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1 **In vitro interaction and biocompatibility of titanate nanotubes with**  
2 **microglial cells**

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15 **Abstract**

16 Titanate nanotubes (TiONts) are promising agents for biomedical applications. Microglial  
17 activation and associated oxidative burst are major challenges in drug delivery applications  
18 across the brain. Here, TiONts were designed for drug delivery systems by functionalizing them  
19 with (3-aminopropyl) triethoxysilane (APTES), their interactions and biocompatibility were  
20 studied *in vitro* using murine microglial BV-2 cells. TiONts-APTES exposure resulted in  
21 increased ROS production and transient mitochondrial hyperpolarization. However, there was

22 no indication of microglial proliferation in BV-2 cells as suggested by cell cycle analysis and  
23 cell count. The internalization process of TiONts-APTES into cells by endocytosis vesicles and  
24 passive diffusion were proved by transmission electron microscopy (TEM) with and without  
25 amiloride, the endocytosis inhibiting agent. In addition, the TiONts-APTES exhibited good  
26 biocompatibility on microglial BV-2 cells as revealed by the morphology and viability analysis.

27 **Keywords:** Titanate nanotubes; reactive oxygen species; microglial activation;  
28 mitochondrial hyperpolarisation; lysosomal integrity; apoptosis

## 29 **Introduction**

30 The central nervous system (CNS), comprising the brain and the spinal cord, is a  
31 highly protected organ, owing to the crucial functions it performs. Due to this fact,  
32 biomedical interventions in the brain are hard to achieve. Nanoparticles being very  
33 small in size can access otherwise protected sites in the body including blood brain  
34 barrier and placenta (Kessler, 2011). With the advancement of nanotechnology, many  
35 nanoparticles have been suggested as diagnostic and therapeutic modalities for  
36 neurobiological applications. Titanate nanotubes (TiONts) are one such particle with a  
37 needle-shaped morphology and surface chemistry (with hydroxides) which permit  
38 further functionalization (Papa *et al.*, 2015). Since their discovery by Kasuga *et al.*  
39 (1998), TiONts have been studied for a variety of applications (Bavykin and Walsh,  
40 2010). Recently, applications of TiONts have turned to the biomedical field (Kijima,  
41 2010) including dopamine detection (Niu *et al.*, 2008), DNA transfection (Papa *et al.*,  
42 2013), bioimaging (Papa *et al.*, 2011), drug delivery (Loiseau *et al.*, 2017), and cancer  
43 cell radiosensitization (Mirjolet *et al.*, 2013, Mirjolet *et al.*, 2017).

44 TiONts have been proposed for glioma therapy (Mirjolet *et al.*, 2013) in addition  
45 to drug delivery and imaging applications (Boudon *et al.*, 2014). Moreover, it is

46 reported that TiONts can be used for the detection of neurotransmitters in the brain (Niu  
47 *et al.*, 2008). Though many neurobiological applications have been proposed for  
48 TiONts, only a little information is available on the biocompatibility and cellular  
49 interaction of these nanoparticles (Rihane *et al.*, 2016, Sallem *et al.*, 2017).

50 One of the main hurdles in the neuro-bio applications of nanoparticles includes  
51 nanoparticle-mediated microglial activation. Microglia are major immune cells of the  
52 brain which are involved in the scavenger functions and cytokine secretion. The  
53 activated microglia can induce oxidative stress in the brain which leads to further  
54 complications. In a recent study (Bussy *et al.*, 2015), it was reported that the carbon  
55 nanotubes toxicity mainly comes from the microglial activation and oxidative stress.

56 In the present study, TiONts were synthesized by hydrothermal synthesis and  
57 functionalized with APTES ((3-aminopropyl) triethoxysilane) for drug delivery  
58 applications. APTES functionalization provides TiONts with additional amine functions  
59 for drug molecule conjugation, making them better drug carriers. Moreover, APTES-  
60 functionalized TiONts exhibit better water dispersion and colloidal stability than bare  
61 TiONts. The interaction and biocompatibility of fully characterized TiONts-APTES  
62 with microglial cells were studied using microglial cells. BV-2 cell line was selected  
63 because it is derived from normal murine microglia and has phenotypic features similar  
64 to that of normal microglia (Blasi *et al.*, 1990). The toxicological responses including  
65 morphological alteration, reactive oxygen species (ROS) production, cell cycle  
66 alteration and cell death were studied using different bio-assays.

## 67 **Methodology**

### 68 *Particle synthesis and characterization*

#### 69 *Titanate nanotubes (TiONts) synthesis:*

70 TiONts were prepared by a classical hydrothermal method in a basic medium  
71 (Papa *et al.*, 2009). TiO<sub>2</sub> rutile precursor powder (1 g) was added to a solution of NaOH  
72 (10 M, 250 mL). The mixture was ultrasonicated (30 min, 375 W, Sonics Vibra-Cells)  
73 and then transferred into a Teflon reactor with mechanical stirring and heating at 155 °C  
74 for 36 h. After cooling, the precipitate was centrifuged (24,000×g for 10 min), dialyzed  
75 four days (Cellu-Sep® tubular membranes of 12-14 kDa) and washed by ultrafiltration  
76 (regenerated cellulose membranes with a MWCO of 100 kDa) with deionized water  
77 until the conductivity reached 2 μS cm<sup>-1</sup>. Finally, the TiONts were freeze dried.

