

1 Piezoelectric Barium Titanate Nanostimulators for the Treatment
2 of Glioblastoma Multiforme

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24 ABSTRACT

25 Major obstacles to the successful treatment of glioblastoma multiforme are mostly related to the
26 acquired resistance to chemotherapy drugs and, after surgery, to the cancer recurrence in
27 correspondence of residual microscopic foci. As innovative anticancer approach, low-intensity
28 electric stimulation represents a physical treatment able to reduce multidrug resistance of cancer
29 and to induce remarkable anti-proliferative effects by interfering with Ca^{2+} and K^+ homeostasis
30 and by affecting the organization of the mitotic spindles. However, to preserve the proliferation
31 and behavior of healthy cells, it is utterly important to direct the electric stimuli only to
32 malignant cells. In this work, we propose a nanotechnological approach based on ultrasound-
33 sensitive piezoelectric nanoparticles to remotely deliver electric stimulations to glioblastoma
34 cells. Barium titanate nanoparticles (BTNPs) have been functionalized with an antibody against
35 the transferrin receptor (TfR) in order to obtain the dual targeting of blood-brain barrier and of
36 glioblastoma cells. The remote ultrasound-mediated piezo-stimulation allowed to significantly
37 reduce *in vitro* the proliferation of glioblastoma cells and, when combined with a sub-toxic
38 concentration of temozolomide, induced an increased sensitivity to the chemotherapy treatment
39 and remarkable anti-proliferative and pro-apoptotic effects.

40 KEYWORDS

41 Barium titanate nanoparticles; piezoelectricity; wireless stimulation; glioblastoma multiforme;
42 blood-brain barrier.

43 INTRODUCTION

44 Despite the dramatic efforts to develop diagnostic and therapeutic tools, the treatment of
45 brain cancer remains a huge challenge in oncology, and successful treatments are still far from
46 being attained. The main obstacles to the successful treatment of brain tumors include *i*) the
47 structural complexity of the central nervous system, *ii*) the recurrence of the tumors, and *iii*) the
48 acquired drug resistance during chemotherapy.¹ The most common and detrimental primary brain
49 tumor among adults is represented by glioblastoma multiforme (GBM), a particularly aggressive
50 malignant astrocytoma. Although various treatments are available for GBM, including surgical
51 resection, chemotherapy, and radiation, prognosis remains extremely poor.² The average survival
52 time following diagnosis of GBM patients is only fourteen months, while the five-year survival
53 rate is about 5%.

54 As alternative anticancer approaches, effective physical treatments based on low-intensity
55 alternating currents (AC) demonstrated great potential for inhibiting the proliferation of different
56 kind of cancer cells without the use of any drug/chemical.^{3,4} Specifically, AC is known to inhibit
57 cell division by interfering with Ca^{2+} and K^+ homeostasis and with the cytoskeletal components
58 involved in cell division. Low-intensity AC resulted able to enhance the efficacy of a standard
59 chemotherapy drug, temozolomide (TMZ), by reducing multidrug resistance,⁵ and have been
60 recently tested in combination with TMZ for the treatment of glioblastoma multiforme in clinical
61 trials.^{6,7} The involved mechanism seems to be mediated by a AC-dependent translocation of the
62 drug transporter P-glycoprotein (P-gp) from the plasma membrane to the cytosol.⁵ However,
63 healthy brain cells (*i.e.*, human astrocytes) are also sensitive to AC-dependent antiproliferative
64 effects³ and, in this context, the local delivery of electrical stimuli to cancer cells is highly
65 desirable.

66 The rapid development of innovative nanotechnological tools is allowing for the targeting
67 of remote physical stimulations (*e.g.*, thermal, electrical, oxidative, ionic, *etc.*) in deep tissues.⁸
68 In the field of nano-oncology, different nanotransducers have been designed to mediate
69 photothermal, photodynamic, or magnetothermal conversion, and to locally deliver anticancer
70 stimuli at tumor level.⁹ These nanotechnology-assisted remote stimulation approaches exploit a
71 non-invasive source of energy, such as, for example, alternated magnetic fields and near-infrared
72 radiations, which penetrates the biological tissues and is finally transduced by the nanomaterial
73 into another potential toxic form of energy (*e.g.*, heat).

74 In this context, our group proposed, for the first time in the literature, the remote electric
75 stimulation of living cells mediated by piezoelectric nanoparticles,^{10,11} an extremely interesting
76 approach for the modulation of cell behavior and activities.^{12,13} Taking advantage of the direct
77 piezoelectric effect, these nanomaterials have been exploited to convert mechanical into
78 electrical energy.^{14,15} Electric potentials can be generated by piezoelectric nanoparticles in
79 remote modality by using ultrasounds (US),¹⁶ mechanical pressure waves that can be safely and
80 efficiently conveyed into deep tissues. Electro-elastic mathematical models¹¹ allowed to estimate,
81 at nanoparticle level, the magnitude of the output voltage ($\varphi_{output} \sim 0.5$ mV) evoked in response
82 to US intensity $I_{US} = 0.8$ W/cm², while electrophysiological recordings¹⁷ and real-time Ca²⁺/Na⁺
83 imaging¹¹ of electrically excitable cells experimentally demonstrated the efficacy of
84 nanoparticle-assisted piezo-stimulation. Recently, our group successfully exploited the
85 antiproliferative effects of nanoparticle-assisted remote electric stimulation as non-invasive
86 “wireless” therapy suitable for inhibiting proliferation of SK-BR3 breast cancer cells.¹⁸ Similarly
87 to low-intensity AC, chronic piezo-stimulations resulted able to inhibit cancer cell cycle
88 progression by interfering with Ca²⁺ homeostasis, by upregulating the gene expression of inward

89 rectifier potassium channels, and by affecting the developing of the mitotic spindles during cell
90 division.¹⁸

91 Associated to the difficulties of treatment of pathologies at the level of the central nervous
92 system, we find the problem of blood-brain barrier (BBB) crossing. The recent growth of
93 nanotechnology promises to revolutionize the delivery of nanomaterials across BBB to brain
94 cancers.¹⁹ At first instance, the delivery of different nanomaterials through the BBB at the tumor
95 site can be efficiently obtained by taking advantage of the enhanced permeability and retention
96 (EPR) effect.²⁰ This phenomenon is associated to a highly fenestrated and permeabilized BBB in
97 correspondence of newly formed tumor vessels. A complementary strategy, that appears to be
98 particularly relevant for diagnostic and therapeutic purposes, is the functionalization of
99 nanomaterials with specific ligands to promote their BBB crossing and their targeting to specific
100 cell types or anatomical districts.²¹ Typical receptors on cancer cell membrane, as the folate, the
101 transferrin, or the epidermal growth factor (EGF) receptors, can be targeted for an efficient
102 delivery of nanostructures to cancer cells. Particular attention has been dedicated to the antibody
103 against the transferrin receptor (anti-TfR Ab), since it can be successfully exploited as a dual-
104 targeting ligand for both enabling the BBB-crossing and the uptake by cancer cells.²²⁻²⁵

105 In this work, we report the preparation of functionalized piezoelectric nanoparticles for *in vitro*
106 BBB crossing, active glioblastoma cell targeting, imaging, and remote electric treatment. To this
107 aim, tetragonal crystalline barium titanate nanoparticles (BTNPs) have been chosen as lead-free
108 piezoelectric nanotransducers²⁶ because of their excellent level of biocompatibility,²⁷ high
109 piezoelectric coefficient ($d_{33} \sim 30$ pm/V),²⁸ peculiar optical properties,²⁹⁻³¹ and possibility to
110 finely control their morphology.³² Finally, the synergic effects of the chronic piezoelectric
111 stimulation combined with sub-toxic TMZ treatment have been *in vitro* investigated.

112 MATERIALS AND METHODS

113 *Nanoparticle functionalization with anti-TfR antibody (AbBTNPs)*

114 Non-centrosymmetric piezoelectric barium titanate nanoparticles were purchased by
115 Nanostructured & Amorphous Materials, Inc (nominal nanoparticle size 300 nm in diameter, as
116 indicated by the provider, purity > 99.9%). In the literature, many different dispersing agents
117 have been adopted to obtain a stable dispersion of these nanoparticles, like
118 poly(vinylpyrrolidone) (PVP),³³ hexamethylenetetramine (HMT),³⁴ ascorbic acid,³⁵ and
119 ethanolamine.³⁵ In this work, a wrapping with the amphiphilic 1,2-distearoyl-*sn*-glycero-3-
120 phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (DSPE-PEG, Nanocs, purity >
121 99%) was carried out, as this copolymer allows for easy and straightforward functionalization
122 with many kinds of targeting moieties¹⁸. DSPE-PEG was mixed with BTNPs (1:1 w/w) in
123 ddH₂O; the mixture underwent sonication with a tip sonicator (8 W for 150 s, Mini 20 Mandelin
124 Sonoplus) and, after a centrifugation step (20 min at 900 rcf, Hettich®Universal 320/320R
125 centrifuge), supernatant containing free DSPE-PEG was discharged. The wrapped nanoparticles
126 were thereafter washed twice in ddH₂O and finally re-dispersed at a 5 mg/ml concentration in
127 ddH₂O (for electron microscopy imaging), in PBS (for estimation of functionalization
128 efficiency), or in complete cell medium (for stability studies and for biological experiments).

