid

disintegration was accompanied with a massive cell death. When analyzing by proteomics for biological phenomena associated to the combined magnetothermal and chemotherapy treatment, we enriched in semantic spaces that, overall, are in line with catabolic paths of cell death (specific gene ontology terms consisted in stimulatory C-type lectin receptor signaling pathway, threonine-type peptidase activity, and proteasome core complex). Moreover, the superior abilities of the anti-TfR Ab functionalized nanovectors to overcome the BBB limitation and to target glioblastoma spheroids were preliminarily demonstrated using multi-cellular *in vitro* models. Future investigations will be devoted to test glioblastoma targeting efficiency and anticancer efficacy of this multifunctional nanosystem on patient-derived orthotopic xenograft models. Concerning the *in vivo* context, although TfR Ab has been already exploited to efficiently delivery nanoparticles to the brain, TfR is also highly expressed in other organs (especially in the liver), where the nanovectors may accumulate and induce relevant side effects. However, taking advantage of an external static magnetic field, it will be likely possible to obtain a successful nanovector accumulation at the anatomical region of the brain where the glioblastoma foci are localized. The main scope of our future works, as previously mentioned, will be focused on the assessment the effects of the combined magnetothermal and chemotherapy treatment in patient-derived xenografts models characterized by different glioblastoma subtypes, therefore addressing the complex heterogeneity of this type of cancer.

## **Experimental Section**

### **Preparation and functionalization of lipid magnetic nanovectors**

Lipid magnetic nanovectors (LMNVs) were fabricated as recently described by our group.<sup>[16]</sup> Briefly, a mix of 2.5 mg of oleic acid (Sigma-Aldrich), 25 mg of 1-stearoyl-rac-glycerol (Sigma-Aldrich), 2.5 mg of 1,2-dipalmitoyl-rac-glycero-3- phosphocholine (Sigma-Aldrich), 4 mg of biotin-conjugated mPEG-DSPE5k (Sigma-Aldrich), and 84.5  $\mu$ l of an ethanol solution with superparamagnetic iron oxide nanoparticles (15 wt%; US Research Nanomaterials Inc.) were sonicated at 70°C until ethanol completely evaporated and lipids melt. After adding 4 ml of a pre-warmed (70°C) Tween<sup>®</sup> 80 (Sigma-Aldrich) solution (1.0 wt%) to the mixture, a successive step of ultrasonic homogenization (Fisherbrand<sup>TM</sup> Q125 Sonicator; amplitude 30%, 120 W, for 20 min) followed by high-pressure homogenization (HPH, EmulsiFlex- B15 from Avestin; 5 repetitions at 100000 psi) was carried out. After stabilization at 4°C for 30

min, nanovectors were purified by centrifugation and washing with ddH<sub>2</sub>O (3 times for 30 min at 4°C). For each preparation, nanovector concentration was estimated by sacrificing part of the sample, lyophilizing and weighting it. Temozolomide-loaded nanovectors (TMZ-LMNVs) were obtained with the same procedure described above, by adding 2.5 mg of TMZ to the lipid mixture. Concerning the detailed morphological, physicochemical and magnetic characterization of the LMNVs, it is possible to refer to our previous work, where the TMZ release profiles were also investigated.<sup>[16]</sup>

The functionalization of nanovectors (both LMNVs and TMZ-LMNVs) with the anti-transferrin receptor antibody (anti-TfR Ab) was obtained by conjugating 30 µl of streptavidin-Ab against TfR (0.5 mg ml<sup>-1</sup>; Abcore) with 100 µl of 5 mg ml<sup>-1</sup> nanovector dispersion containing mPEG-DSPE-biotin (shacking for 1 h at 4°C), similarly as described for other nanoparticles.<sup>[20]</sup> The Ab-functionalized nanovectors (respectively named AbLMNVs and TMZ-AbLMNVs) were then purified by dialysis (overnight under stirring with ddH<sub>2</sub>O as eluent; molecular weight cut off 300 KDa; Spectrum laboratories, Inc.).

For the fluorescence imaging of nanovectors, 100 µl of 5 mg ml<sup>-1</sup> LMNVs and AbLMNVs were stained with 5 µl of Vybrant DiO (3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolyl- idene)-1-propenyl]-, perchlorate; ThermoFisher). After 40 min of incubation under shacking, samples were centrifuged twice (15000 rpm for 80 min at 4°C) and the pellet with nanovectors was re-dispersed in ddH<sub>2</sub>O. Both the stained and the non-stained nanovectors were centrifuged and dispersed in the complete medium before performing the experiments. The same procedure was carried out for the staining of the AbLMNVs with the lipophilic 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil; 5 µl of Dil were used for 100 µl of 5 mg ml<sup>-1</sup> AbLMNVs).

Dynamic light scattering analysis was carried out at 25°C on a 100 µg ml<sup>-1</sup> AbLMNV dispersion in water and in complete medium by using a Zeta-sizer NanoZS90 (Malvern Instruments Ltd).

### **TEM imaging and functionalization analysis**

Nanovectors have been imaged with a JEOL JEM1011 equipped with a thermionic electron source (tungsten) operating at 100 kV. TEM images were acquired with an 11 Mp Orius 1000 CCD camera (Gatan). To prepare the sample, a 10 µl drop of the solution has been drop-casted onto a carbon-coated Cu grid and dried in air. For negative staining, the grid has been treated with a 1% uranyl acetate solution for 30 s.

Concerning the analysis of the functionalization efficiency, samples were diluted with the 4X Laemmli Sample Buffer (Bio-Rad), denatured for 10 min at 95°C for 10 min, and then run on a 4-15% Mini-PROTEAN® TGX™ Gel (Bio-Rad) at 100 V for 1 h. The PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific) was run in parallel with the samples. Subsequently, 1 h of staining with Coomassie Brilliant Blue R (Sigma-Aldrich) was carried out. The gel was washed and transferred in MilliQ water for image acquisition. Finally, the band intensities were measured in terms of pixel values by using Fiji software (<https://imagej.net/Fiji>). The quantity of Ab in AbLMNV sample was then calculated owing to a calibration curve available as Supplementary Information (**Figure S1**).

### **3D glioblastoma (GBM) spheroids coupled to a multicellular model of the blood-brain barrier**

Cancer spheroids were derived from U-87 MG cells (ATCC® HTB-14TM) by exploiting the hanging drop approach.<sup>[50]</sup> The cell medium used consisted in high-glucose Dulbecco Modified Eagle's Medium (DMEM; Sigma- Aldrich), supplemented with 10% fetal bovine serum (FBS; Gibco), 1% sodium pyruvate, 1% L-glutamine, 1% non-essential amino acids (Gibco), 100 IU ml<sup>-1</sup> penicillin (Gibco), 100 µg ml<sup>-1</sup> streptomycin (Gibco). 25 µl drops of cell suspension (1·10<sup>6</sup> cells ml<sup>-1</sup>) were disposed upside down on lids of 75 mm cell culture dishes to promote cell-cell aggregation (24 h into humid chamber). Afterwards, the obtained 2D cell aggregates were transferred and cultured for 4 days on 75 mm cell culture dishes pre-coated with agarose hydrogel (1% in PBS). Spheroids were stained with Hoechst 33342 (1 µg ml<sup>-1</sup>; Invitrogen), calcein (ThermoFisher) and ethidium homodimer-1 (ethd-1; ThermoFisher), following the standard procedures indicated by the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (ThermoFisher); 3D imaging of stained spheroids was then performed with confocal laser scanning microscopy (CLSM; C2s system, Nikon) and the equivalent diameter was measured by using the NIS-Elements software (Nikon).

A multicellular *in vitro* model of blood-brain barrier (BBB) separating a luminal compartment from an abluminal chamber with 3D GBM spheroids was obtained. The BBB model was prepared by firstly seeding C8D1A brain astrocytes (ATCC® CRL-2541™) on the abluminal side of the transwell inserts (3 µm diameter porous; Corning Incorporated) at a density of 2·10<sup>4</sup> cells cm<sup>-2</sup> and, subsequently, by plating brain-derived endothelioma bEnd.3 cells (ATCC® CRL-2299™) on the luminal side of the inserts at the density of 8·10<sup>4</sup> cells cm<sup>-2</sup> (the second seeding was carried out ~ 15 h after the first one). The cells were co-cultured for additional 4

days by using the same medium composition described for U-87 MG cells. Co-cultures were maintained in a humidified atmosphere, at 37°C, with 5 % CO<sub>2</sub>. The barrier functionality was assessed in terms of transendothelial electrical resistance (TEER) with a Millipore Millicell ERS-2 Volt-Ohmmeter, and by analyzing with immunofluorescence the expression of a specific marker of tight junctions, the *zonula occludens-1* (ZO-1); the details of the immunocytochemistry procedures are indicated in the “Immunofluorescence assays” paragraph of the “Experimental Section”. Finally, for assessing the dual targeting of AbLMNVs, 3D spheroids were transferred to the abluminal compartment of the multicellular BBB model.