#### 78 *Functionalization of TiONts with a stabilizing electrostatic agent (APTES):*

79 Silane coupling agents present high reactivity with hydroxyl groups on the  
80 surface of metal oxide materials. Therefore, TiONts were modified with APTES (in  
81 large excess) *via* hydrolysis and condensation in a solution of water and ethanol (50:50  
82 V:V) under magnetic stirring for 24 h to yield TiONts-APTES nanohybrids (Loiseau *et*  
83 *al.*, 2017). After the reaction, a part of the solvent was removed with a rotary evaporator  
84 (80 °C; 250 mbar) then the resulting suspension was ultrafiltered (100 kDa) to eliminate  
85 the unreacted APTES. Finally, TiONts-APTES were freeze-dried.

#### 86 *Nanoparticles characterization:*

87 The TEM (Transmission Electron Microscopy) used was a JEOL JEM 2100

88 LaB6 microscope with an acceleration voltage of 200 kV and equipped with a high tilt  
89 pole-piece achieving a point-to-point resolution of 0.25 nm to study nanotube  
90 morphology and their agglomeration state. The samples were prepared by dropping a  
91 dilute suspension of powders on carbon-coated copper grids.

92 ThermoGravimetric Analysis (TGA, TA instrument, Discovery TGA) was used  
93 to determine the amount of grafted molecules on the surface of TiONts: water  
94 desorption and organic decomposition. The measurements were carried out under air  
95 atmosphere ( $25 \text{ mL min}^{-1}$ ) with a heating rate of  $10 \text{ }^\circ\text{C min}^{-1}$  from  $50^\circ\text{C}$  to  $800^\circ\text{C}$ .

96 Specific surface area measurements were performed with a Micromeritics  
97 Tristar II apparatus and were calculated with the BET (Brunauer Emmett Teller)  
98 method ( $S_{\text{BET}}$ ) from  $\text{N}_2$  gas adsorption. Samples were first outgassed *in situ* under a  
99 pressure of 20 mTorr at  $100^\circ\text{C}$  (16 h) before  $\text{N}_2$  adsorption/desorption cycles.

100 Zeta potentials ( $\zeta$ -potentials) were measured with a Malvern Nano ZS  
101 instrument supplied by DTS Nano V7.11 software. The pH of the suspensions was  
102 adjusted from 3 to 11 by adding HCl or NaOH solutions. The Smoluchowski equation  
103 was used for zetametry measurements. For each measurement, powders were dispersed  
104 in 12 mL of aqueous NaCl solution (the final concentration of NaCl is  $10^{-2} \text{ M}$ ). pH  
105 titrations were performed using aqueous solutions of HCl (0.1 M), NaOH (0.1 M), or  
106 NaOH (0.01 M).

107 UV-visible absorbance measurements were carried out using Shimadzu UV-  
108 2550 at 600 nm. Stability measurements were made in PBS 0.1 M at  $\text{pH} = 7.4$  and  $T =$   
109  $25^\circ\text{C}$  (one measurement/5 min) during 120 min to analyze the colloidal stability.

110 The equipment of Fourier-Transform InfraRed spectroscopy (FT-IR) was a  
111 Bruker Vertex 70v using OPUS version 3.1. FT-IR spectra were obtained from

112 transmission measurements of thin pellets which consist of 2 mg of nanohybrids powder  
113 finely ground with dried KBr (198 mg) before being pressed into a pellet.

114 X-ray Photoelectron Spectroscopy (XPS) analyses were recorded by a PHI 5000  
115 Versaprobe apparatus with a monochromatic Al  $K_{\alpha 1}$  X-ray source (energy of 1486.7 eV  
116 with a 200  $\mu\text{m}$  spot size, accelerating voltage of 12 kV, and power of 200 W). The  
117 resolution was 2.0 eV for global spectra and 1.3 eV for windows corresponding to  
118 selected lines. The powders were deposited on an indium sheet then pressed.  
119 Photoemission peak areas were calculated after subtracting background using a Shirley  
120 routine. The titanium 2p peak (458.7 eV) was used as reference and allowed the  
121 correction of charge effects. Gauss (70%)–Lorentz (30%) profiles were used and data  
122 were analyzed with CasaXPS software for curve fitting and MultiPak software for  
123 quantitative analysis.

#### 124 ***Cell culture and nanoparticles treatment***

125 The BV-2 cells were cultured in RPMI medium with 10% heat inactivated fetal  
126 calf serum (FCS) and antibiotic/antimycotic solution as previously described (Nury *et*  
127 *al.*, 2017). For the nano-interaction studies, cells were seeded in appropriate seeding  
128 density and cultured overnight. The cells were exposed to TiONts-APTES for different  
129 times (6 and 24 h) at concentrations of 1, 5, 10, 20, 40 and 80  $\mu\text{g}/\text{mL}$  by diluting the  
130 stock solution (4 mg/mL).