129 Concerning the nanoparticle functionalization with the antibody against the transferrin
130 receptor, BTNPs were firstly coated with biotin-DSPE-PEG (20 % w/w, Nanocs, purity > 95%)
131 and DSPE-PEG (80 % w/w), and subsequently conjugated to streptavidin-Ab anti-TfR (2.5 µg of
132 Ab / mg of BTNPs, Abcore), similarly as described in a previous work.¹⁸ Ab-functionalized
133 BTNPs will be indicated in the text as AbBTNPs. DPSE-PEG-coated BTNPs have been used as
134 control and will be indicated in the following as BTNPs for easiness of reading. The non-

135 functionalized plain BTNP powder will be indicated as plain BTNPs. The quantification of Ab
136 functionalization efficiency was carried out through the bicinchoninic acid (BCA) assay
137 following the manufacturers' procedures (enhanced test tube protocol, Thermo Fisher).
138 Nanoparticle size, Z-potential, and polydispersity of AbBTNP and BTNP suspension (100 µg/ml)
139 were characterized by using dynamic light scattering (DLS, Nano Z-Sizer 90, Malvern
140 Instrument); the dynamic measurements of size and polydispersity were performed every ten
141 minutes for two hours. Fourier-transformed infrared spectroscopy (FT-IR) was performed using a
142 Shimadzu Miracle 10 as previously described.³⁶

143 *Multimodal imaging of BTNPs*

144 Imaging of BTNPs was performed by using scanning electron microscopy (SEM), second
145 harmonic generation (SHG) microscopy, and confocal laser scanning microscopy (CLSM). A
146 drop of the diluted BTNP dispersion (100 µg/ml) was deposited and let dry on a glass coverslip.

147 SHG imaging of tetragonal crystal lattice of piezoelectric BTNPs was carried out with a
148 multimodal custom-made non-linear microscope using a femtosecond pulsed laser source
149 (Discovery, Coherent Inc.) for excitation. Images were acquired using an excitation wavelength
150 of 800 nm and a 20X water immersion objective lens (XLUM 20X 0.95 NA, Olympus
151 Corporation). SHG signal at 400 nm was collected in the epi-direction using a dichroic filter.
152 Emission spectrum was obtained exciting with a pump-and-probe beam at 810 nm and a Stokes
153 beam at 1060 nm.

154 BTNPs were also detected by CLSM (C2s system, Nikon) with a 642 nm laser (emission
155 collected at $670 \text{ nm} < \lambda_{em} < 750 \text{ nm}$), as showed elsewhere.^{11,18,31} BTNP signal from SHG and
156 CLSM images related to the same region of the glass coverslip were obtained and then merged
157 with ImageJ software (<https://imagej.nih.gov/ij/>).

158 For SEM, the coverslip with the deposited nanoparticles was gold-sputtered at 60 nA for 25 s,
159 and imaging was carried out by using a Helios NanoLab 600i FIB/SEM, FEI.

160 *Characterization of the blood-brain barrier model*

161 Cultures of immortalized brain-derived endothelioma bEnd.3 cell line (ATCC® CRL-2299™)
162 were seeded at high confluence (seeding density $8 \cdot 10^4$ cells/cm²) and maintained in proliferative
163 conditions on 3 µm porous transwells (Corning Incorporated) in order to obtain a functional
164 endothelial barrier mimicking the BBB.³⁷ In this configuration, endothelial layer separates the
165 luminal compartment (on the top) from the abluminal compartment (on the bottom). Both the
166 abluminal and luminal compartments were incubated with complete cell medium, composed by
167 Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific), 10% fetal bovine
168 serum (FBS, Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco).

169 The development of a functional biological barrier was assessed at day 1, 3 and 6 of
170 proliferation by measuring both FITC-dextran permeability and transendothelial electric
171 resistance (TEER). BBB model permeability was analyzed by incubating the luminal
172 compartment with 200 µg/ml of FITC-dextran (Sigma, molecular weight 70 KDa) and measuring
173 the fluorescence emission ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm, Perkin Elmer Victor X3 UV-Vis
174 spectrophotometer) of the abluminal compartment at different time points (10, 20, 30, 60, 120
175 min). TEER was assessed with a Millipore Millicell ERS-2 Volt-Ohmmeter device. Resistance
176 across the plain transwell (blank) was subtracted to all the TEER measurements. After the
177 quantitative BBB model characterizations, all the subsequent experiments reported in the text
178 were performed on BBB models at day 3. The qualitative morphological integrity of the BBB
179 models at day 3 and the expression of a specific marker of tight junctions (*zonula occludens-1*)
180 were respectively verified by Coomassie® Brilliant Blue Staining (BioRad, 0.2% for 5 minutes)

181 and by immunocytochemistry (please refer to the Materials and methods “Immunofluorescence
182 staining”).

183 *Investigations of nanoparticle-cell interactions and BBB model crossing*

184 BTNPs associated to bEnd.3 cells were observed with SEM imaging combined with energy-
185 dispersive X-ray spectroscopy (EDX). Samples incubated for 30 min with 100 µg/ml BTNPs
186 were washed twice in PBS and fixed with paraformaldehyde (PFA, 4 % in PBS). Subsequently,
187 cells were washed twice with ddH₂O and treated with glutaraldehyde solution (2.5 % in ddH₂O
188 for 30 min at 4°C) and dehydrated by using progressive ethanol gradients (0 %, 25 %, 50 %, 75
189 %, and 100 % in ddH₂O). Before SEM/EDX imaging (Helios NanoLab 600i FIB/SEM), samples
190 were gold-sputtered as described above.

191 Concerning TEM imaging, samples incubated for 30 min with BTNPs or AbBTNPs were
192 washed twice with PBS, fixed with a solution of 1.5 % glutaraldehyde in sodium cacodylate
193 buffer (0.1 M, pH 7.4) and the pellet treated for epoxy resin embedding. Briefly, cells were post-
194 fixed in 1% osmium tetroxide plus 1% K₃Fe(CN)₆ at room temperature; then cells were *en bloc*
195 stained with 3 % solution of uranyl acetate in 20 % ethanol; finally, they were dehydrated and
196 embedded in epoxy resin (Epon 812, Electron Microscopy Science). Polymerization has been
197 performed for 48 h at 60°C. Samples were then sectioned with a UC7 Leica ultramicrotome
198 equipped with a 45° diamond knife (DiATOME), and the slices of 80-90 nm were collected on
199 300 mesh copper grids. The ultrastructural analysis was performed by using a Zeiss Libra 120
200 Plus instrument operating at 120 kV equipped with an in-column omega filter.

201 Fluorescence staining of plasma membranes and acidic organelles in living bEnd.3 cells was
202 carried out. For these experiments, bEnd.3 cells were seeded on 35 mm µ-dish (Ibidi) at 8·10⁴
203 cells/cm² density for 3 days and then incubated with 100 µg/ml BTNPs / AbBTNPs for 24 and 72

204 h. After nanoparticle treatment, cells were washed in PBS and stained with CellMask Green
205 Plasma Membrane Stain (1:1000 dilution; Invitrogen) or with LysoTracker (50 nM; Invitrogen)
206 following the manufacturers' procedures. Nuclear staining was performed with Hoechst 33342 (1
207 $\mu\text{g}/\text{ml}$, Invitrogen) in all samples. Finally, cells were washed and incubated with HEPES-
208 supplemented (25 mM) phenol red-free DMEM (Thermo Fisher) supplemented with 10% of FBS
209 for CLSM imaging (C2s system, Nikon). Images were acquired by using the same acquisition
210 parameters for the different experimental classes and were subsequently analyzed with NIS-
211 Elements software (Nikon). Concerning the analysis of nanoparticle internalization, signals of
212 plasma membranes and nanoparticles were selected and then measured upon intensity
213 thresholding. Intersections between the areas of BTNPs / AbBTNPs and plasma membranes or of
214 nanoparticles and intracellular regions were then obtained and expressed as percentages of the
215 total nanoparticle area. Co-localization between acidic organelles and nanoparticles was
216 investigated by assessing Mander's overlap coefficient. 3D reconstruction of z-stack images was
217 carried out by using NIS-Elements software (Nikon).

218 Investigations of nanoparticle internalization were also performed on U-87 cells (ATCC®
219 HTB-14), a cell line derived from a human primary glioblastoma that is well characterized and
220 commonly used in brain cancer research.³⁸ The composition of the medium used for culturing U-
221 87 cells was the same of that for bEnd.3 cells (U-87 seeding density $2 \cdot 10^4$ cells/cm²).
222 Internalization studies were performed by incubating 100 $\mu\text{g}/\text{ml}$ of nanoparticles directly on U-
223 87 cells seeded on 35 mm μ -dish (Ibidi). Alternatively, U-87 cells were seeded in the abluminal
224 compartment of the transwell and 100 $\mu\text{g}/\text{ml}$ of nanoparticles were dispersed in cell medium of
225 the luminal compartment. Staining, CLSM imaging, and image analysis were carried out as
226 described above for bEnd.3 cells. SHG imaging of nanoparticle internalization was carried out

227 with the multimodal custom-made non-linear microscope described above, exploiting a pump-
228 and-probe beam at 800 nm and a Stokes beam at 1040 nm. Coherent anti-Stokes Raman
229 spectroscopy (CARS) signal at 650 nm and SHG signal from pump beam at 400 nm were
230 acquired simultaneously in epi-direction.

231 BBB model-crossing was investigated through flow cytometry (CytoFLEX, Beckman
232 Coulter). 100 µg/ml of nanoparticles were incubated in the luminal compartments of a BBB
233 model. At 4 h, 24 h and 72 h of nanoparticle treatment, concentrations of BTNPs / AbBTNPs
234 were assessed in the abluminal compartments. The number of events measured by flow
235 cytometry was then converted to nanoparticle concentrations thanks to a calibration curve
236 obtained at different known concentrations of BTNPs ($R^2 = 0.997$, Figure S1).

237 *Chronic ultrasound (US) stimulations and temozolomide (TMZ) treatment*

238 US were generated by a KTAC-4000 device (Sonidel) through a tip transducer (S-PW 3 mm
239 diameter tip). Chronic US stimulations were applied with 1 W/cm² intensity and 1 MHz
240 frequency. Single US stimuli lasted 200 ms and were delivered every 2 s, 1 h *per* day, for 4 days.
241 This protocol of US treatment was chosen since was not able to detectably increase the
242 temperature of the cell medium neither to affect cell behavior / proliferation.^{17,18}

243 Concerning TMZ treatment, different concentrations of the drug (0-400 µg/ml) were assessed
244 at 24 and 72 h in order to evaluate TMZ effects. The highest non-toxic concentration (50 µg/ml)
245 was then tested in combination with US stimulations.