### **Analysis of the blood-brain barrier crossing and of the spheroid targeting**

The crossing of DiO-stained nanovectors from the luminal to the abluminal chamber through the multicellular BBB system was investigated in the presence of or without a static magnetic field (SMF; NdFeB magnet with 10 mm diameter and 8 mm height; 2.9 kg of attraction force; Italfit Magneti S.r.l.); specifically, magnets have been fixed under the multicellular system through a custom-made multi-magnet support (**Figure S6**). Subsequently, 160 µg of DiO-stained nanovectors (160 µl of 1 mg ml<sup>-1</sup> dispersion) were incubated in the luminal compartment of the transwell inserts in the presence of or without the SMF and the fluorescence of the medium of the abluminal compartment (600 µl) was measured at different time points (24, 48 and 72 h) by using a Perkin Elmer Victor X3 UV-Vis spectrophotometer ( $\lambda_{ex} = 485 \text{ nm}$ ,  $\lambda_{em} = 535 \text{ nm}$ ). The fluorescence intensity was then converted to the nanovector concentration thanks to a calibration curve available as Supplementary Information (**Figure S7**). The same experiment was performed with GBM spheroids in the abluminal compartment; after 72 h of nanovector incubation in the luminal compartment, the nanovector fluorescence in the spheroids was assessed by CLSM imaging and by flow cytometry. Concerning CLSM, spheroids were washed twice with PBS, fixed with paraformaldehyde (PFA; 4% in PBS at 4°C for 25 min) and stained with Hoechst 33342 (1 µg ml<sup>-1</sup>; Invitrogen); 3D volume analysis of nanovector signal was performed by NIS-Elements software (Nikon), and the volume occupied by nanovectors was normalized and expressed as % of the spheroid volume. Regarding flow cytometry, spheroids were washed twice and treated with trypsin for 10 min at 37°C; subsequently, spheroids were dissociated to single cells by pipetting, samples were centrifuged, and finally cells resuspended in PBS for flow cytometry analysis of fluorescence emission (Beckman Coulter CytoFLEX;  $\lambda_{ex} 488 \text{ nm}$ ,  $\lambda_{em} 525$

± 40 nm). The highest value of fluorescence emission in non-treated control spheroids was used for thresholding the population nanovector-positive cells.

The nanovector targeting to spheroids was moreover investigated by directly incubating spheroids with 167 µg ml<sup>-1</sup> of nanovector concentration (corresponding to the maximum concentration of nanovectors that was able to cross the BBB). Spheroids were washed twice with PBS, fixed with PFA (4% in PBS at 4°C for 25 min) and then stained with Hoechst 33342 (1 µg ml<sup>-1</sup>) and TRITC-phalloidin (100 µM; Millipore). The study of nanoparticle targeting and internalization was then carried out by 3D volume analysis (NIS-Elements software, Nikon), as described above, and by focused ion beam milling combined with scanning electron microscopy (FIB-SEM).

Concerning ultrastructure FIB-SEM imaging, U87-derived spheroids were prepared using the reduced osmium – thiocarbohydrazide - osmium (RO-T-O) ultra-thin plasticization protocol as previously presented.<sup>[51,52]</sup> Briefly, samples were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, EMS) in 0.1 M sodium cacodylate buffer (EMS) at 4°C overnight, then washed (3 times for 5 min) in the same buffer and incubated in 20 mM chilled glycine (Sigma-Aldrich) for 20 min. After 3 washing in chilled buffer, specimens were post-fixed in a 2% osmium tetroxide (EMS) / 1% potassium ferrocyanide (EMS) solution for 1 h on ice (RO step), and then washed 3 times in chilled buffer. During the T step, samples were incubated with 1% thiocarbohydrazide aqueous solution (EMS) at room temperature for 20 min and washed in distilled water before the incubation with 2% osmium tetroxide aqueous solution (O step) for 1 h at room temperature. To enhance the contrast specimens, samples were *en bloc* stained overnight at 4°C in 0.5% uranyl acetate (EMS), washed in chilled deionized water, and incubated in 0.15% tannic acid. After 3 washes in water at 4°C, spheroids were dehydrated by treating at 4°C with increasing ethanol concentrations (30%, 50%, 75%, 95% and 100%, at 4°C, 10 min of incubation for each solution). Finally, samples were infiltrated with increasing concentration of Spurr's resin (EMS) in 100% ethanol (1:3 dilution for 2 h; 1:2 for 2 h; 1:1 overnight; 2:1 for 2 h; 3:1 for 2 h; absolute resin overnight). To remove the excess of resin, each spheroid was mounted in vertical position for 3 h and washed for a few seconds in absolute ethanol. After the polymerization in oven at 70°C, spheroids were then cut in 4 parts, mounted with colloidal silver paste (RS Company) to 12 mm pin stub, and sputtered with 25 nm gold layer. SEM was acquired applying a voltage of 3-10 kV and a current of 20-400 pA. The secondary electrons (SE) detector was used for the imaging of the whole surface

of the spheroids, while backscattered electrons (BSE) were collected for the imaging of the cross sections after the FIB milling. A first trench-out was performed by ion milling with voltage at 30 kV and current at 65 nA. Then, the section was polished by ion beam milling fixing the voltage at 30 kV and the current at 9.3 nA.

Similarly, brain endothelial bEnd.3 cell line at 24 h of nanovector incubation were fixed with 2.5% of glutaraldehyde (GA; Sigma-Aldrich, 2.5% in ddH<sub>2</sub>O for 30 min at 4°C) and then processed with FIB-SEM as above described for spheroids.

### **Immunofluorescence assays**

The internalization and transcytosis pathway of the nanovectors in the endothelial cells of the multicellular BBB model was investigated by immunostaining of different vesicles involved in the intracellular trafficking. Specifically, the signal co-localization of DiO-stained nanovectors with three markers, caveolin-1, clathrin, and rab11, respectively expressed in caveosomes, clathrin-coated organelles, and transcytosis vesicles were investigated after 24 h of nanovector incubation. Immunofluorescence staining was carried out as previously described.<sup>[53]</sup> Briefly, cells were fixed with PFA (4% in PBS at 4 °C for 25 min), permeabilized with Triton X-100 (Sigma-Aldrich; 0.1% in PBS room temperature for 25 min) and incubated with blocking solution (10% goat serum in PBS at room temperature for 1 h). For primary antibody, samples were treated with primary rabbit anti-clathrin antibody (Abcam; 1:200 in 10% goat serum), mouse anti-caveolin-1 antibody (Abcam; 1:100 in 10% goat serum), or rabbit anti-rab11 antibody (Abcam; 1:100 in 10% goat serum), for 60 min at 37°C. Afterwards, cells were washed 5 times with 10% goat serum in PBS and then incubated with a staining solution of 10% goat serum in PBS containing Hoechst 33342 (Invitrogen; 1:1000 dilution,) and a TRITC-conjugated secondary antibody: a TRITC-conjugated secondary anti-rabbit antibody (1:150 dilution; Invitrogen) was used for the staining of clathrin and rab11, while a TRITC-conjugated secondary anti-mouse antibody (1:75 dilution; Millipore) was used for staining the caveolin-1. Subsequently, CLSM imaging was performed (C2s system, Nikon) and the Pearson's correlation coefficients were calculated with NIS-Elements software (Nikon). The ZO-1 marker expression in BBB was revealed by immunofluorescence following the same procedures indicated above, with a IgG primary antibody against ZO-1 (1:120 dilution; Invitrogen) followed by a staining solution consisting in goat Alexa Fluor 488-IgG anti-rabbit secondary antibody (1:200 dilution; Invitrogen), Hoechst 33342 (1 µg ml<sup>-1</sup>; Invitrogen) and TRITC-conjugated phalloidin (100 µM; Millipore).

## Cell temperature and propidium iodide time-lapse imaging during acute magnetothermal stimulation

Considering the superior performances of Ab-functionalized nanovectors in BBB crossing and in GBM cell targeting, the following experiments were performed just by using AbLMNVs. The intracellular temperature imaging during stimulation with alternating magnetic fields (AMF) was carried out by exploiting the ER-thermo yellow fluorescence thermometer during fluorescence time-lapse microscopy similarly as described in previous works.<sup>[20-21]</sup> Briefly, U-87 MG cells were seeded on WillCo<sup>®</sup> glass bottom dishes, pre-treated for 24 h with  $167 \mu\text{g ml}^{-1}$  of AbLMNVs, stained with 300 nM ER-yellow, and incubated with phenol red-free HEPES-supplemented complete medium; samples were positioned on a plastic support of the microscope stage (CLSM C2 system; Nikon), in the center of an electromagnetic coil of a MagneTherm<sup>™</sup> equipment (NanoTherics; 16 mT, 753 kHz). CLSM time-lapse imaging was carried out by exploiting perfect focus system (Nikon) and, in order to prevent objective heating during AMF generation, the microscope revolver was automatically lowered after each acquisition thanks to the escape function (NIS-Elements software); before each acquisition, the revolver was raised up and the perfect focus activated. The same procedures were performed for the control cultures that were not incubated with nanovectors. After fluorescence imaging, regions of interest (ROI) were thresholded and the fluorescence intensity ( $F$ ) was measured for each ROI and normalized for the fluorescence intensity at  $t = 0$  min ( $F_0$ ). The  $F/F_0$  time course was then converted into  $\Delta T$  ( $^{\circ}\text{C}$ ) by using a previously reported calibration curve (*i.e.*, an increase of temperature of  $\Delta T = 1^{\circ}\text{C}$  corresponds to a fluorescence decrease of  $\Delta F = -2.7\%$ ).<sup>[19]</sup>

To evaluate the membrane integrity / disruption in response to the AbLMNV-assisted magnetothermal stimulation, three experimental classes were considered: cells pre-incubated with AbLMNVs but not stimulated with AMF, cells non-treated with AbLMNVs but stimulated with AMF, and finally cells pre-incubated with AbLMNVs and stimulated with AMF. After 24 h of nanovector treatment, cells were washed and then incubated with  $1.5 \mu\text{M}$  propidium iodide (PI) in phenol red-free HEPES-supplemented complete medium. The fluorescence time-lapse imaging during AMF exposure was carried out following the same procedures indicated above for the ER-thermo yellow staining. After time-lapse experiments, the acquisition of the whole samples was performed by image stitching through the “Large Image” function (NIS-Elements software, Nikon).