#### 131 ***Cytotoxicity and dose response: MTT assay***

132 Cell viability was assessed as a function of mitochondrial activity using MTT (3-  
133 (4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded  
134 in 12 well plates at an initial seeding density of  $5 \times 10^4$  and incubated overnight at

135 37°C, 5% CO<sub>2</sub>. After TiONts-APTES exposure, cells were harvested by centrifugation  
136 at 200×g for 5 min and then incubated with MTT (0.05 mg/mL) for 3 h in the dark. The  
137 formazan crystals formed were solubilized using 200 µL DMSO and absorbance was  
138 read at 540 nm. Final results are obtained by subtracting the absorbance of control wells  
139 (without cells) from the absorbance of treated samples in order to avoid nanoparticles  
140 interference in MTT results.

#### 141 ***Cell morphology: Phase contrast microscopy***

142 Cells were seeded in 12-well plates at an initial seeding density of  
143  $5 \times 10^4$  cells/well. Cells were exposed to TiONts-APTES for 24 h. Cellular morphology  
144 was assessed under a phase contrast microscope (Axiovert 40 CFL, Zeiss). Digital  
145 images were taken with an AxioCam ICm1 camera (Zeiss).

#### 146 ***Particle uptake:***

#### 147 ***Flow cytometry***

148 Uptake of TiONts-APTES by microglia was analyzed using flow cytometry  
149 following the method of Suzuki *et al.* (2007). Particle uptake by cells leads to an  
150 increase in granularity which is revealed by an increase in the side-scattered light  
151 (SSC), while the forward-scattered light (FSC) remains the same. In this experiment,  
152 cells were seeded in 6-well plates at an initial density of  $1 \times 10^5$  and kept overnight for  
153 incubation. Cells were then exposed to 5, 20, and 80 µg/mL of TiONts-APTES for 6 h.  
154 Cells, harvested by centrifugation, were resuspended in 500 µL PBS and analyzed by  
155 flow cytometry using a Dako/Partec/GALAXY flow cytometer (Partec, Münster,  
156 Germany) equipped with a solid blue laser (50 mW) and controlled by 'FloMax'  
157 software (version 2.4; Partec).

158

159

160 ***Transmission electron microscopy (TEM):***

161           The mechanism of nanoparticles uptake by BV-2 cells was studied using TEM.  
162   The cells were seeded at an initial density of  $1.175 \times 10^5$ /mL and incubated overnight.  
163   Cells were subsequently exposed to 20  $\mu$ g/mL of TiONts-APTES for 24 h without and  
164   with 100  $\mu$ M amiloride, a macropinocytosis inhibitor (added 1h prior to nanoparticles  
165   exposure). At the end of treatment, cells were harvested and fixed for 1 h at 4 °C in 4%  
166   paraformaldehyde and 2.5% of glutaraldehyde in Sorensen phosphate buffer (0.1 mM,  
167   pH 7.3). After fixation, samples were washed by Sorensen phosphate buffer. The post-  
168   fixation treatment was realized with 1% osmium tetroxide at room temperature for 1h.  
169   Dehydration and resin impregnation of the samples were performed with a Leica EM  
170   AMW automatic microwave tissue processor: dehydration was done by increasing  
171   degrees of ethyl alcohol (50°, 70°, 95°, 100°), the substitution of ethanol by resin was  
172   progressively done by three absolute ethanol/Embed-812 resin mixtures ( $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{3}$   
173   ethanol), followed by two impregnations in pure Embed-812 resin. The polymerization  
174   of samples was performed with a mixture Embed-812/3% benzyldimethylamine  
175   (BDMA, polymerisation catalyst) in gelatin capsule maintained for 48 h at 60 °C.  
176   Blocks were cut on a Reichert ultramicrotome and slices (thickness of 70 nm) were  
177   deposited on copper and palladium grid. After drying, grids were contrasted with uranyl  
178   acetate and lead citrate. TEM observations of cells were realized on a HITACHI H-  
179   7500 operating at 80 kV. For each test carried out, one grid per sample was observed,  
180   which corresponds to a total of 30 to 50 cells. The photos presented are representative  
181   of the cells observed by samples.

182 ***Reactive oxygen species analysis***

183 *Superoxide radicals: DHE probe*

184           Superoxide anions production in presence of TiONts-APTES was measured  
185 using a cell permeable molecule, dihydroethidium (DHE; Life Technologies, St Aubin,  
186 France). DHE, mainly upon reaction with superoxide anions ( $O_2^{\bullet-}$ ), forms a red  
187 fluorescent product, 2-hydroxyethidium, which intercalates with DNA. The cells were  
188 seeded in 12-well plates at a density of  $5 \times 10^4$  cells/well and kept overnight in the  $CO_2$   
189 incubator. After TiONts-APTES exposure, the cells were harvested by centrifugation.  
190 The pellets were resuspended in 1 mL PBS containing 1.6 mM DHE and incubated for  
191 15 min at  $37^\circ C$ . The cells were analyzed by Dako/Partec/GALAXY flow cytometer  
192 (Partec) through a 590/10 nm band pass filter.