246 *Cell viability assays*

247 Metabolism of cell cultures after the treatment with temozolomide (TMZ, Sigma-Aldrich) and
248 after chronic US stimulation was assessed with WST-1 Assay Reagent ((2-(4-iodophenyl)-3-(4-
249 nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium sodium salt, BioVision), as previously

250 described.³⁹ Samples were washed twice with PBS and then incubated with the WST-1 reagent
251 (1:10 dilution in complete medium with phenol red-free DMEM, 50 minutes at 37°C). The
252 absorbance of the collected supernatants was measured with a multiplate reader (Perkin Elmer
253 Victor X3 UV-Vis spectrophotometer); the blank, corresponding to the non-specific absorbance
254 of the WST-1 dilution in phenol red-free DMEM, was subtracted from all measurements. Finally,
255 all data were normalized with respect to the non-treated controls.

256 *Immunofluorescence staining*

257 Immunofluorescence was carried out to detect the expression of the tight junction marker
258 *zonula occludens-1* (ZO-1) in the BBB model. PFA-fixed cells were incubated with a 0.1%
259 Triton X-100 solution in PBS (25 min at room temperature) for membrane permeabilization and
260 with 10% goat serum in PBS (1 h at room temperature) as a blocking solution. Subsequently,
261 samples were treated with rabbit IgG primary antibody against ZO-1 (Invitrogen, 1:100 dilution
262 in PBS supplemented with 10% goat serum, 3 h at room temperature) and, after 5 washes with
263 PBS supplemented by 10% goat serum, were incubated with goat Alexa Fluor 488-IgG anti-
264 rabbit secondary antibody (Invitrogen, 1:200 dilution in PBS supplemented with 10% goat
265 serum, 2 h at room temperature). TRITC-conjugated phalloidin (100 μ M, Millipore) and Hoechst
266 33342 (1 μ g/ml, Invitrogen) were also included in solution with the secondary antibody in order
267 to stain f-actin and nuclei, respectively.

268 Double immunofluorescence was performed to analyze the expression of the Ki-67
269 proliferation marker and of the p53 tumor suppressor marker on U-87 cells after 4 days of remote
270 chronic piezoelectric stimulation and TMZ treatment. Immunocytochemistry was performed as
271 described above with a primary mouse monoclonal anti-p53 antibody (Abcam, 1:200), a primary
272 rabbit IgG anti-Ki-67 antibody (Millipore, 1:150), a TRITC-conjugated secondary anti-rabbit

273 antibody (1:200, Millipore), and a FITC-conjugated secondary anti-mouse antibody (1:75,
274 Millipore).

275 *Ca²⁺ imaging*

276 Ca²⁺ imaging was performed during US stimulation, with or without piezoelectric AbBTNPs,
277 taking advantage of Fluo-4 AM Ca²⁺-sensitive fluorescence dye, similarly as in a previous
278 work.¹¹ Before US stimulation, U-87 cells were stained with Fluo-4 AM (Invitrogen, 1 μM in
279 DMEM for 30 min at 37°C), washed twice with PBS and incubated with HEPES-supplemented
280 (25 mM) phenol red-free DMEM (Thermo Fisher). Fluorescence time-lapse imaging was
281 performed with CLSM (C2s system, Nikon), and obtained images were processed by using
282 ImageJ (<http://rsbweb.nih.gov/ij/>). The average intracellular fluorescence intensity was defined
283 as F_0 at time $t = 0$ s, and as F for $t > 0$ s. F/F_0 values were calculated for both US and
284 AbBTNPs+US experimental groups and reported in the graph.

285 *Statistics*

286 For multiple sample comparisons, ANOVA followed by Tukey's HSD *post-hoc* test was
287 performed by using *R* software (<https://www.r-project.org/>); regarding the analysis of
288 nanoparticle internalization in bEnd.3 and U-87 cells, independent two-sample *t*-tests were
289 carried out by using Excel software. Statistically significant differences among distributions were
290 indicated for $p < 0.05$. Finally, data were plotted in histograms as average \pm standard error by
291 using Excel software.

292 RESULTS

293 The scheme of the experimental design is represented in Figure 1. In Figure 1a the strategy of
294 nanoparticle functionalization is depicted: piezoelectric BTNPs (showed in red) are wrapped
295 with DSPE-PEG and DSPE-PEG-biotin, and subsequently conjugated with streptavidin-Ab

296 against human TfR to finally obtain AbBTNPs. In Figure 1b the luminal (in red) and abluminal
297 (in light blue) compartments of the *in vitro* BBB model are showed, where bEnd.3 and U-87
298 cells are respectively seeded (cell membranes are shown in green, nuclei in blue, and AbBTNPs
299 in red). After 72 h of nanoparticle incubation in the luminal compartment, U-87 cells exposed to
300 nanoparticles that crossed the BBB model have been piezoelectrically stimulated with chronic
301 US treatments, as schematically indicated in Figure 1c.

302 *Characterization and imaging of piezoelectric BTNPs*

303 Imaging of piezoelectric BTNPs is presented in Figure S2. Figure S2a reports a representative
304 SEM image of the sample. Figure S2b represents the emission spectrum obtained by illuminating
305 the tetragonal crystal lattice of piezoelectric BTNPs with a pair of spatially- and temporally-
306 overlapped laser beams at 810 nm and 1060 nm (pump-and-probe beam and Stokes beam,
307 respectively). Figure S2c shows the multi-modal imaging of piezoelectric BTNPs; signal of
308 BTNPs observed by SHG of the pump beam (in red) co-localizes with that one detected by
309 CLSM (in green).

310 Fourier transformed infrared spectroscopy (FT-IR) was performed in order to verify the
311 successful functionalization of the BTNPs. Starting from the low wavelengths, the peaks in the
312 range $530\text{-}600\text{ cm}^{-1}$ (Figure S3) can be attributed to the Ti-O stretching bond and they are
313 characteristic of the BaTiO_3 compound.⁴⁰ Shifting to higher wavelengths, the peak at 1450 cm^{-1}
314 that can be seen in spectrum *i*) of plain BTNPs can be attributed to an impurity of BaCO_3 as it
315 has been reported elsewhere.⁴⁰ The peaks between $1000\text{-}1100\text{ cm}^{-1}$ (spectrum *ii*)) are attributed to
316 the C-O-C and C-O-H stretching⁴¹ vibration of the aliphatic chain of poly(ethylene glycol)
317 (PEG), while the peaks in the range $1600\text{-}1670$ and $1300\text{-}1460\text{ cm}^{-1}$ (spectrum *iii*)) can be
318 attributed to the Amide I (C=O stretching)⁴² and Amide III⁴⁰ vibrations of the attached anti-

319 transferrin antibody. The peaks at 2280-2400 and 2850-3000 cm^{-1} (spectra *ii*) and *iii*) are
320 attributed to the C-H stretching bond of the DSPE-PEG while the peak at 3320 cm^{-1} (spectrum
321 *iii*) can be attributed both to the O-H stretching vibration of the DSPE-PEG/TfR antibody as
322 well as to the Amide A (N-H stretching)⁴³ of the TfR antibody. The corresponding vibrations and
323 wavelengths are summarized in Table S1. A small yet significant difference in Z-potential was
324 observed between BTNPs (-29.6 ± 0.8 mV) and AbBTNPs (-22.0 ± 0.6 mV), thus further
325 supporting the hypothesis of the successful functionalization of BTNPs with the Ab.

326 Quantitative measurements of functionalization efficiency indicated an amount of 1.1 ± 0.4 μg
327 of Ab *per* mg of BTNPs ($\sim 44\%$ of the Ab used for the reaction successfully linked to BTNPs).
328 Considering the molecular weight of the Ab (~ 90 KDa) and the number of BTNPs *per* mg of
329 powder ($1.2 \cdot 10^{10}$ particles / mg), about 624 ± 227 molecules of Ab were conjugated to each
330 BTNP.

331 Polydispersity index (PDI) and hydrodynamic diameter (*Rd*) of BTNPs and AbBTNPs were
332 investigated (Figure S4). The PDI was found stable over time for both BTNPs and AbBTNPs
333 (Figure S4a; 1 measurement / 10 min for 110 min total; for $t = 0$ min, 0.29 ± 0.05 for BTNPs and
334 0.25 ± 0.02 for AbBTNPs; for $t = 110$ min, 0.37 ± 0.04 for BTNPs and 0.37 ± 0.04 for
335 AbBTNPs); in both cases $0.2 < \text{PDI} < 0.4$, thus indicating a moderate dispersivity.⁴⁴
336 Furthermore, hydrodynamic diameter *Rd* of both samples were stable along the experiment
337 (Figure S4b; 1 measurement / 10 min for 110 min total; for $t = 0$ min, $Rd = 274 \pm 1$ nm for
338 BTNPs and $Rd = 252 \pm 11$ nm for AbBTNPs; for $t = 110$ min, $Rd = 304 \pm 13$ nm for BTNPs and
339 $Rd = 280 \pm 2$ nm for AbBTNPs).