### **Temperature measurement inside the AbLMNVs in response to AMF**

The intraparticle temperature was monitored by using the lipophilic 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) fluorescence dye.

Firstly, the temperature sensitivity of the DiI was characterized by measuring the fluorescence emission spectra of the DiI solution in dimethyl sulfoxide (1:100 in DMSO) at different temperatures (ranging from 26 to 43 °C) with a spectrofluorimeter (Cary Eclipse fluorescence spectrophotometer, Agilent Technologies). Fluorescence emission spectra were collected during two temperature cycles to evaluate the reversibility of the thermo-sensitive process. Subsequently, U-87 MG cells were stained with ER-thermo yellow or were incubated with 167  $\mu\text{g ml}^{-1}$  of DiI-stained AbLMNVs for 24 h, and then heated with a focused 1475 nm infrared laser. Specifically, cells were heated to obtain different temperature increments ( $\Delta T_1 = 0.0^\circ\text{C}$ ;  $\Delta T_2 = 3.5^\circ\text{C}$ ;  $\Delta T_3 = 7.8^\circ\text{C}$ ;  $\Delta T_4 = 10.9^\circ\text{C}$ ;  $\Delta T_5 = 12.3^\circ\text{C}$ ;  $\Delta T_6 = 14.3^\circ\text{C}$ ), induced by increasing IR laser powers ( $LP$ ; nominal  $LP$  were, respectively  $LP_1 = 0$  mW;  $LP_2 = 100$  mW;  $LP_3 = 200$  mW;  $LP_4 = 300$  mW;  $LP_5 = 400$  mW;  $LP_6 = 500$  mW). During heating, a time-lapse fluorescence imaging was performed with a confocal fluorescence microscope (Leica, SP8; HC PL APO CS2 63X/1.40 oil objective lens; 552 nm excitation; 560-647 nm emission) equipped with an IR-LEGO system for the heating by the focused IR laser; the fluorescence sensitivity of the DiI-stained particles to the temperature was expressed as linear function of  $\Delta F/F_0$  over  $\Delta T$ . Finally, the intraparticle temperature measurement was carried out in spheroids pre-incubated for 24 h with 167  $\mu\text{g ml}^{-1}$  DiI-stained AbLMNVs. After incubation, spheroids were transferred and settled on gelatine-coated WillCo<sup>®</sup> glass bottom dishes for CLSM imaging during AMF exposure. The values of  $F/F_0$  were finally converted to  $T$  depending to the linear temperature sensitivity of the particle fluorescence.

### **Investigations on apoptosis, necrosis and spheroid size after chronic magnetothermal stimulation**

Spheroids were grouped in 8 experimental classes: non-treated controls (w or w/o AMF), cells treated with AbLMNVs (w or w/o AMF), TMZ-AbLMNVs (w or w/o AMF) or free drug (TMZ, w or w/o AMF). Chronic AMF stimulations were carried out with a MagneTherm<sup>™</sup> equipment (NanoTherics; 20 mT, 753 Hz), 2 h *per* day, for 4 days. During AMF exposure, temperature data were collected by using a fiber optic temperature sensor (Osensa). After 4 days, spheroids were collected, washed twice in PBS, treated with trypsin (10 min at 37°C), and dissociated to single cells by pipetting; samples were centrifuged and cells resuspended

in annexin V binding buffer (1×) supplemented with 2.5  $\mu\text{M}$  of annexin V-FITC and 1  $\mu\text{g ml}^{-1}$  of propidium iodide (PI). The staining solution was incubated for 15 min at 37°C protected from light. Fluorescence intensity of the cells stained for annexin V-FITC/PI was evaluated using a Beckman Coulter CytoFLEX (for V-FITC,  $\lambda_{ex}$  488 nm;  $\lambda_{em}$  525  $\pm$  40 nm were used; for PI,  $\lambda_{ex}$  488 nm;  $\lambda_{em}$  690  $\pm$  50 nm were used). The percentages of early / late apoptotic, necrotic and healthy cell populations were analyzed using the CytoFLEX software and subsequently reported on column graphs. Moreover, after the chronic treatment, spheroids were imaged by transmission light (CLSM; C2 system; Nikon) and their equivalent diameter was plotted on the graph.

### **Proteomic analysis**

Samples were solubilized in 25  $\mu\text{l}$  2% sodium deoxycholate (SDC), 40 mM chloroacetamide, 10 mM tris(2-carboxyethyl)phosphine (TCEP) and 100 mM Tris HCl pH 8 at 100°C for 10 min and sonicated with a Ultrasonic Processor UP200St (Hielscher), 3 cycles of 30 s. Lysates samples were digested with 0.7  $\mu\text{g}$  trypsin and 0.3  $\mu\text{g}$  LysC over night at 37°C. Then nanovectors were separated from the samples by a DynaMag-2 magnetic particle concentrator (Invitrogen) and treated with 30  $\mu\text{l}$  of 5%  $\text{NH}_4\text{OH}$ . The supernatant separated from the nanovectors was concentrated and joined with the rest of the sample and processed by iST protocol.<sup>[54]</sup>

The samples were analyzed as described in Gaggero *et al.*<sup>[55]</sup> with fewer changes. Briefly, the tryptic mixture is loaded from the sample loop directly into a 75- $\mu\text{m}$  ID  $\times$  50 cm 2  $\mu\text{m}$ , 100 Å C18 column maintained at 60°C and then peptides are separated at a flow rate of 250  $\text{nl min}^{-1}$  using with a non-linear gradient of 5-45 % solution B (75% acetonitrile, 20%  $\text{H}_2\text{O}$ , 5% DMSO and 0.1% formic acid) in 140 min. Eluting peptides were analyzed using an Orbitrap Fusion Tribrid mass spectrometer (ThermoScientific). Orbitrap detection was used for MS1 at resolving power of 120 K, while Ion Trap detection was used for MS2 measurements with Rapid Ion Trap Scan Rate. Data dependent MS/MS analysis was performed in top speed mode with a 2 s cycle time, during which precursors detected within the range of  $m/z$  375–1500 were selected for activation in order of abundance. Quadrupole isolation with a window of 1.8  $m/z$  was used, and dynamic exclusion was enabled for 30 s. Automatic gain control targets were 4 $\cdot$ 10<sup>5</sup> for MS1 and 1 $\cdot$ 10<sup>4</sup> for MS2, with 50 and 45 ms maximum injection times, respectively. The signal intensity threshold for MS2 was 1 $\cdot$ 10<sup>4</sup>. HCD was performed using 28% normalized collision energy. One microscan was used for both MS1 and MS2

events. The mass spectrometry proteomics data, containing MaxQuant results, have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014652.<sup>56</sup> Reviewer account details are Username:

[reviewer04949@ebi.ac.uk](mailto:reviewer04949@ebi.ac.uk), Password: NkxEW4Ef).

MaxQuant software<sup>57</sup> version 1.6.5.0 was used to process the raw data, setting a false discovery rate (FDR) of 0.01 for the identification of proteins, peptides and PSM (peptide-spectrum match); a minimum length of 6 amino acids for peptide identification was required. Andromeda engine, incorporated into MaxQuant software, was used to search MS/MS spectra against Uniprot human database (release UP000005640\_9606 April 2019). In the processing, the variable modifications are Acetyl (Protein N-Term), Oxidation (M), Deamidation (NQ), on the contrary the Carbamidomethyl (C) was selected as fixed modification. The intensity values were extracted and statistically evaluated using the ProteinGroup Table and Perseus software.<sup>[58]</sup> Algorithm MaxLFQ was chosen for the protein quantification with the activated option “match between runs” to reduce the number of the missing proteins.