193 *Peroxide radicals: DHR123 probe*

194           Hydrogen peroxide production in the presence of TiONts-APTES was estimated  
195 using dihydrorhodamine 123 (DHR123, Sigma-Aldrich, USA). DHR 123 is an  
196 uncharged and non-fluorescent molecule that can passively diffuse across plasma  
197 membranes where it is oxidized to cationic rhodamine 123 by hydrogen peroxide  
198 ( $H_2O_2$ ) to give green fluorescence. The cells were seeded on 24 well plates at an initial  
199 density of  $2 \times 10^4$  cells/well and exposed to 5, 20, 40 and 80  $\mu g/mL$  of TiONts-APTES  
200 for 6 and 24 h. The cells were harvested after centrifugation and resuspended in 1 mL  
201 PBS. 2mM DHR123 were added to each tube at a final concentration of 6  $\mu M$  and  
202 incubated for 15 min at  $37^\circ C$ . Fluorescence were measured using  
203 Dako/Partec/GALAXY flow cytometer (Partec) with a 520/20 nm band pass filter.

204 ***Transmembrane mitochondrial potential ( $\Delta\Psi_m$ ): DiOC<sub>6</sub>(3) probe***

205           Alteration in transmembrane mitochondrial potential ( $\Delta\Psi_m$ ) was analyzed using  
206 a fluorescent probe: 3,3'-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>(3) (Life Technologies,  
207 Molecular Probes). DiOC<sub>6</sub>(3) is a cell-permeant, green-fluorescent, lipophilic dye,  
208 which is selective for the mitochondria of live cells when used at a nanomolar  
209 concentration. DiOC<sub>6</sub>(3) allows measuring  $\Delta\Psi_m$ . The cells were seeded at an initial  
210 density of  $2 \times 10^4$  cells/well of 24 well plates and incubated overnight. They were  
211 exposed to TiONts-APTES of concentration 1, 10, 20, 40 and 80  $\mu\text{g/mL}$  for 6 h and  
212 24 h. Cells incubated with 4% paraformaldehyde (in DPBS for 10 min at 37 °C) were  
213 used as positive control. The cells washed in PBS were incubated with DiOC<sub>6</sub>(3)  
214 (0.1  $\mu\text{M}$  in PBS) for 15 min at 37°C. The flow cytometry analysis was done using the  
215 DAKO/GALAXY flow cytometer (Partec, Germany) with a 520/20 nm band pass filter.

216 ***Lysosomal membrane integrity: Acridine orange staining***

217           Lysosomal destabilization in presence of nanoparticles was analyzed using  
218 acridine orange (AO). AO is a lipophilic, cationic and metachromatic fluorochrome  
219 capable of permeating cells and organelle membrane structure. Under a blue light  
220 excitation, the dye differentially stains cytoplasm and acid vesicles (Pierzyńska-Mach *et*  
221 *al.*, 2014). In normal cells, the dye gets protonated inside acidic vesicles and emits  
222 orange fluorescence whereas the cytoplasm remains green in color. When the cells have  
223 altered lysosomes, leading to an alteration of the proton pump, the resulting  
224 alkalisation of the lysosomes is associated with a green/yellow fluorescence of these  
225 vesicles. The cells, seeded at an initial density of  $2 \times 10^4$  cells/well of 24 well plates,  
226 were exposed to TiONts-APTES (5, 20, 40, and 80  $\mu\text{g/mL}$ ) for 6 h and 24 h. The cells,  
227 harvested by direct pipetting, were washed with PBS and incubated in 1 mL AO

228 solution (2  $\mu\text{g/mL}$  in PBS) for 15 min at 37°C. The analysis was carried out using a  
229 Dako/Partec/ GALAXY flow cytometer (Partec) with a 590/10 nm band pass filter.

### 230 ***Plasma membrane integrity: Propidium Iodide (PI) staining***

231 The plasma membrane integrity on TiONts-APTES exposure was analyzed by  
232 Propidium Iodide (PI) staining. The principle underlying the PI staining is dye exclusion  
233 by living cells (Lizard *et al.*, 1995). Cells seeded at an initial density of  $2 \times 10^4$   
234 cells/well of 24 well plates were exposed to TiONts-APTES (5, 20, 40, and 80  $\mu\text{g/mL}$ )  
235 for 6 and 24 h. Harvested cells were washed and stained with PI (1  $\mu\text{g/mL}$ ) for 15 min  
236 at 37°C. The flow cytometry analysis was done using Dako/Partec/GALAXY flow  
237 cytometer (Partec) with a 630 nm long pass filter.