340 *AbBTNPs efficiently target endothelial-like cells and cross BBB model*

341 In order to obtain a functional biological barrier mimicking the BBB, confluent cultures of
342 bEnd.3 cells were maintained in proliferative conditions on 3 μm porous transwell for 1, 3 and 6
343 days (BBB model characterization is reported in Figure S5). Transendothelial electric resistance
344 (TEER) was measured to assess the performances of the barrier at the different time points
345 (Figure S5a). After 3 and 6 days of maturation, BBB model showed similar TEER levels ($41.9 \pm$
346 $8.9 \Omega \cdot \text{cm}^2$ and $48.5 \pm 7.4 \Omega \cdot \text{cm}^2$ at day 3 and day 6 of culture, respectively), in both cases
347 significantly higher with respect to the 1 day culture ($20.1 \pm 0.9 \Omega \cdot \text{cm}^2$; $p < 0.05$). The crossing
348 of FITC-dextran through the BBB model (at day 1, 3 and 6 of maturation) is shown in Figure
349 S5b and has been expressed as % of the maximum theoretically achievable fluorescence intensity
350 in the abluminal compartment at different time points (10, 20, 30, 60 and 120 min). BBB model
351 at day 3 and day 6 showed similar permeability to FITC-dextran after 20 min of incubation (19.8
352 $\pm 0.6\%$ at day 3 and $17.8 \pm 0.4\%$ at day 6), while a significant lower permeability at day 6 of
353 maturation ($35.1 \pm 1.7\%$) was observed after 120 min of dextran treatment with respect to both
354 day 1 and day 3 ($101.2 \pm 2.7\%$ at day 1 and $52.3 \pm 2.4\%$ at day 3; $p < 0.05$). The developing of
355 cell multilayers was also observed at day 6. Considering the good performances of the BBB
356 model at day 3 as well as the scarce mechanical stability and resistance to the shear forces of
357 BBB immediately after day 6 (delamination and cell layer detachments were observed in
358 different cultures starting from day 7-8), nanoparticle-crossing through barrier was tested on
359 BBB starting from day 3. In Figure S5c the Coomassie (left image) and the immunofluorescence
360 staining (right image, ZO-1 in green and nuclei in blue) of the 3-day BBB model are reported; it
361 is possible to appreciate the complete maturation of functional junctions among bEnd.3 cells,
362 that develop a endothelial layer separating the luminal from abluminal compartment of the
363 transwell.

364 Analysis of BTNP / AbBTNPs interacting with bEnd.3 cells and assessment of BBB model
365 crossing are shown in Figure 2. In Figure 2a the SEM imaging and the energy dispersive X-ray
366 analysis (EDX) of BTNPs associated to the plasma membranes of bEnd.3 cells are reported.
367 Qualitatively, TEM observations (Figure 2b) highlighted a higher amount of AbBTNPs
368 associated to plasma membranes and up-taken by bEnd.3 cells with respect to the non-
369 functionalized BTNPs. CLSM of immunofluorescence staining against the ZO-1 marker of tight
370 junctions after 72 h of BTNP / AbBTNP treatment is showed in Figure 2c (nuclei in blue, f-actin
371 in red, ZO-1 in green, nanoparticles in white). CLSM imaging revealed that both BTNPs and
372 AbBTNPs were internalized in bEnd.3 cells; however, increased nanoparticle internalization can
373 be appreciated in samples treated with AbBTNPs. Plasma membrane imaging was carried out at
374 72 h of nanoparticle treatment (Figure 2d) and showed a higher amount of nanoparticles
375 internalized in cell body with respect to those associated to the plasma membranes (this was
376 observed for both BTNPs and AbBTNPs). Histograms of Figure 2e and 2f show the cell
377 membrane and intracellular areas (%) of bEnd.3 cells co-localizing with BTNPs / AbBTNPs at
378 24 and 72 h of nanoparticle incubation, respectively. The quantitative analysis demonstrates a
379 significantly higher amount of AbBTNPs, both associated to membranes ($1.06 \pm 0.27\%$) and
380 internalized by bEnd.3 cells ($2.04 \pm 0.30\%$), with respect to BTNPs ($0.30 \pm 0.14\%$ associated to
381 plasma membranes and $0.55 \pm 0.31\%$ internalized in cells; $p < 0.05$) at 24 h. Furthermore, the
382 amount of intracellular nanoparticles (both AbBTNPs and BTNPs) decreased from 24 to 72 h
383 (AbBTNPs and BTNPs internalized in cells for 72 h correspond, respectively, to $1.02 \pm 0.14\%$
384 and $0.24 \pm 0.05\%$), likely due to the active transport mechanisms through the bEnd.3 cells (*e.g.*,
385 transcytosis and exocytosis). Despite this decrement, the amount of functionalized nanoparticles
386 internalized in bEnd.3 cells remained significantly higher with respect to BTNPs at 72 h ($p <$

387 0.05). 3D reconstructions of nanoparticles (BTNPs / AbBTNPs in red) and bEnd.3 plasma
388 membranes (in green) are available in Figure S6 (72 h of incubation). Moreover, co-localization
389 analysis of nanoparticles and acidic cell compartments (*i.e.*, late endosomes and lysosomes) at 4,
390 24 and 72 h of BTNP / AbBTNP incubation is reported in Figure S7, and showed a progressive
391 accumulation in the acidic organelles of the cells. Higher AbBTNPs co-localization with acidic
392 cell compartment was found with respect to BTNPs at both 24 h (Mander's coefficients were
393 0.56 ± 0.06 for AbBTNPs and 0.36 ± 0.03 for BTNPs; $p < 0.05$) and 72 h (Mander's coefficients
394 were 0.78 ± 0.14 for AbBTNPs and 0.36 ± 0.07 for BTNPs; $p < 0.05$), presumably as a
395 consequence of the higher internalization level with respect to the non-functionalized ones.

396 In order to measure BBB model-crossing, BTNP / AbBTNP were incubated in the luminal
397 compartment of the BBB model and nanoparticle concentrations in the abluminal compartment
398 were measured at 4, 24 and 72 h of nanoparticle treatment. Results reported a progressive BBB-
399 crossing of BTNPs / AbBTNPs at the different time points. Similar BTNP and AbBTNP
400 concentrations were found at 4 h (3.25 ± 0.01 $\mu\text{g/ml}$ and 2.96 ± 0.26 $\mu\text{g/ml}$ respectively for
401 BTNP and AbBTNP) and 24 h (6.26 ± 0.83 $\mu\text{g/ml}$ and 6.72 ± 0.10 $\mu\text{g/ml}$ respectively for BTNP
402 and AbBTNP). Instead, a significantly higher BBB-crossing ability of AbBTNPs was observed at
403 72 h with respect to non-functionalized nanoparticles ($\sim 34\%$ increase: 8.01 ± 0.03 $\mu\text{g/ml}$ and
404 10.69 ± 0.17 $\mu\text{g/ml}$ respectively for BTNP and AbBTNP; $p < 0.05$). All together, these results
405 indicated a higher BBB-targeting and BBB-crossing efficiency of the functionalized nanosystem.

406 *Dual targeting of AbBTNPs*

407 Additionally to the measurements of nanoparticle concentration in the abluminal compartment,
408 the ability of AbBTNPs to efficiently bind glioblastoma cells was tested (Figure 3). CLSM
409 analysis of U-87 cells that were incubated for 24 h with 100 $\mu\text{g/ml}$ BTNPs or AbBTNPs is

410 shown in Figures 3a-b. Interestingly, a higher level of AbBTNPs ($1.00 \pm 0.23\%$ intracellular
411 nanoparticles and $1.32 \pm 0.42\%$ associated to membranes) were found with respect to BTNPs
412 ($0.16 \pm 0.03\%$ intracellular nanoparticles and $0.26 \pm 0.11\%$ associated to membranes; $p < 0.05$).
413 Qualitative observations with CARS / SHG scans confirmed the higher level of AbBTNP
414 internalization (Figure 3c). Moreover, the CLSM analysis of U-87 cells exposed to nanoparticles
415 that crossed the BBB model was carried out; Figure 3d shows representative CLSM images of
416 U-87 cells cultured in the abluminal compartment after a 72 h treatment with BTNPs or
417 AbBTNPs in the luminal compartment. In Figure 3e, the quantitative co-localization analysis
418 revealed a higher amount of AbBTNPs in the abluminal compartment that are associated to the
419 plasma membranes ($1.11 \pm 0.35\%$) and internalized by U-87 cells ($0.96 \pm 0.25\%$) compared to
420 BTNPs ($0.38 \pm 0.15\%$ associated to plasma membranes and $0.16 \pm 0.03\%$; internalized by U-87
421 cells; $p < 0.05$), thus demonstrating as the AbBTNPs resulted a successful nanosystem able to
422 cross the *in vitro* BBB model and to target U-87 cells with higher efficiency with respect to the
423 non-functionalized BTNPs. For this reason, the following experiments have been performed just
424 by using AbBTNPs.

425 *Chronic piezoelectric stimulation inhibits proliferation of human glioblastoma cells*

426 Inhibitory effects of chronic piezoelectric stimulation on U-87 proliferation are shown in
427 Figure 4. Two concentrations of AbBTNPs have been investigated: $100 \mu\text{g/ml}$, already
428 successfully tested on SK-BR3 breast cancer cells,¹⁸ and $10 \mu\text{g/ml}$, the concentration of
429 nanoparticles able to cross the BBB model after 72 h. The expression of the nuclear proliferation
430 marker Ki-67 has been analyzed through immunofluorescence assays combined with CLSM
431 imaging (Figure 4a). Qualitatively, it is possible to appreciate a lower Ki-67 expression in
432 piezoelectrically-stimulated cells (AbBTNPs+US) with respect to the control cultures (non-

433 stimulated and non-incubated controls, cells incubated with AbBTNPs but non-stimulated with
434 US, cultures stimulated with US without the presence of AbBTNPs); quantitative analysis of Ki-
435 67⁺ nuclei (%) are presented in Figure 4b and reported as the lowest proliferation rate was found
436 for AbBTNPs+US cultures incubated with 100 µg/ml of nanoparticles ($28.7 \pm 2.5\%$), followed
437 by AbBTNPs+US cultures incubated with 10 µg/ml of nanoparticles ($51.1 \pm 4.5\%$). Both these 2
438 experimental conditions resulted characterized by a significantly lower proliferation rate with
439 respect to all the other control groups ($72.3 \pm 3.7\%$ for non-stimulated and non-incubated
440 controls, $77.4 \pm 3.2\%$ for cells incubated with AbBTNPs but non-stimulated with US, $86.8 \pm$
441 1.9% for cultures stimulated with US without the presence of AbBTNPs; $p < 0.05$).