Using local scripting, we produced lists of DRPs for each comparison. Three types of lists were generated for every comparison, namely a set of up-regulated proteins, one of down-regulated proteins, and a list containing both types of factors. To study the biological effects of each experimental variable, DRP lists of the latter type were intersected one another in multiple ways. Experimental classes were named from 1 to 8 (1 = negative control; 2 = AMF; 3 = TMZ; 4 = AMF + TMZ; 5 = LMNVs; 6 = LMNVs + AMF; 7 = TMZ-LMNVs; 8 = TMZ-LMNVs + AMF). Single variables were AMF, TMZ and LMNVs; each of them was studied by four comparisons, respectively: 2 vs. 1, 4 vs. 3, 6 vs. 5, and 8 vs. 7; 3 vs. 1; 4 vs. 2; 7 vs. 5, and 8 vs. 6; 5 vs. 1, 6 vs. 2, 7 vs. 3, and 8 vs. 4. Each of these sets of four comparisons can be seen as a group of all and only the comparisons that, in different environments, measure the response to a given stimulus. We rendered them as four-way Venn diagrams. Compound variables can be investigated with analogous modalities. These are AMF & TMZ, AMF & LMNVs, and TMZ & LMNVs; they are each represented by two comparisons (respectively 4 vs 1 and 8 vs 5, 6 vs 1 and 8 vs 3, 7 vs 1 and 8 vs 2), so we illustrated them as two-way Venn diagrams. A further option is provided by the combination of all single variables at once, AMF & TMZ & LMNVs, which was studied by a single comparison (8 vs 1) and that we depicted as a simple set.

Concerning evaluation of coherence and second-order intersections, whenever DRPs were found at any intersection, we evaluated their coherence. Within a given Venn diagram, we defined a protein as coherent only when exclusively up- or exclusively down-regulated in all parent sets generating the subset in which the protein was found. Most relevant proteins were further skimmed by intersecting comparison 8 vs 1 with coherent factors for the other most impactful compound variable. We presented the outcome as a two-way, second-order Venn diagram.

To unveil GO terms significantly associated to our treatment, we performed GOrilla analyses<sup>[59]</sup> on all coherent DRPs found at the intersection between comparison 8 vs 1 and the coherent DRPs for a second most relevant compound variable. GOrilla input files were an unranked list of UniProt identifiers for the selected proteins and a background list of all UniProt identifiers within our entire dataset. GOrilla searches were performed for GO process, GO function and GO component terms. Results were trimmed for significance (on  $p$ - and  $q$ -values,  $\alpha = 0.05$ ), and sent to REVIGO for final plotting.<sup>[60]</sup>

### **Magnetothermal stimulation of post-mortem brain tissue injected with LMNVs**

A *post-mortem* brain tissue from cow was purchased from local butcher and dedicated to AbLMNV-assisted hyperthermia experiment. 300  $\mu\text{l}$  of a 7  $\text{mg ml}^{-1}$  AbLMNV dispersion was injected into a brain tissue of 4.9 g; the sample was then transferred into a 15 ml plastic tube and placed in the center of the electromagnetic coil of the MagneTherm<sup>TM</sup> equipment (NanoTherics; 20 mT, 753 Hz). The temperature time course was monitored with a fiber optic temperature sensor (Osensa), and values were reported in the graph.

*Statistical analysis:* Statistical analyses were performed with R software (<https://www.r-project.org/>). ANOVA test followed by Tukey's HSD *post-hoc* test was carried out for multiple sample comparisons, while independent  $t$ -tests were performed in case of two-sample comparisons. Statistically significant differences among distributions were indicated with \* in graphs for  $p < 0.05$ . Finally, data were plotted in graphs as average  $\pm$  standard error. Refer to "Proteomic analysis" Section for the details about the statistical analysis carried out on proteomic data.

### **Supplementary Information**

Supplementary Information (ESI) is available.

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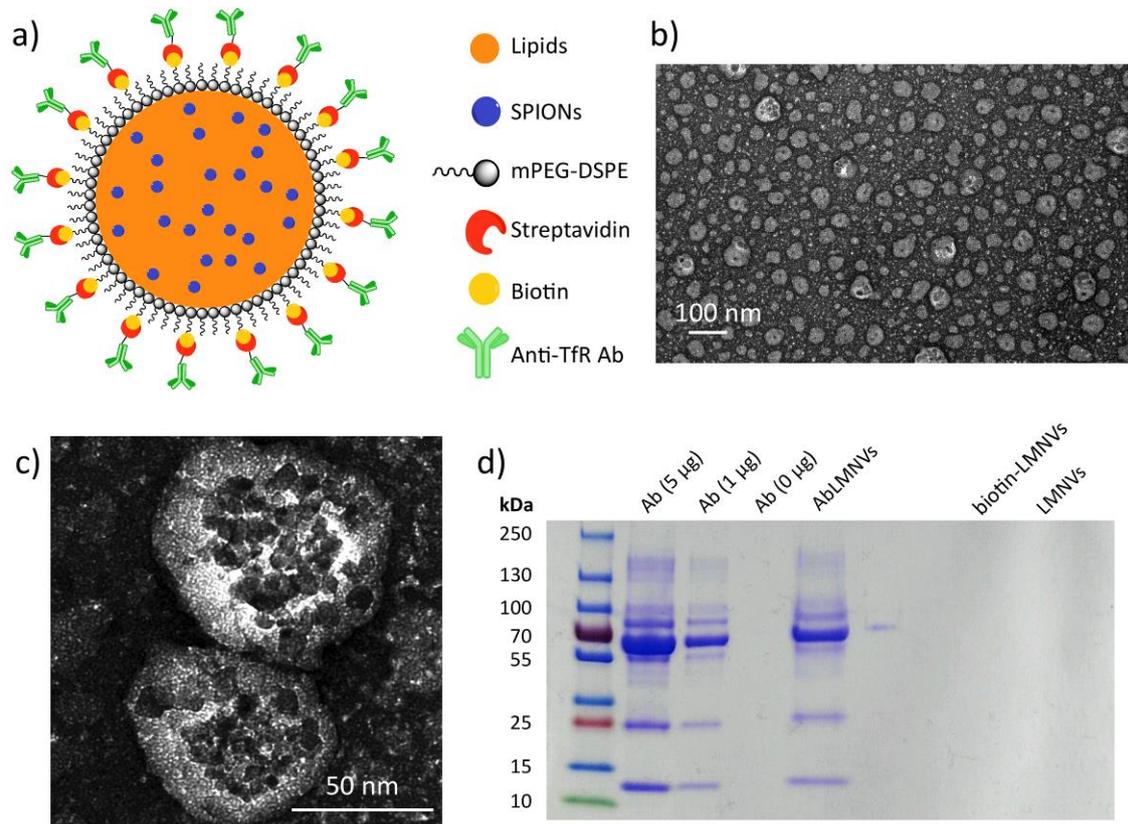
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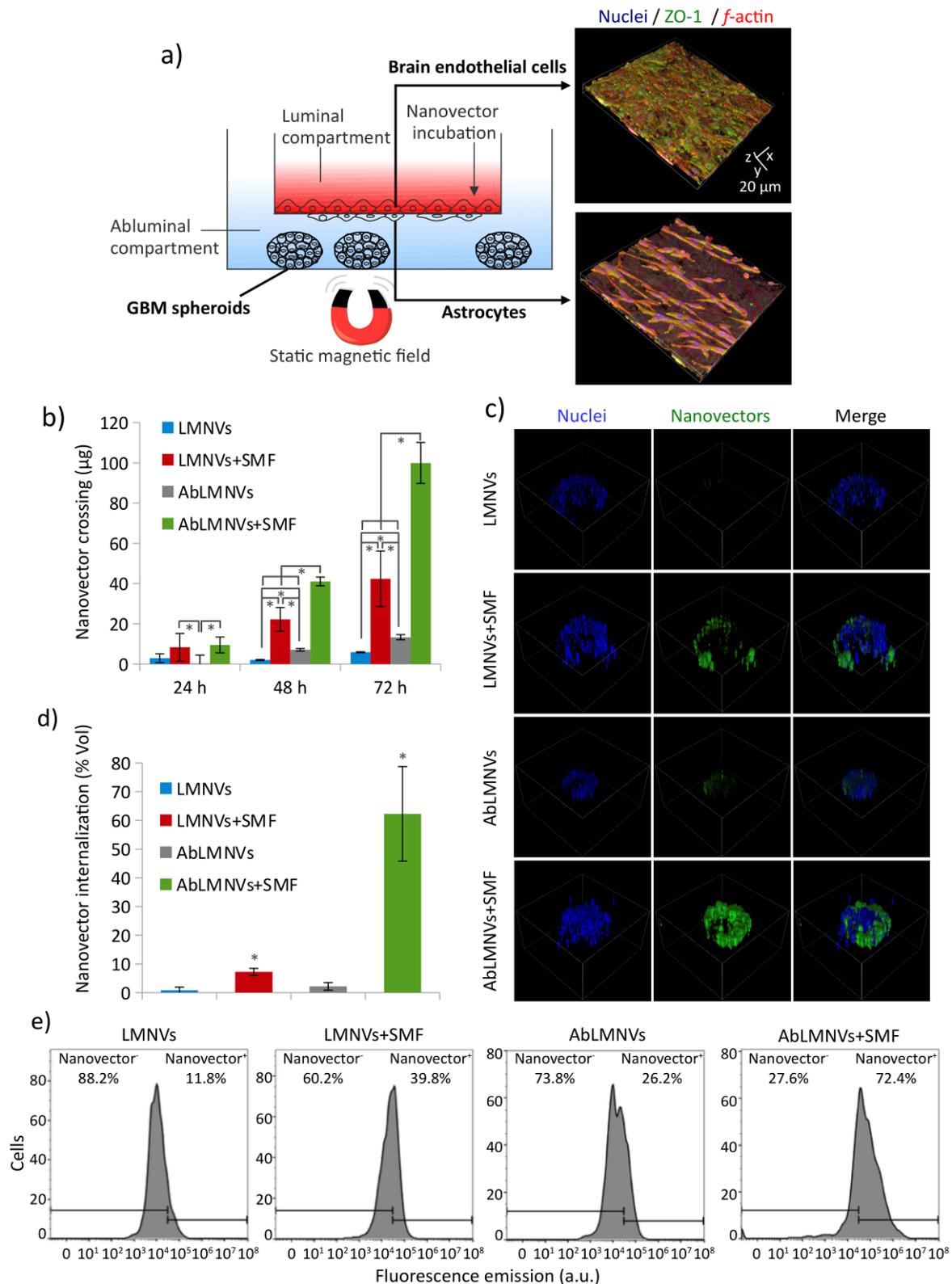
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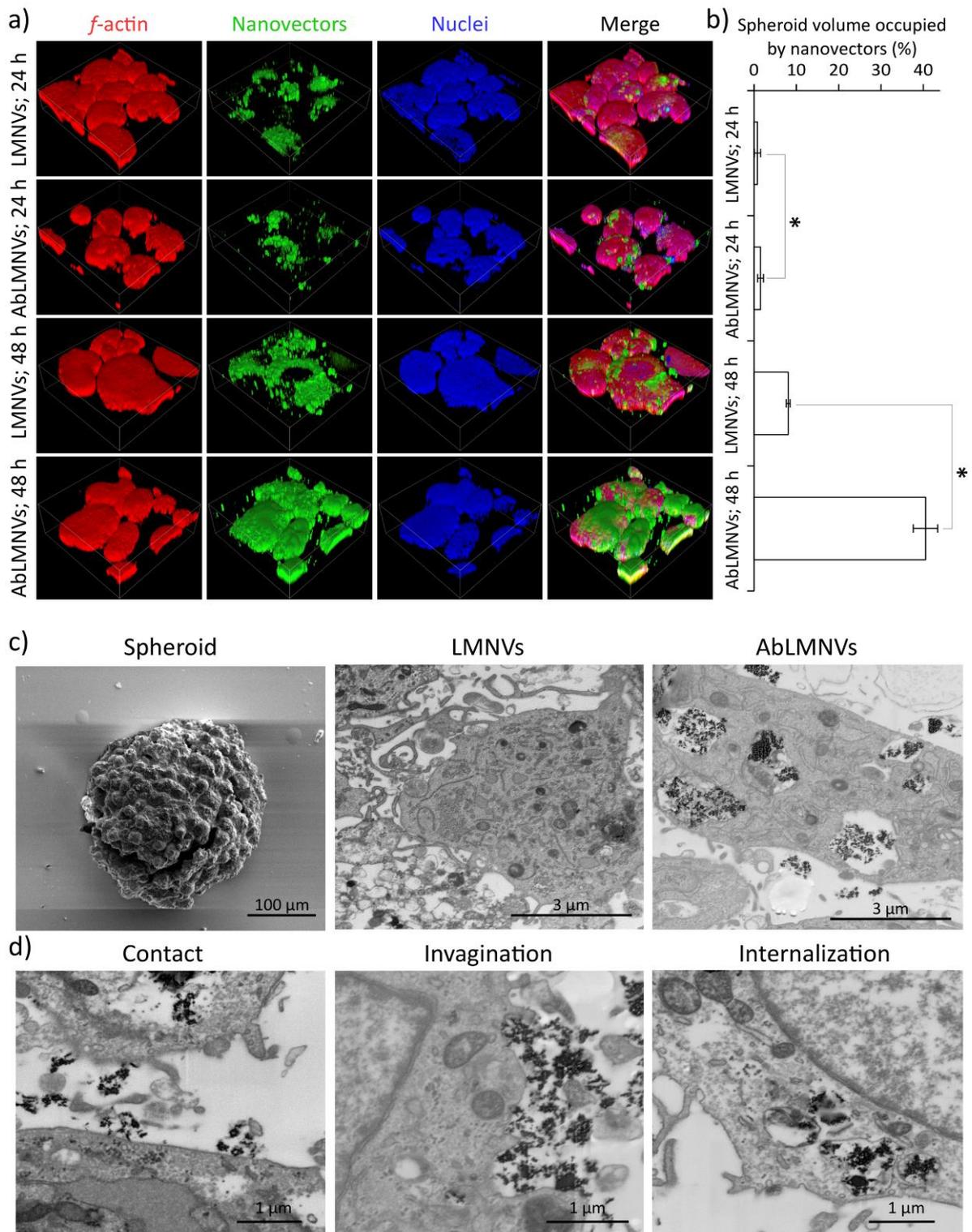