### 238 ***Poly(ADP-ribose) polymerase (PARP) cleavage assay***

239 The cells were seeded at an initial density of  $2 \times 10^4$  cells/well of 24 well plates  
240 and incubated overnight. The cells were exposed to 20  $\mu\text{g/mL}$  and 80  $\mu\text{g/mL}$  of TiONts-  
241 APTES for 24 h. Untreated cells were used as control. After treatment cells were  
242 harvested and centrifuged at 200 $\times$ g, 4°C for 5 min.

### 243 ***Sample lysis and protein quantification***

244 The cell pellets were transferred to fresh tubes and washed with 1 mL cold PBS.  
245 After discarding supernatant, the pellets were incubated with 80  $\mu\text{L}$  of RIPA buffer  
246 containing 1 $\times$  anti-protease for 30 min on ice. The cell suspension was centrifuged at  
247 12,000 $\times$ g for 20 min at 4°C. Supernatant was collected in a fresh tube (this solution can  
248 be stored at -20°C for weeks and thawed at room temperature) and the pellet was  
249 discarded. The concentration of protein solution was measured by BCA (Bicinchoninic

250 acid assay) with 2% copper sulfate solution. 25  $\mu$ L of BSA was used as protein standard  
251 and concentration was calculated from the standard graph obtained.

#### 252 *Loading and running gel*

253 The isolated protein was loaded in 8% polyacrylamide gel at a concentration of  
254 70  $\mu$ g/well. Electrophoresis was carried out for 2h (30 min at 70 V followed by 1.5 h at  
255 120 V).

#### 256 *Gel to membrane transfer of protein*

257 The polyvinylidene difluoride (PVDF) membrane was activated with methanol  
258 for 1 min and rinsed with transfer buffer. Gel stack was prepared by sandwiching the  
259 gel and PVDF membrane between filter papers and sponges and keeping them in  
260 transfer buffer for 1 h.

#### 261 *Antibody revelation by chemiluminescence*

262 The protein attached membrane was blocked using 5% milk solution for 1 h at  
263 room temperature and washed 3 times with PBST (PBS and Tween 20, 0.1%) for 5 min  
264 each. It was transferred into a solution containing primary antibody (1:1,000) and  
265 incubated overnight at 4°C. Antibody for actin (1: 10,000) was used as a standard. The  
266 solution was washed 5 times with PBST (5 min each). The membrane was then  
267 incubated with secondary antibody (in 1% milk) for 1 h at 25°C and covered in a  
268 transparent plastic wrap after removing excess reagent. The image was acquired by  
269 chemiluminescence method.

## 270 *Cell cycle analysis: propidium iodide staining*

271 The changes in cell cycle patterns were analyzed by staining with propidium  
272 iodide (PI) (Marel *et al.*, 2008). In brief, the cells were seeded at an initial density of  
273  $2 \times 10^4$  cells/well on 24 well plates and exposed to TiONts-APTES (5, 20, 40, and  
274 80  $\mu\text{g/mL}$ ) for 24 h. Cells, harvested by direct pipetting, were washed with PBS. The  
275 pellets were fixed using 1 mL cold 80% ethanol (kept at  $-20^\circ\text{C}$ ), by vortexing, to avoid  
276 cell aggregation and kept at  $-20^\circ\text{C}$  for at least 2 h (cells can be kept in this condition for  
277 few days.). The cells were washed ( $350\times g$  for 5 min) and resuspended in 300  $\mu\text{L}$  PBS  
278 containing 80  $\mu\text{g/mL}$  PI and 200  $\mu\text{g/mL}$  RNase. After 1h incubation at  $37^\circ\text{C}$ , the  
279 solution was made up to 1 mL by adding 700  $\mu\text{L}$  PBS and analyzed using flow  
280 cytometry on a Dako/Partec/GALAXY flow cytometer (Partec) with a 630nm long pass  
281 filter.

282

## 283 *Statistical analysis*

284 All the experiments were done in triplicates and the values were expressed as  
285 mean  $\pm$  SD. Statistical comparison between the control and experimental values were  
286 done using one-way ANOVA test. In result, analyses with  $p < 0.05$  were considered as  
287 statistically significant.

## 288 **Results**

### 289 *Nanotubes synthesis, functionalization, and characterizations*

290 TEM images were used to highlight the synthesis of TiONts after the  
291 hydrothermal treatment. They present a spiral-shaped morphology, with internal cavity  
292 (Figure 1a,b) and a high specific surface area ( $S_{\text{BET}} = 174 \pm 1 \text{ m}^2 \text{ g}^{-1}$ ). They are ( $10 \pm 1$ )

293 nm in outer diameter and  $(170 \pm 50)$  nm in length.