442 Time-lapse Ca²⁺ imaging on AbBTNPs+US (10 µg/ml) cultures demonstrated the successful
443 remote activation of the cells (Figure 4c): remarkable long-term Ca²⁺ waves are observed in
444 response to the US stimulation in the presence of the nanoparticles. The peak of the Ca²⁺ wave
445 was detected ~ 5 min after starting the US stimulations, and the Ca²⁺ concentrations remain
446 higher than the basal levels even after 25 min of stimulation. The time lapses video of Ca²⁺
447 imaging performed on US and AbBTNPs+US cultures are available as Supplementary
448 Information (Video S1 and S2, respectively). The stability of Ca²⁺ levels and the regular
449 proliferation rate observed in response to the plain US stimulation (*i.e.*, without the presence of
450 AbBTNPs) support the safeness of the proposed stimulation method.

451 *Synergic efficacy of remote piezoelectric stimulation with temozolomide treatment*

452 The ability of nanoparticle-assisted piezoelectric stimulation to improve the anticancer efficacy
453 of temozolomide (TMZ) treatment was investigated (Figure 5). Toxic effects of TMZ were
454 assessed by testing different concentrations of the chemotherapy treatment (0-400 µg/ml) at two
455 different time points (24 and 72 h) through WST-1 assay (data are normalized and expressed as

456 percentage of WST-1 absorbance values measured at 24 h on control cultures). Metabolism of U-
457 87 cultures at 72 h of treatment was significantly affected when treating with concentrations at
458 least of 200 $\mu\text{g/ml}$ (Figure 5a): the best anti-proliferative effects were observed with the highest
459 tested TMZ concentration (400 $\mu\text{g/ml}$), while first significant effects were observed by using 200
460 $\mu\text{g/ml}$. The hypothesis that piezoelectric stimulation could increase the sensitivity of TMZ was
461 investigated by using 50 $\mu\text{g/ml}$ of this chemotherapy drug, the highest concentration that was not
462 effective in our testing conditions (Figure 5b-e). Experimental classes we represented by control
463 cultures, cultures incubated with 10 $\mu\text{g/ml}$ AbBTNPs, cultures incubated with 50 $\mu\text{g/ml}$ TMZ,
464 cultures incubated with 50 $\mu\text{g/ml}$ TMZ and 10 $\mu\text{g/ml}$ AbBTNPs, cultures chronically stimulated
465 with US, cultures stimulated with US in the presence of 10 $\mu\text{g/ml}$ AbBTNPs, cultures stimulated
466 with US in the presence of 50 $\mu\text{g/ml}$ TMZ, and, finally, cultures stimulated with US in the
467 presence of 10 $\mu\text{g/ml}$ AbBTNPs and of 50 $\mu\text{g/ml}$ TMZ. WST-1 assay (Figure 5b) was performed
468 at day 4 on control cultures ($100.0 \pm 7.2\%$), cultures incubated with AbBTNPs ($101.3 \pm 1.7\%$),
469 cultures incubated with TMZ ($97.4 \pm 2.4\%$), cultures incubated with TMZ and 10 $\mu\text{g/ml}$
470 AbBTNPs ($95.3 \pm 0.9\%$), cultures chronically stimulated with US ($94.9 \pm 4.5\%$), cultures
471 stimulated with 10 $\mu\text{g/ml}$ AbBTNPs+US ($87.8 \pm 1.3\%$), cultures stimulated with US and TMZ
472 ($94.7 \pm 4.1\%$), and, finally, cultures stimulated with 10 $\mu\text{g/ml}$ AbBTNPs+US in the presence of
473 TMZ (TMZ+AbBTNPs+US; $72.1 \pm 1.7\%$). Results confirmed the anti-proliferative effects of
474 nanoparticle-assisted piezoelectric stimulation (AbBTNPs+US), that was able to significantly
475 decrease the metabolic activity without the presence of TMZ with respect to the other control
476 conditions (control, AbBTNPs, TMZ, AbBTNPs+TMZ, US, US+TMZ; $p < 0.05$). However, the
477 major effects were observed by synergistically combining piezo-stimulation with TMZ
478 (TMZ+AbBTNP+US; $p < 0.05$).

479 The expression of the Ki-67 proliferation marker and of the p53 tumor suppressor marker in
480 response to 50 µg/ml TMZ, 10 µg/ml AbBTNPs+US, and of 10 µg/ml AbBTNPs+US with 50
481 µg/ml TMZ (TMZ+AbBTNPs+US) were compared with control cultures and are showed in
482 Figure 5c. Qualitatively, a decreased number of cells and a lower Ki-67 expression were found in
483 both AbBTNP+US and TMZ+AbBTNP+US experimental classes, compared to both control and
484 TMZ. This observation is in line with the lowest metabolism levels reported in response to these
485 treatments. Moreover, a higher amount of p53⁺ nuclei was detected in response to
486 TMZ+AbBTNP+US treatment with respect to the other experimental groups. Quantitatively, Ki-
487 67⁺ nuclei in control (72.4 ± 2.8%) and in TMZ-treated (65.8 ± 5.7%) cultures were significantly
488 higher with respect to the cultures treated with AbBTNP+US (49.2 ± 3.7%; *p* < 0.05) and with
489 TMZ+AbBTNP+US (27.7 ± 2.5%; *p* < 0.05), the last of which resulted the strongest
490 antiproliferative treatment (*p* < 0.05; Figure 5d). Higher levels of p53⁺ nuclei were found in
491 response to the combined TMZ+AbBTNP+US therapy (28.3 ± 6.6%; *p* < 0.05; Figure 5e) with
492 respect to all the other treatments (1.2 ± 1.3% for AbBTNP+US; 3.4 ± 1.1% for TMZ) and
493 control cultures (1.0 ± 0.7%).

494 Overall, these results indicate that piezoelectric stimulation affects proliferation of U-87 cells
495 and increases their sensitivity to TMZ. Indeed, TMZ therapy at non-toxic concentrations, when
496 combined with chronic piezoelectric treatment, was able to promote cell apoptosis and reducing
497 cell proliferation.

498 DISCUSSION

499 Recent advances in nanobiotechnology are directed to the development of smart and
500 biocompatible sensors / actuators that are able to detect and respond to specific physicochemical
501 conditions in the human body.⁴⁵⁻⁴⁷ Piezoelectric nanomaterials are a promising class of

502 nanostructures, that have been successfully exploited both as mechanical sensors for energy-
503 harvesting and mechanobiology studies, and as nanostimulators for indirect electrical activation
504 of excitable cells.^{48,49}

505 In this work, we report for the first time the successful crossing of a piezoelectric nanomaterial
506 through a BBB model. Piezoelectric barium titanate nanoparticles used in this study are
507 characterized by a 300 nm diameter size, and resulted able to cross a BBB model with a quite
508 good efficiency; crossing was however improved of ~30% by promoting nanoparticle targeting
509 to BBB cells thanks to surface functionalization with anti-TfR Ab. These results are in line with
510 observations of Wohlfart *et al.*, that reviewed various nanoparticles adopted for the delivery of
511 different drugs into the brain and reported as most of the successfully ones are characterized by a
512 size ranging from 150 to 300 nm.⁵⁰ Moreover, nanoparticles of 300 nm size are still small
513 enough to passively cross the large fenestrations of the tumor-associated vessels developed
514 during aberrant angiogenesis.^{11,51} Indeed, the cutoff size of porous blood vessels in most of
515 tumors is 380-780 nm, and 400 nm size nanoparticles are known to efficiently accumulate in the
516 brain tumors.⁵² These considerations are extremely important in view of exploiting piezoelectric
517 BTNPs for *in vivo* and preclinical studies, especially considering the potential impact of these
518 nanomaterials in nanomedicine, not only for brain cancer treatment, yet also for the non-invasive
519 electric deep brain treatment of different neurodegenerative pathologies that are characterized by
520 a fenestrated vasculature, such as Parkinson's and Alzheimer's diseases.⁵³

521 The higher levels of AbBTNPs associated to plasma membranes and internalized in cell body
522 with respect to BTNPs confirm the efficacy of the dual targeting strategy mediated by the
523 antibody against TfR, a receptor highly expressed by the endothelial cells of the
524 neurovasculature⁵⁴ and by different cancer cells (*i.e.*, glioma, lymphoma, leukemia, breast, lung,

525 bladder).^{55,56} In agreement with our observations, Cui *et al.* and Chang *et al.* exploited TfR
526 targeting to promote the targeting of poly(lactic-co-glycolic acid) (PLGA) nanoparticles to
527 glioblastoma cells, both *in vitro* and *in vivo*. In these cited works, PLGA nanoparticles were
528 functionalized with Tf. However, recent researches reported a decrease of specificity of Tf-
529 functionalized nanosystems in biological environment due to the high levels of endogenous free
530 Tf.⁵⁷ Therefore, following an approach adopted also by other groups,^{58,59} we performed
531 nanoparticle functionalization with anti-TfR Ab, that does not compete with endogenous Tf for
532 TfR binding. The cell-targeting efficiency of our nanopatform was investigated by exploiting
533 different imaging techniques, as SEM/EDX, TEM, CLSM and SHG, the last of which represents
534 an advanced imaging technique allowing detecting the crystal asymmetry of BTNP tetragonal
535 lattice. Taking advantage of these imaging approaches, nanomaterial was detected in biological
536 samples without the need of any kind of surface modification (*e.g.*, with fluorophore
537 functionalization or quantum-dot decoration) that can potentially interfere with nanomaterial-cell
538 interaction and with its internalization fate. In this regard, thanks to their peculiar optical
539 properties, non-centrosymmetric BTNPs display a potential impact for cancer theranostics.³¹

540 Concerning piezoelectric stimulation, AbBTNP+US treatment resulted able to affect the
541 proliferation of different types of cancer cells, thus suggesting a high versatility of this anticancer
542 approach. Particularly, we observed a remarkable decrease of proliferative U-87 cells after 4 days
543 of chronic piezo-stimulation (from $86.8 \pm 1.9\%$ of Ki-67⁺ nuclei, observed in control cultures, to
544 $28.7 \pm 2.5\%$ of Ki-67⁺ nuclei, when stimulating with 100 $\mu\text{g/ml}$ AbBTNPs+US); the decrease in
545 U-87 cell proliferation in response to piezoelectric stimulation was even more pronounced with
546 respect to that observed on SK-BR-3 cells (from $80 \pm 8\%$ of Ki-67⁺ nuclei observed in control
547 cultures to $56 \pm 13\%$ of Ki-67⁺ nuclei when stimulating with 100 $\mu\text{g/ml}$ AbBTNPs+US).