**Figure 1.** a) Scheme showing lipid magnetic nanovectors (LMNVs) loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and functionalized with an anti-transferrin receptor antibody (anti-TfR Ab). b) Low magnification and c) high magnification transmission electron microscopy (TEM) imaging of functionalized nanovectors (AbLMNVs). d) Identification of the anti-TfR Ab on AbLMNVs: SDS-PAGE stained with Coomassie Brilliant Blue R.



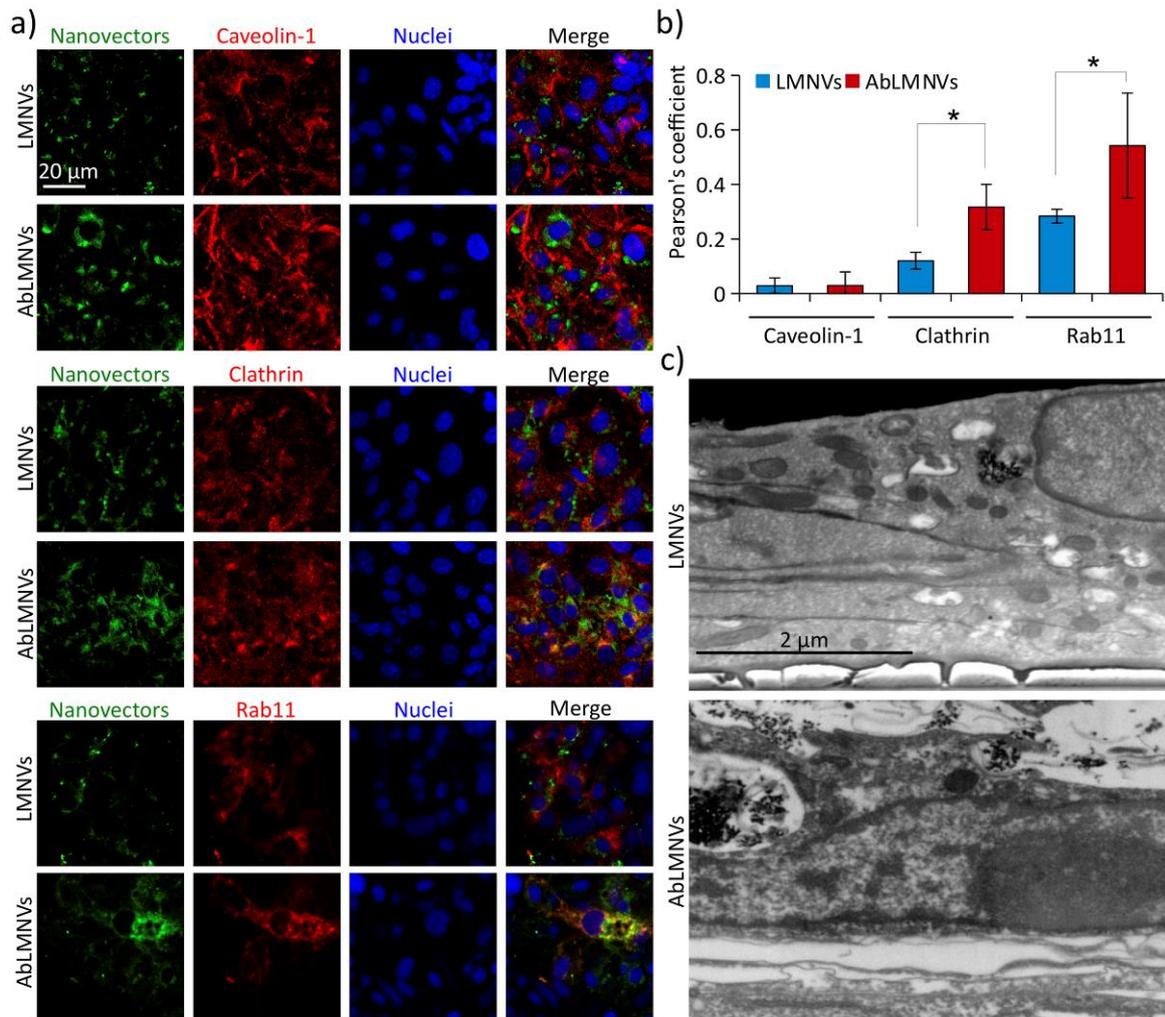
**Figure 2.** Blood-brain barrier (BBB) crossing and glioblastoma multiforme (GBM) cell targeting of DiO-stained nanovectors (LMNV and AbLMNV) in a multicellular organoid model. a) Schema of the multicellular model (left) of the BBB (with brain endothelial cells and astrocytes) and of the 3D GBM spheroids; on the right, 3D rendering of fluorescence confocal images of the endothelial monolayers (top) and of astrocytes (bottom; nuclei in blue, ZO-1 in green, f-actin in red). b) Nanovector crossing through the BBB with or without the presence of a static magnetic field (SMF), at different time points (24, 48 and 72 h). The presence of