294 The functionalization of TiONts with APTES (Figure 1e,f) seems to reduce  
295 nanoparticles agglomeration and improve their dispersion in comparison to bare TiONts  
296 (Figure 1c,d). The grafting ratio of APTES on the surface of TiONts was determined by  
297 TGA (Figure 2). The amount of hydroxyl groups on the surface of bare TiONts was  
298 calculated based on water weight loss from TGA curve. Indeed, the release of one  
299 molecule of chemisorbed water involves the condensation of two hydroxyl groups. As  
300 the weight loss below  $175^{\circ}\text{C}$  is due to physisorbed water (Sabzi *et al.*, 2009), only the  
301 weight loss related to chemisorbed water between  $175^{\circ}\text{C}$  and  $800^{\circ}\text{C}$  is taken into  
302 account for hydroxyl and grafting ratio estimation. Consequently, a greater weight loss  
303 is obtained after APTES grafting (Figure 2 and Table 1). The results show a hydroxyl  
304 density of about  $(10.6 \pm 1.5)$   $\text{OH}\cdot\text{nm}^{-2}$  and APTES grafting ratio of  $(7.3 \pm$   
305  $0.5)$   $\text{APTES}\cdot\text{nm}^{-2}$ . It should be noted that the specific surface area of TiONts decreases  
306 after their functionalization with APTES ( $S_{\text{BET}} = 147 \pm 2$   $\text{m}^2 \text{g}^{-1}$ ). APTES may form an  
307 envelope all around TiONts and hinder, at least partially, access to internal cavities.

308  $\zeta$ -potential measurements of TiONts-APTES indicated an isoelectric point (IEP)  
309 at pH 8.5 (Figure 3). This value is greater than that of bare TiONts (IEP at pH 3.3) due  
310 to the presence of amine functions (Paris *et al.*, 2015). Moreover, an inversion in  
311  $\zeta$ -potential sign is also observed at physiological pH, where it is positive *ca.* + 10 mV  
312 for APTES-functionalized TiONts and largely negative (*ca.* -25 mV) for bare TiONts.

313 X-ray Photoelectron Spectroscopy (XPS) analyses are realized to evaluate the  
314 chemical composition of the surface of TiONts-APTES. Besides to the chemical  
315 elements that are present in bare TiONts, some additional specific elements, such as  
316 nitrogen and silicon, are also found in TiONts-APTES (Table 2), thus proving the  
317 presence of APTES at the surface of nanotubes.

318 The FT-IR spectrum of TiONts-APTES proves the grafting of APTES molecule  
319 onto the surface of TiONts (Figure 4). APTES characteristic bands are located at  
320  $1000\text{ cm}^{-1}$  ( $\nu$  Si-O-Si),  $1100 - 1200\text{ cm}^{-1}$  ( $\nu$  C-N) and  $3250\text{ cm}^{-1}$  ( $\nu$  NH<sub>2</sub>).

### 321 ***Cytotoxicity and dose response: MTT assay***

322 After 6 h, the exposure to TiONts-APTES does not markedly alter cells viability,  
323 which is evaluated with the MTT assay by measuring the activity of succinate  
324 dehydrogenase, except for 80  $\mu\text{g/mL}$  treated wells. A statistically significant increase in  
325 absorbance (% control) is observed for 80  $\mu\text{g/mL}$  treated samples at 6h of exposure  
326 which subsided by 24h. The absorbance is significantly reduced in 20 ( $93.80 \pm 5.84\%$ ),  
327 40 ( $90.12 \pm 4.17\%$ ) and 80 ( $84.24 \pm 4.12\%$ )  $\mu\text{g/mL}$  TiONts-APTES treated wells at  
328 24 h of incubation (Figure 5a).

### 329 ***Cell morphology***

330 The cellular morphology in presence of TiONts-APTES is evaluated using a  
331 phase contrast microscopy. The treated cells do not show any alteration in their  
332 morphology like membrane rupture, swelling, blebbing and/or cytoplasmic  
333 disintegration which are morphological features usually associated to cytotoxic effects.  
334 The cells size and morphology are similar to those of the negative control cells (Figure  
335 6a).

### 336 ***Particle uptake***

337 TiONts-APTES uptake by BV-2 cells is analyzed from the SSC changes in flow  
338 cytometry. As shown in Figure 6b the TiONts-APTES exposed cells exhibit a dose-  
339 dependent increase in SSC (5  $\mu\text{g/mL}$ : 5.04% and 80  $\mu\text{g/mL}$ : 35%) compared to negative

340 (2.56%) and positive controls (12.5%) suggesting an internalization and/or an  
341 interaction of nanoparticles with the plasma membrane.

342 To define whether the nanoparticles were internalized and to determine by  
343 which mechanism – the passive (diffusion through the plasma membrane) or the active  
344 one (endocytose) – they can accumulate inside the cells, the nanoparticles uptake was  
345 studied by TEM. Cells are incubated with TiONts-APTES (20  $\mu\text{g/mL}$ , 24 h), without  
346 and with amiloride (100  $\mu\text{M}$ ), an endocytosis inhibiting molecule introduced in the  
347 culture medium 1 h before nanoparticles (Figure 7). In TiONts-APTES-treated cells, the  
348 presence of nanoparticles was revealed in large vesicles (average diameters of the  
349 vesicles:  $0.85 \pm 0.32 \mu\text{m}$ ) in all the observed cells (Figure 7b,b1,b2). However, when  
350 BV-2 cells were cultured with amiloride, only few cells with internalized nanoparticles  
351 (around 10 %) were found: this data supports TiONts-APTES internalization mainly by  
352 endocytosis (Figure 7c,c1). In these conditions, the interactions of nanoparticles with  
353 the plasma membrane are however observed, and their diffusion through the plasma  
354 membrane are detected (Figure 7c2,c3,c4). It is noteworthy that the size of the  
355 endocytic vesicles in amiloride-treated cells ( $0.50 \pm 0.16 \mu\text{m}$ ) are smaller than those in  
356 cells treated with nanoparticles only. Altogether, these data support that nanoparticles  
357 enter in cells mainly by endocytosis and show that a passive diffusion does exist. In the  
358 latter case, more individualized TiONts are observed into the cytoplasm and some of  
359 them are closer to nucleus.