548 Moreover, the anti-proliferative effects of piezoelectric stimulation resulted preserved, albeit to a
549 lesser extent, when reducing nanoparticle concentration to 10 $\mu\text{g/ml}$ (corresponding to the
550 concentration of nanoparticles that crossed the BBB model after 72 h). No significant increases
551 of apoptotic glioblastoma cells were observed when treating cells only with the piezoelectric
552 stimulation. Instead, the piezo-stimulation approach, when combined with sub-toxic TMZ
553 treatment, was able to significantly increase the percentage of apoptotic cells of about 25% and
554 to further reduce the proliferation rate of the cells with respect to the piezo-stimulation alone.
555 These results demonstrated as the nanoparticle-assisted remote piezoelectric stimulation
556 increases the sensitivity of glioblastoma cells to TMZ treatment. The synergic attack (chemical,
557 thanks to the chemotherapy drug, and physical, thanks to the remote electric stimulation)
558 remarkably reduced the cell number and the metabolic activity of glioblastoma cultures. The
559 remote piezo-stimulation has the potential to improve the therapeutic success by overcoming the
560 main obstacles for brain tumor treatment indicated in the Introduction, and will be tested in more
561 complex *in vivo* models and in preclinical studies. Particularly intriguing is the future perspective
562 to target also small microscopic foci of the GBM, that are the main cause of the recurrence of the
563 disease. A further point worth of investigation will be the analysis of the effects of nanoparticle
564 size / morphology on anticancer effects; indeed, the size / shape also affect the values of
565 piezoelectric and dielectric susceptibility coefficients,⁶⁰ thus not allowing to easily and
566 independently control the nanomaterial morphology and its piezoelectric behavior.

567 Summarizing, the main novelties of this research consist in the preparation of piezoelectric
568 nanoceramics able to cross a BBB model, to target glioblastoma cells, and to provide remote
569 electric stimulations for increasing GBM sensitivity to TMZ-based chemotherapy; on the other
570 hand, it is also necessary to underline the limits characterizing the present work, where *in vitro*

571 models of BBB and GBM were adopted, thus highlighting once more the needing for future *in*
572 *vivo* experiments.

573 CONCLUSIONS

574 We presented for the first time the preparation of functionalized piezoelectric BTNPs for BBB-
575 crossing, active cancer cell targeting, imaging, and remote US-driven electric treatment.

576 Moreover, we demonstrated the versatility of this nanotechnological approach, that allows the
577 successful delivery of antiproliferative stimuli to glioblastoma cells. Furthermore, the chronic
578 piezoelectric stimulation, in synergic combination with a sub-toxic concentration of TMZ,
579 induced an increased sensitivity to chemotherapy treatment and remarkable anticancer effects.

580 All together, these findings open new interesting perspectives in nanomedicine, with a
581 potential positive impact for the remote therapy of brain cancer and neurodegenerative
582 conditions. Future works will be focused on investigating the efficacy of nanoparticle-assisted
583 piezo-stimulation in xenograft models, in order to explore the realistic translation of these
584 nanomaterials in the future clinical practice. The possibility to fabricate piezoelectric BTNPs
585 with different size and higher piezoelectric coefficient by maintaining the same level of
586 biocompatibility will be assessed, and the effects of nanoparticle morphology on BBB crossing
587 and on piezo-stimulation efficiency will be evaluated. Moreover, the anticancer performances of
588 remote piezo-stimulation approach will be tested in combination with TMZ for the treatment of
589 TMZ-resistant glioblastoma cells, analyzing the molecular mechanisms at the base of TMZ
590 resistance and sensitivity. Finally, the combination of piezo-stimulation with different anticancer
591 drugs, radiotherapy and hyperthermia is envisaged in order to develop an efficient anticancer
592 protocol for pre-clinical studies.

593

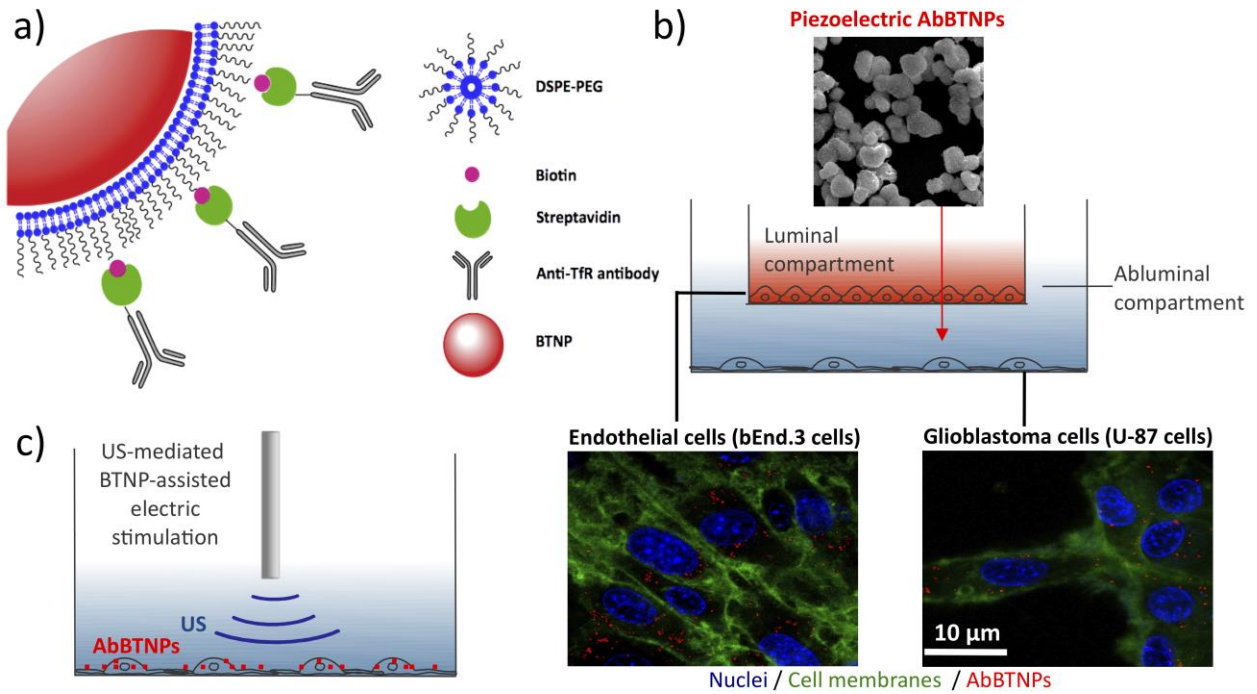
594 AUTHOR STATEMENT OF CONTRIBUTIONS

595 A.M. performed the nanomaterial functionalization, the piezoelectric stimulation experiments
596 and the Ca²⁺ imaging and contributed to write the manuscript. E.A. carried out the tests
597 concerning the BBB crossing, and performed immunochemistry and statistical analysis. S.M.
598 performed viability studies and CLSM imaging. C.T. carried out nanomaterial characterization
599 (analysis of nanoparticle size, stability and BCA assay to evaluate the efficiency of nanomaterial
600 functionalization). M.B. developed and characterized the BBB model (TEER analysis, ZO-1
601 expression and Coomassie® Brilliant Blue Staining). V.C. performed TEM analysis. F.S.P. and
602 R.C. supervised the SHG acquisition. R.C. and M.M. built the SHG setup. M.M. and G.d.V.
603 performed the SHG acquisition and analyzed SHG data. G.C. supervised and planned the whole
604 work and contributed to write the manuscript. All authors have given approval to the final
605 version of the manuscript.