SMF and the functionalization with anti-TfR Ab are able to independently and synergistically promote the BBB crossing. c) 3D rendering of fluorescence confocal images of nanovectors targeting the GBM spheroids after BBB crossing (72 h of incubation in the luminal compartment, with or without SMF); scan volume is 600  $\mu\text{m}$  ( $x$  axis)  $\times$  600  $\mu\text{m}$  ( $y$  axis)  $\times$  320  $\mu\text{m}$  ( $z$  axis). d) Graph reporting the percentage of the spheroid volume occupied by nanovectors. e) Flow cytometry analysis of fluorescence emission of cells dissociated from spheroids, and corresponding percentage of nanovector-positive (nanovector<sup>+</sup>) cells.

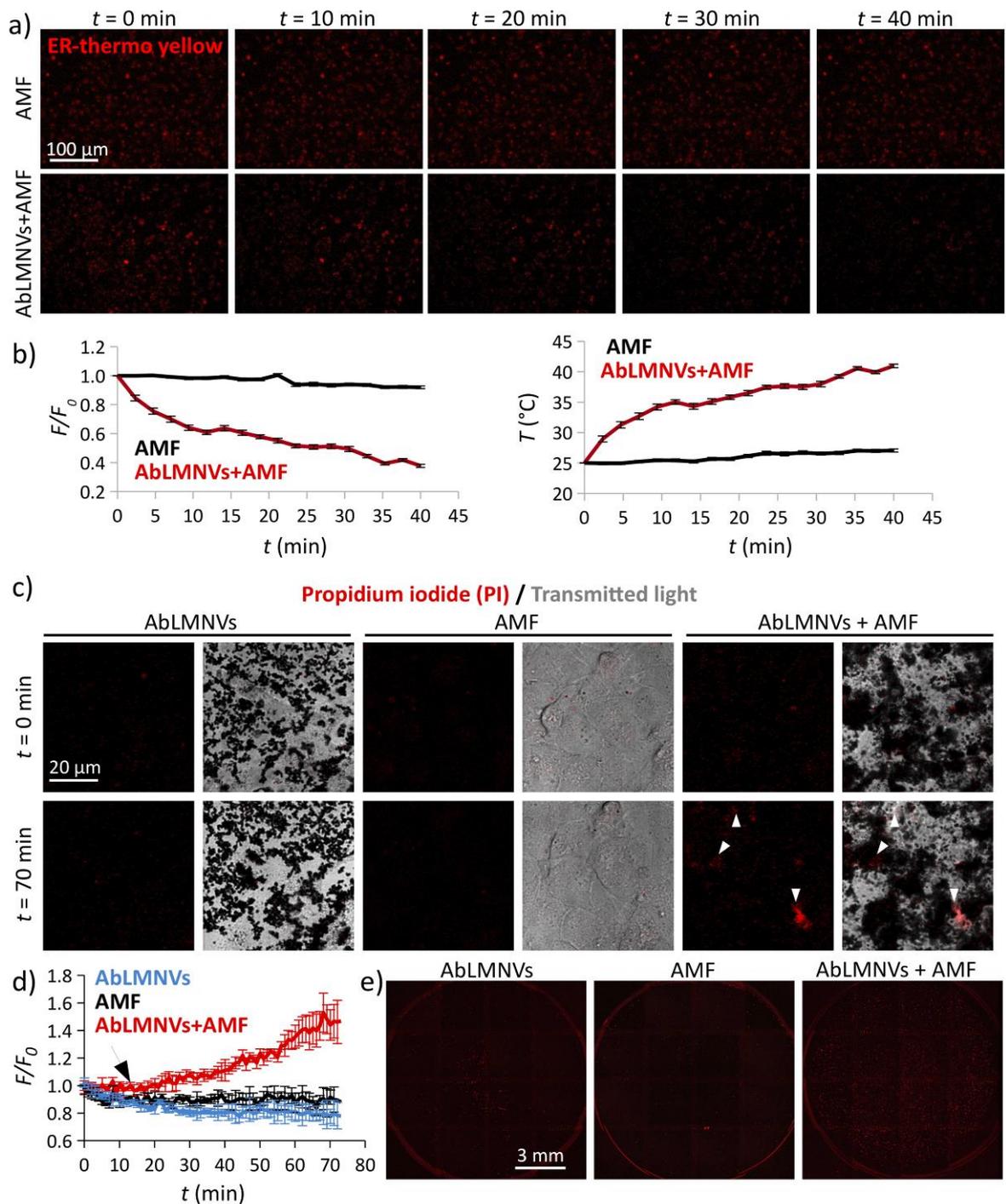


**Figure 3.** Nanovector targeting and penetration in spheroids (direct incubation of LMNVs or AbLMNVs in spheroid medium). 3D rendering of fluorescence confocal images of spheroids incubated for 24 and 48 h with DiO-stained LMNVs or AbLMNVs; *f*-actin in red, nanovectors in green, nuclei in blue; scan volume is 1270  $\mu\text{m}$  (*x* axis)  $\times$  1270  $\mu\text{m}$  (*y* axis)  $\times$  185  $\mu\text{m}$  (*z* axis). b) Graph reporting the percentage of spheroid volume occupied by the nanovectors. c) Scanning electron microscopy (SEM) images of a representative spheroid before FIB milling (left); SEM after FIB sectioning of a spheroid incubated for 24 h with LMNVs (middle) or with AbLMNVs (right). d) FIB-SEM of different phases of the nanovector internalization: the

nanovector-plasma membrane contact (left), the cell membrane invagination (middle), and the nanovector internalization in intracellular vesicles (right). \*  $p < 0.05$ .