360 On the other hand, a change in the mitochondrial behavior is observed in cells  
361 uptaking nanoparticles. Indeed, in the control cells, mitochondria are usually situated  
362 near to nucleus, in the majority of cells, however, in contact with TiONts-APTES, many  
363 mitochondria are located near the cellular membrane (Figure c2) or near the endocytic  
364 vesicles containing nanoparticles (Figure d,e). In addition, bigger mitochondria are

365 observed in nanoparticles-treated cells ( $0.94\pm 0.57 \mu\text{m}$ ) compared to control ( $0.64\pm 0.29$   
366  $\mu\text{m}$ ). This change in mitochondrial size could be induced by the presence of TiONts-  
367 APTES nanoparticles and may affect the mitochondrial function.

### 368 ***ROS overproduction***

#### 369 *Superoxide radicals*

370 Superoxide anion production in the presence of TiONts-APTES is studied using  
371 the probe DHE. The cells exhibited a concentration dependent increase in DHE  
372 fluorescence at 6h of incubation. However, at 24h of treatment the particular trend was  
373 not observed in the treated wells, where the percentage of cells exhibiting DHE  
374 fluorescence declined. No concentration dependent increase in DHE positive cells were  
375 observed at 24h except for  $80 \mu\text{g/mL}$  TiONts-APTES (Figure 8a).

#### 376 *Hydrogen Peroxide*

377 Hydrogen peroxide production in the presence of TiONts-APTES is studied  
378 using the probe DHR 123 which is a cellular indicator of peroxides and peroxynitrite  
379 production. The results obtained shows little increase in the percentage of cells  
380 overproducing peroxide radicals' at 6 h except for the highest concentration of TiONts-  
381 APTES ( $80 \mu\text{g/mL}$ ) (Figure 8a). Cells treated with  $80\mu\text{g/mL}$  showed statistically  
382 significant increase in peroxide radical production at 6h. On the other hand, TiONts-  
383 APTES exposure at 24h showed a concentration-dependent and statistically significant  
384 increase in peroxides production (Figure 8b).

385 ***Transmembrane mitochondrial potential ( $\Delta\Psi_m$ )***

386 A possible  $\Delta\Psi_m$  change in response to TiONts-APTES is evaluated using  
387 fluorescent probe DiOC<sub>6</sub>(3). Cells exhibit an initial mitochondrial hyperpolarization  
388 after 6 h of exposure, as revealed by the increase in the green intensity of DiOC<sub>6</sub>(3).  
389 7-ketocholesterol (7-kc)-treated cells (positive control) also exhibits similar response.  
390 However, after 24 h, the  $\Delta\Psi_m$  in treated cells reverts to normal state and becomes  
391 similar to controls (Figure 9).

392 ***Lysosomal membrane integrity***

393 Lysosomal membrane integrity in the presence of TiONts-APTES is analyzed by  
394 staining with acridine orange (AO). Flow cytometry analysis of AO stained cells does  
395 not indicate any signs of lysosomal membrane permeabilization (LMP) in TiONts-  
396 APTES-treated cells. AO signals increase in the treated cells for both 6 and 24 h  
397 suggesting the absence of TiONts-APTES mediated LMP in BV-2 cells (Figure 10).

398 ***Plasma membrane integrity***

399 Plasma membrane integrity in the presence of TiONts-APTES is analyzed using  
400 PI staining. Flow cytometry analysis of the PI stained cells indicates no significant  
401 increase in the PI positive cells for 24 h with respect to control. As shown in Figure 11,  
402 cells treated with 80  $\mu\text{g/mL}$  of TiONts-APTES show only 4.5% PI positive cells  
403 compared to 65.5% PI positive cells in 7-kc treated wells (positive control).

404 ***Mechanism of cell death***

405 PARP cleavage assay is carried out to study the mechanism of TiONts-APTES  
406 mediated cell death in BV-2 cells. The little cells death observed in the present study is

407 *via* apoptosis as evidenced by PARP cleavage assay. A light band of cleaved PARP  
408 could be visible in both 20 and 80  $\mu\text{g/mL}$  of TiONts-APTES treated cells at 24h (Figure  
409 12).