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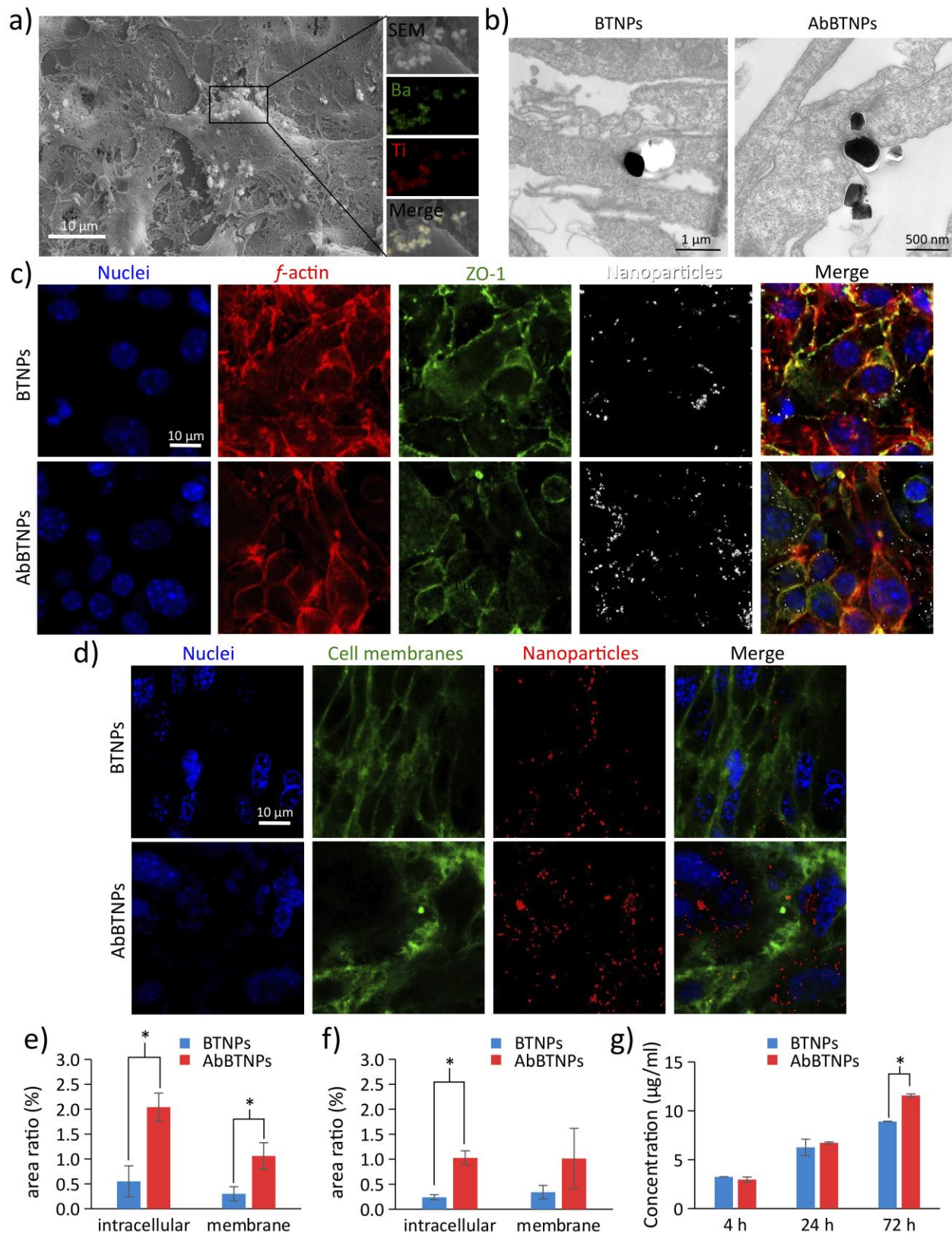
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608 Union's Horizon 2020 research and innovation program (grant agreement N°709613, SLaMM).

609



610 **Figure 1.** Experimental scheme of a) BTNP functionalization with antibody against transferrin
 611 receptor (TfR), b) nanoparticle crossing through a static 2D model of the BBB (nuclei in blue,
 612 cell membranes in green and AbBTNPs in red), and c) chronic piezoelectric stimulation of
 613 glioblastoma cells.

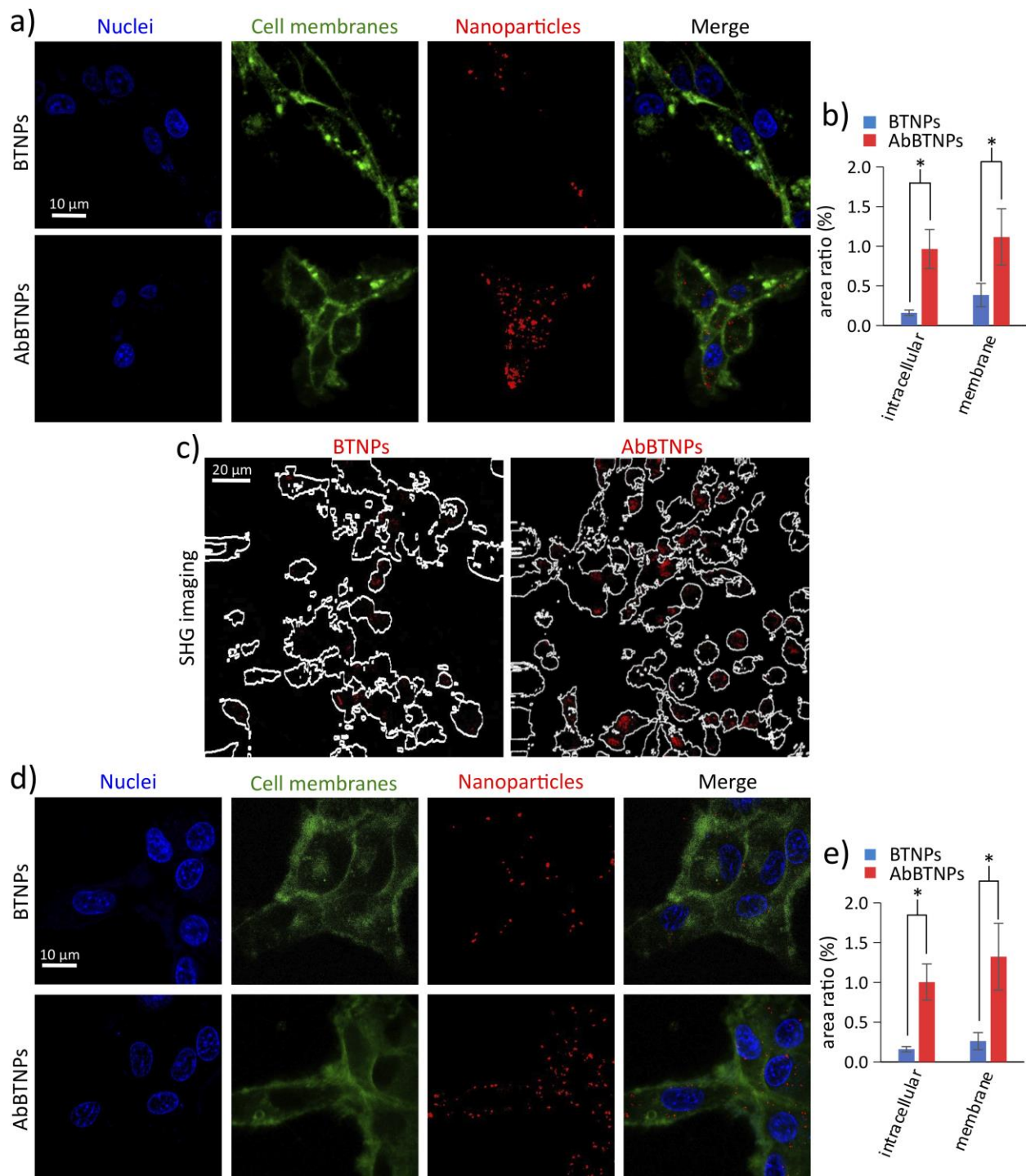
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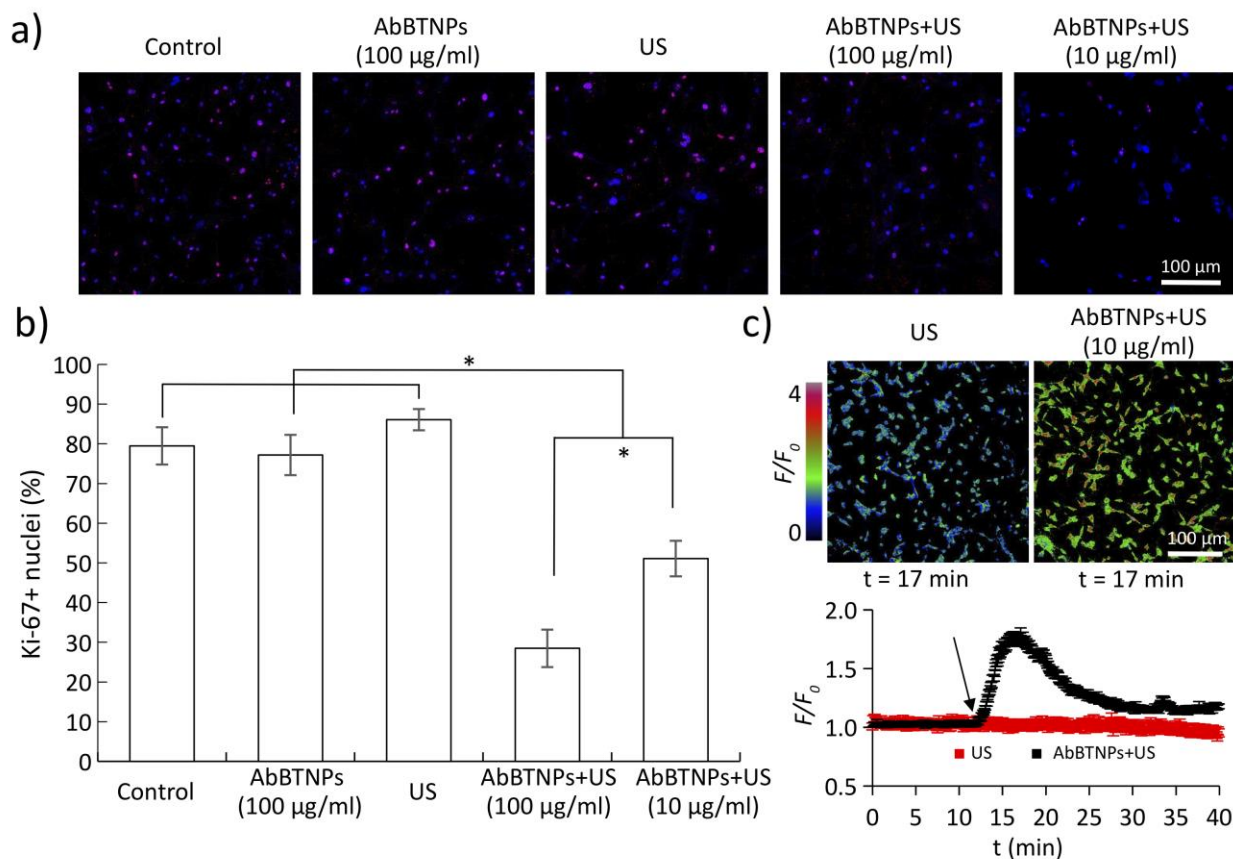
615 **Figure 2.** Analysis of BTNPs and AbBTNPs interaction with bEnd.3 cells and assessment of the
616 BBB model crossing. a) SEM imaging and EDX analysis of BTNPs associated to the plasma

617 membranes of bEnd.3 cells (Ba in green and Ti in red). b) TEM image highlighting a higher
618 amount of AbBTNPs associated to plasma membranes and up-taken by bEnd.3 cells with respect
619 to the non-functionalized BTNPs. c) CLSM of immunofluorescence staining of bEnd.3 cells
620 against the ZO-1 marker after 72 h of BTNP / ABTNP treatment (nuclei in blue, f-actin in red,
621 ZO-1 in green and nanoparticles in white). d) CLSM imaging of bEnd.3 plasma membranes (in
622 green), nanoparticles (in red) and nuclei (in blue), after 72 h of nanoparticle treatment. e-f)
623 Histograms reporting intracellular and cell membrane areas (%) co-localizing with BTNPs /
624 AbBTNPs after 24 and 72 h of nanoparticle incubation, respectively. g) Concentrations of
625 BTNPs / AbBTNPs measured in the abluminal compartment after BBB crossing at different time
626 points (4, 24 and 72 h). * $p < 0.05$.