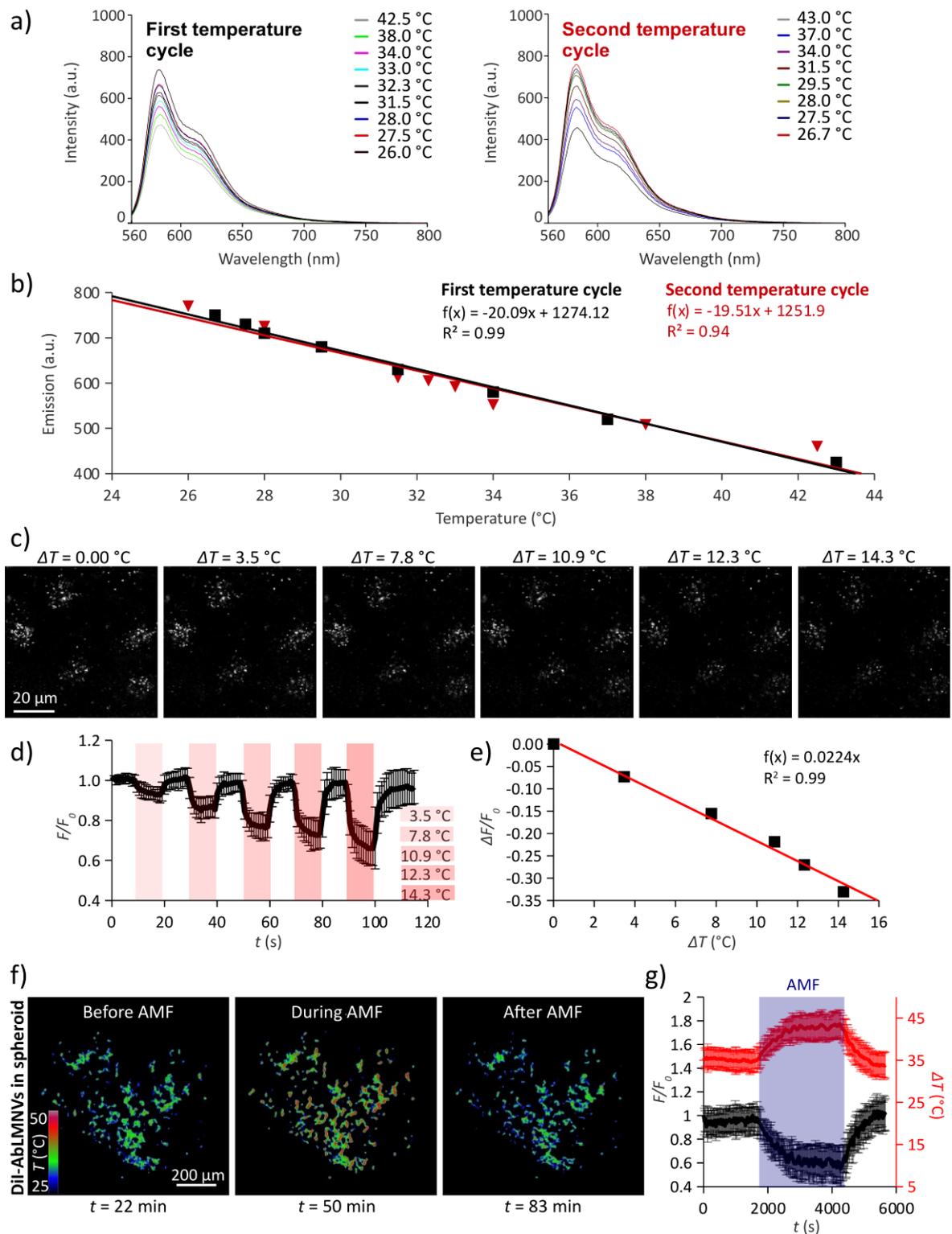


**Figure 4.** LMVN functionalization promotes clathrin-dependent endocytosis and transport to transcytosis vesicles. a) Fluorescence confocal images of caveolin-1, clathrin and rab11 markers (in red) in endothelial cells incubated with nanovectors (DiO-stained LMNVs or AbLMNVs; in green); nuclei are shown in blue. b) Graph reporting the co-localization of the nanovectors with the signal of caveolin-1, clathrin and rab11; \*  $p < 0.05$ . c) FIB-SEM of endothelial cells during the uptake of LMNVs (top) and AbLMNVs (bottom).



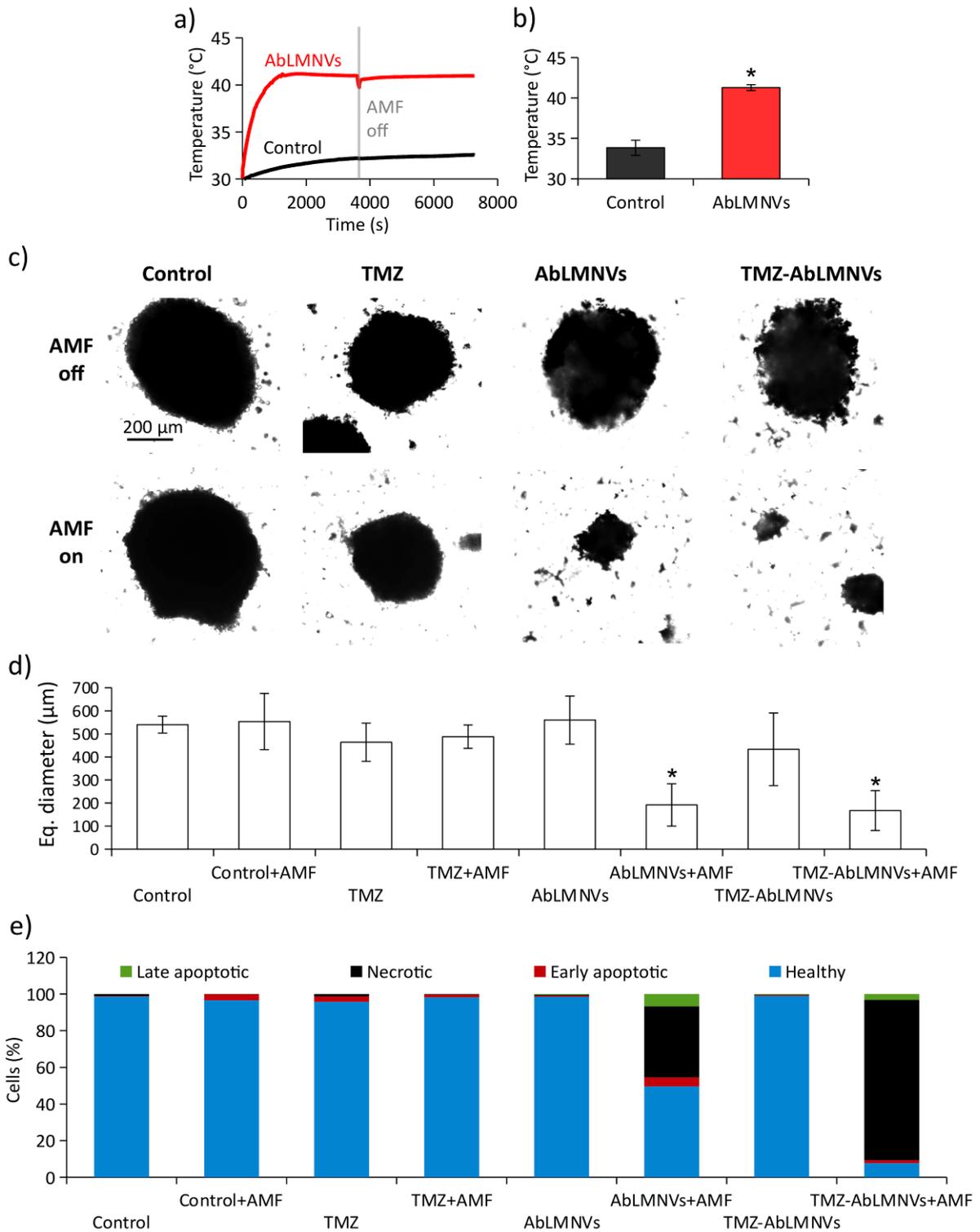
**Figure 5.** AbLMNVs-assisted magnetothermal stimulation affects plasma membrane integrity in GBM cells. a) Intracellular temperature imaging during AMF stimulation by ER-thermo yellow fluorescent thermometer, the fluorescence emission of which proportionally decreases in response to the temperature increment. Representative fluorescence images of GBM cells non-incubated (top) or pre-incubated (bottom) with AbLMNVs that underwent AMF stimulation are reported. b) The graphs respectively depict fluorescence ( $F/F_0$ ; left) and temperature ( $T$ ; right) time courses for cells non-incubated (black trace) or pre-incubated (red trace) with AbLMNVs, that underwent AMF stimulation. c) Propidium iodide (PI) / transmission light imaging before ( $t = 0$  min) and during ( $t = 70$  min) AMF stimulus (starting at  $t = 12$  min) of AbLMNVs-incubated cells non-stimulated with AMF (AbLMNVs), cultures non-incubated with AbLMNVs that underwent AMF stimulation (AMF), and of cultures incubated

with AbLMNVs and stimulated with AMF (AbLMNVs+AMF). White arrows indicate the cell internalization of PI due the loss of cell membrane integrity. d) PI fluorescence ( $F/F_0$ ) time courses of the three experimental conditions (the black arrow indicate the starting of the AMF stimulus); AbLMNVs in blue, AMF in black, AbLMNVs+AMF in red. e) Fluorescence imaging of the whole samples at the end of the experiment.