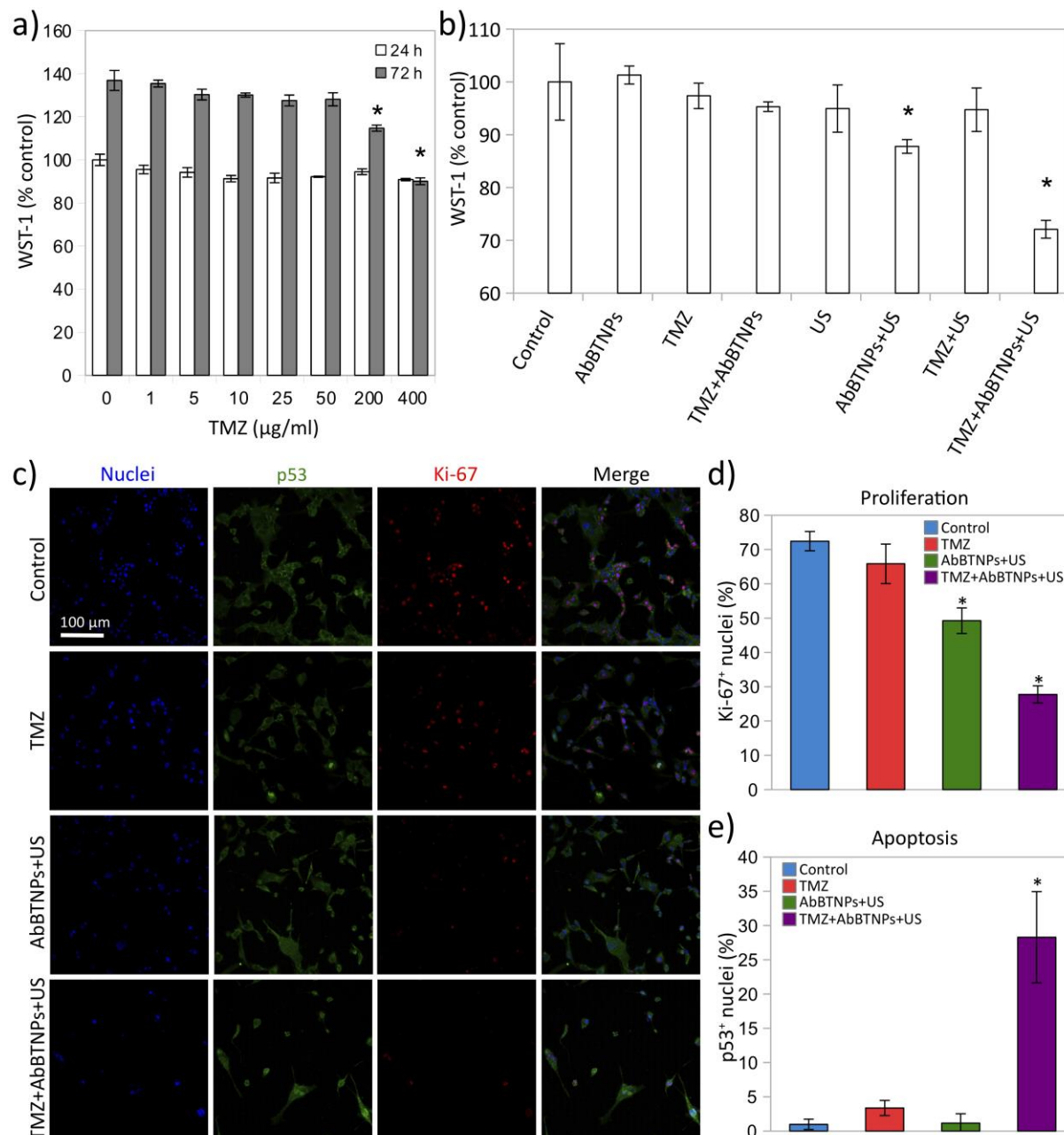
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628 **Figure 3.** BTNP / AbBTNP targeting to glioblastoma cells. a-c) U-87 cells incubated for 24 h
 629 with 100 $\mu\text{g}/\text{ml}$ BTNPs or AbBTNPs. a) CLSM imaging (plasma membranes in green,
 630 nanoparticles in red and nuclei in blue), b) histogram of nanoparticle localization, and c) SHG
 631 signal from nanoparticles (in red) overlaid on the outlines of the cells generated from the CARS
 632 images. d-e) CLSM analysis of U-87 cells exposed to nanoparticles after BBB model crossing, d)
 633 representative CLSM images of U-87 cells cultured in the abluminal compartment after 72 h of
 634 BTNP or AbBTNP treatment in the luminal compartment; e) quantitative analysis of experiment
 635 depicted in d). * $p < 0.05$.



636 **Figure 4.** Inhibitory effects of chronic piezoelectric stimulation on U-87 proliferation by testing
 637 different AbBTNP concentrations (100 µg/ml and of 10 µg/ml, the latter corresponding to the
 638 concentration of nanoparticles crossing the BBB model after a 72 h treatment). a) CLSM
 639 analysis of Ki-67 proliferation marker on control cultures, AbBTNPs-treated cells, US-stimulated
 640 cells, and on AbBTNPs+US treated cultures. b) Histogram reporting Ki-67⁺ nuclei (%). c) Time-
 641 lapse Ca^{2+} imaging in response to plain US and to AbBTNPs+US (10 µg/ml). Images at the top
 642 show F/F_0 signal of cells after 5 min of US (top left) and AbBTNP+US (top right) stimulations.
 643 At the bottom, the graph reports F/F_0 traces of cultures stimulated with US (in red) and with
 644 US+AbBTNP (in black). * $p < 0.05$.



645 **Figure 5.** Nanoparticle-assisted piezoelectric stimulation (AbBTNPs+US) improves anticancer
 646 efficacy of temozolomide (TMZ). a) WST-1 assay on U-87 cells incubated for 24 h and 72 h with
 647 different concentrations of drug (0-400 $\mu\text{g/ml}$; data are normalized and expressed as percentage
 648 of WST-1 absorbance values measured at 24 h on control cultures). b) WST-1 assay respectively
 649 performed on control cultures, cultures incubated with 10 $\mu\text{g/ml}$ AbBTNPs, cultures incubated
 650 with 50 $\mu\text{g/ml}$ TMZ, cultures incubated with 50 $\mu\text{g/ml}$ TMZ and 10 $\mu\text{g/ml}$ AbBTNPs, cultures
 651 chronically stimulated with US, cultures stimulated with US in the presence of 10 $\mu\text{g/ml}$
 652 AbBTNPs, cultures stimulated with US in the presence of 50 $\mu\text{g/ml}$ TMZ, and, finally, cultures
 653 stimulated with US in the presence of 10 $\mu\text{g/ml}$ AbBTNPs and of 50 $\mu\text{g/ml}$ TMZ. c) CLSM
 654 imaging of Ki-67 and p53 expression in the different experimental conditions. The histograms
 655 reporting the Ki-67⁺ and p53⁺ nuclei are respectively showed in d) and e). * $p < 0.05$.

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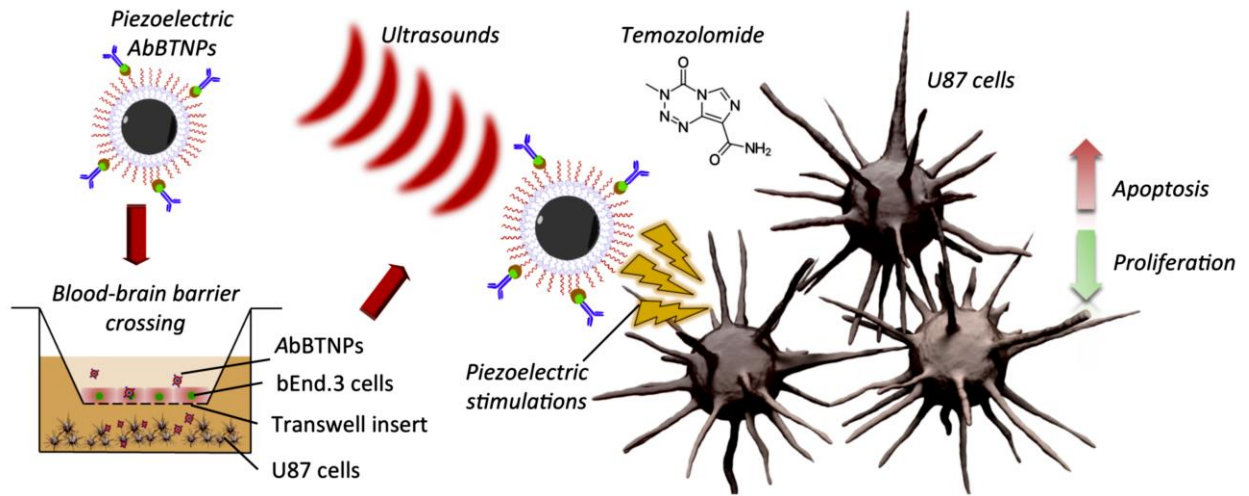
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