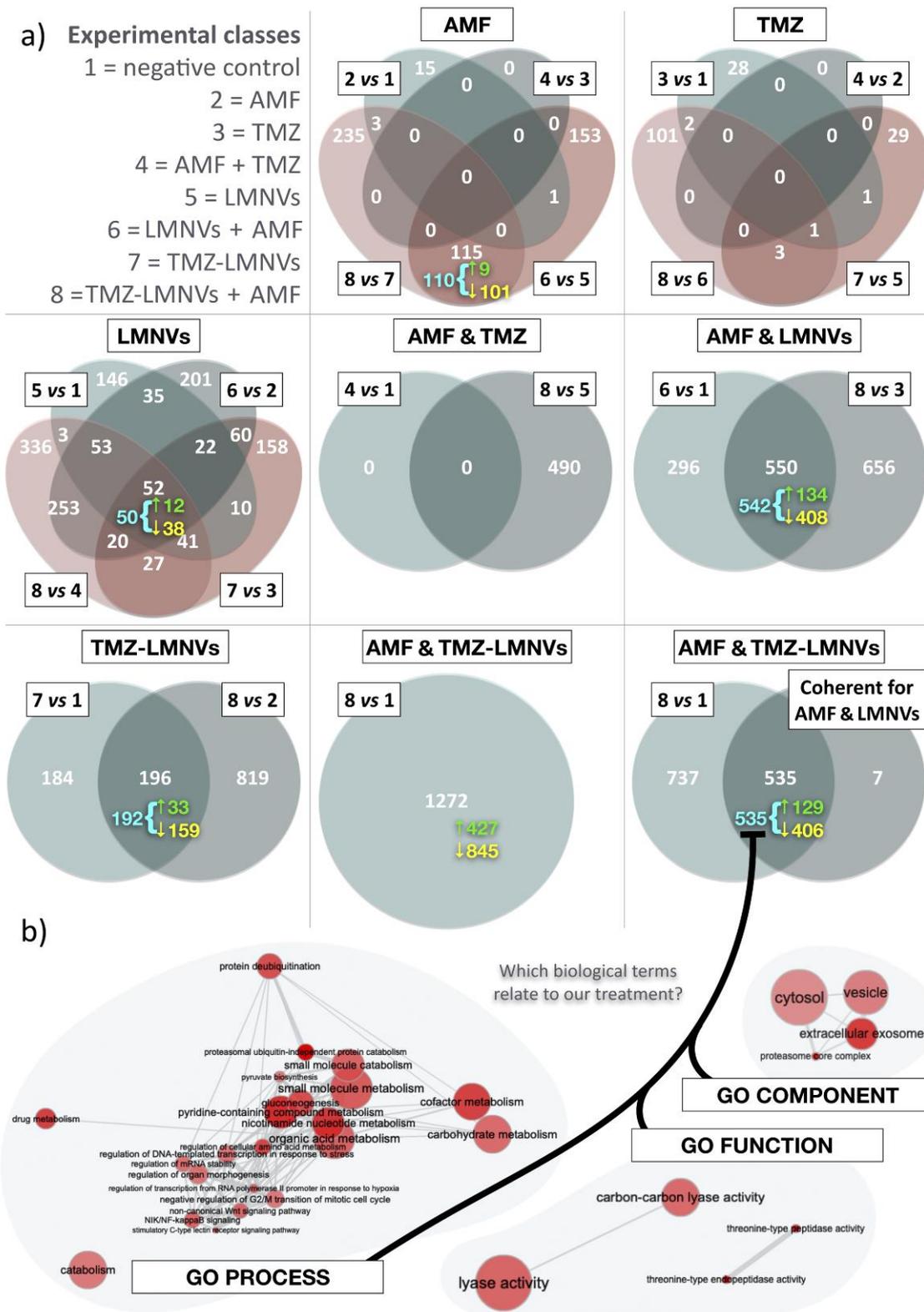
**Figure 6.** Intraparticle temperature monitoring during AMF stimulation. a) Thermosensitivity of fluorescence of the Dil dye in DMSO; fluorescence emission of Dil during two temperature cycles, shown in the left and right graph, respectively. b) Linear dependency between the Dil fluorescence emission peak (at  $\lambda = 583$  nm) and the temperature during the two temperature cycles (respectively shown in black and in red). c) Fluorescence imaging of the Dil-labeled AbLMNVs during heating ( $\Delta T_1 = 0.0^\circ\text{C}$ ;  $\Delta T_2 = 2.4^\circ\text{C}$ ;  $\Delta T_3 = 5.4^\circ\text{C}$ ;  $\Delta T_4 = 7.5^\circ\text{C}$ ;  $\Delta T_5 = 8.6^\circ\text{C}$ ;  $\Delta T_6 = 9.9^\circ\text{C}$ ) induced by an infrared (IR) laser source. d) Fluorescence intensity ( $F/F_0$ ) of the Dil-labeled AbLMNVs during heating. e) Linear relation between  $\Delta F/F_0$  and  $\Delta T$  in Dil-labeled

AbLMNVs. f) Temperature imaging of Dil-labeled AbLMNVs internalized into a GBM spheroid before, during, and after the AMF stimulation. g) Graph reporting the  $F/F_0$  and the  $\Delta T$  time course of Dil-AbLMNVs in GBM spheroids during AMF stimulation (the period of AMF exposure is highlighted in blue).



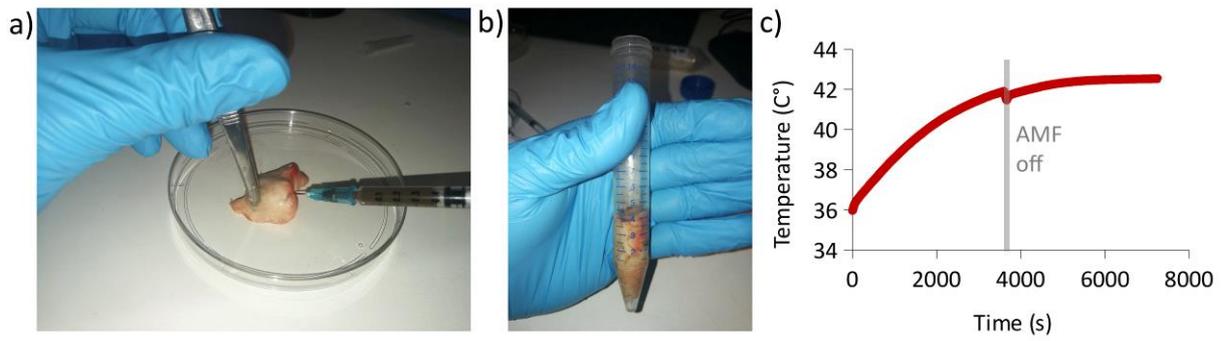
**Figure 7.** Disaggregation of GBM spheroids following magnetothermal stimulation and drug treatment. a) Temperature curves and b) average temperatures at the equilibrium of the medium in proximity of the spheroids, that were not incubated (Control) or pre-incubated (AbLMNVs) with functionalized nanovectors, during AMF stimulation. c) Transmission light images and d) size analysis of non-stimulated (AMF off; top) and stimulated (AMF on; 4 days of chronic treatment, 2 h per day; bottom) spheroids, without pre-incubation with AbLMNVs (Control), treated with the drug (TMZ), pre-incubated with AbLMNVs (AbLMNVs), or pre-incubated with TMZ-loaded AbLMNVs (TMZ-AbLMNVs). e) Plots reporting the percentage of

healthy (light blue), early apoptotic (red), late apoptotic (green), and necrotic (black) cells dissociated from the spheroids and analyzed by flow cytometry.



**Figure 8.** Apoptotic effects of nanopharmacological treatment are supported by proteomics. a) Venn diagrams for comparisons among differentially represented proteins (DRPs) for different experimental classes (reported on top, left); AMF = alternate magnetic field; LMNVs = lipid-based magnetic nanovectors; TMZ = temozolomide. The number of DRPs in each subset is reported in white; for selected subsets, the amount of coherent DRPs is added below (in cyan). Up- and down-regulated DRPs are also reported for some subsets, respectively in green (preceded by the symbol  $\uparrow$ ) or yellow (preceded by the symbol  $\downarrow$ );

when coherent DRPs are highlighted, up- and down-regulated proteins refer only to these (as implied by a cyan brace). Experimental variables are evaluated singularly in four-way Venn diagrams (either AMF, TMZ or LMNVs); two experimental variables can also be studied as a whole, in two-way Venn diagrams (AMF + TMZ, LMNVs + AMF, or TMZ-LMNVs). The combined effects of TMZ-LMNVs + AMF are shown as a single set; this is also intersected with coherent genes for LMNVs + AMF. b) REVIGO interactive graphs for gene ontology (GO) terms enrichment for biological processes, functions and components, within 535 coherent DRPs found at the intersection between comparison 8 vs. 1 and coherent DRPs yielded by the LMNVs + AMF intersection. Single GO terms are represented by red circles, the broadness of which indicating how general a GO term is; color saturation correlates instead with *p*-value. Gray lines connect similar GO terms, with line width reflecting the level of similarity. Circle positions also mirror semantic analogies, but these were adjusted as needed for graphical reasons.



**Figure 9.** AbLMNVs-assisted AMF-mediated magnetothermal stimulation of a post-mortem animal brain tissue. a) Injection of 300  $\mu$ l of a 7 mg/ml AbLMNV dispersion in the brain tissue. b) Transfer of the injected tissue in a plastic tube for the AMF stimulation. c) Graph reporting the temperature levels at the injection site during 2 h of AMF stimulation.

**Table 1.** Differentially represented proteins (DRPs). Number of DRPs for different comparisons (among experimental classes), grouped according to the Venn diagram to which they belong (as depicted in Figure 8a). DRP compositions in terms of up- and down-regulated factors is also reported.

<b>Venn diagram</b>	<b>Comparison</b>	<b>DRPs</b>	<b>Up-regulated</b>	<b>Down-regulated</b>
<b>AMF</b>	2 vs 1	19	2	7
	4 vs 3	0	0	0
	6 vs 5	269	74	195
	8 vs 7	353	90	263
<b>TMZ</b>	3 vs 1	31	19	12
	4 vs 2	0	0	0
	7 vs 5	34	15	19
	8 vs 6	107	52	55
<b>LMNVs</b>	5 vs 1	362	87	275
	6 vs 2	696	186	510
	7 vs 3	390	78	312
	8 vs 4	785	187	598
<b>AMF &amp; TMZ</b>	4 vs 1	0	0	0
	8 vs 5	490	141	349
<b>AMF &amp; LMNVs</b>	6 vs 1	846	250	596
	8 vs 3	1206	382	824
<b>TMZ &amp; LMNVs</b>	7 vs 1	380	76	304
	8 vs 2	1015	282	733
<b>AMF &amp; TMZ &amp; LMNVs</b>	8 vs 1	1272	427	845

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The proposed nanoplatform represents an efficient tool for a synergic treatment based on hyperthermia and chemotherapy against glioblastoma.