#### 410 ***Cell cycle analysis***

411 TiONts-APTES mediated cell cycle changes are evaluated by staining the cells  
412 with PI. The flow cytometry analysis of PI-stained cells indicates no alteration in cell  
413 cycle pattern in the presence of TiONts-APTES. Moreover, SubG1 population  
414 indicative of apoptotic cells (Ormerod *et al.*, 1992) are not visible in TiONts-APTES  
415 treated cells for 24 h of incubation (Figure 13).

#### 416 **Discussion**

417 TiONts have been proposed for a variety of biomedical applications including  
418 drug delivery, bioimaging, cancer therapy, biosensing and disease diagnosis (Papa *et*  
419 *al.*, 2011, Mirjolet *et al.*, 2013, Papa *et al.*, 2013, Boudon *et al.*, 2014, Loiseau *et al.*,  
420 2017, Mirjolet *et al.*, 2017). TiONts are ideal candidates for neuro-bio applications  
421 because of their low toxicity. In the present study, TiONts-APTES nanoparticles,  
422 synthesized for drug delivery applications across BBB, are evaluated for their toxicity  
423 using microglial cells line BV-2.

424 APTES grafting onto TiONts surface is confirmed by several chemical  
425 characterization techniques such as zeta potential measurement, XPS and IR  
426 spectroscopy. Indeed, the increase in the zeta potential value and the isoelectric point  
427 after APTES grafting prove the presence of positively charged functional group on the  
428 surface (amine groups). The increase in carbon percentage and the appearance of new  
429 chemical elements such as nitrogen and silicon, observed by XPS analysis, confirm the

430 APTES grafting. Moreover, IR spectroscopy shows the characteristic vibration bands of  
431 APTES in TiONts-APTES spectrum. The grafting density of this molecule is estimated  
432 by thermogravimetric analysis to  $(7.3 \pm 0.5)$  APTES/nm<sup>2</sup> of nanotubes, based on the  
433 difference in the weight losses between bare and grafted TiONts.

434 Cytotoxicity and dose response are studied in terms of mitochondrial activity by  
435 MTT assay. The mitochondrion is a highly sensitive organelle and hence early stage  
436 changes in the cellular health are reflected in mitochondrial activity. Dose-dependent  
437 alteration in mitochondrial activity after cells exposure to TiONts has been reported in  
438 some studies (Iavicoli *et al.*, 2011). Despite the change in mitochondria location and  
439 morphology observed in the present study through TEM images, no marked reduction in  
440 mitochondrial activity is observed after TiONts-APTES exposure. The statistically  
441 significant reduction observed in the mitochondrial activities are not beyond 30% to be  
442 called as cytotoxic according to the ISO 10993-5 guidelines for the *in vitro* cytotoxicity  
443 evaluation of medical devices. It is reported that TiO<sub>2</sub> nanoparticles interfere with MTT  
444 to give false positive results (Lupu and Popescu, 2013). In the present study, absorbance  
445 values of controls (without cells) are subtracted from the MTT results to rule out  
446 nanoparticles interference.

447 MTT results are just an indicator of cell metabolic state. Changes in MTT  
448 signals can be due to a cytotoxic effect, a cytostatic effect (Riss *et al.*, 2016) or to a loss  
449 of cells during the assay procedure. Therefore it is important to correlate the MTT  
450 results with cellular morphology and total cell count (Quent *et al.*, 2010). For this  
451 reason, Trypan blue exclusion assay is carried out to count the cells (data not shown  
452 here). In the present study, even high concentrations of TiONts-APTES does not elicit  
453 any morphological alteration or reduction in cell size or density after 24 h of exposure.  
454 It can be concluded from the obtained results, that the decrease in mitochondrial

455 activity, observed in MTT assay, cannot be due to the cytotoxic effect of TiONts-  
456 APTES. It is reported that TiO<sub>2</sub> nanoparticles induce size reduction and morphological  
457 alterations in cells (Saquib *et al.*, 2012). However, it is shown in the current result that  
458 TiONts-APTES do not elicit any morphological alterations in BV-2 cells.

459 Cellular uptake of nanoparticles is an important event in bio-nano interaction.  
460 Drug delivery applications require nanoparticles internalization. Moreover, the low  
461 toxicity, indicated in nanotoxicological studies, can be due to the failure of cells to  
462 internalize particles. In the present study cellular internalization of TiONts-APTES is  
463 proved by SSC granularity measurements and TEM observations. On the one hand, the  
464 dose dependent increase in SSC indicates nanohybrids uptake by BV-2 cells. Indeed,  
465 the results of the current study are in corroboration with those of Chen *et al.* (2010)  
466 where cellular internalization of TiO<sub>2</sub> nanorods was shown. As the increase in SSC may  
467 be also the result of apoptotic vesicles increase, the cells are exposed to a cytotoxic  
468 agent, 7-kc, to differentiate if the observed increase in granularity is due to TiONts-  
469 APTES uptake or apoptosis. The SSC changes in 7-kc treated cells are not as  
470 pronounced as those observed for TiONts-APTES which confirms the latter's uptake by  
471 BV-2 cells. On the other hand, TEM images confirm the internalization of TiONts-  
472 APTES and show two uptake processes; the first and main one is endocytosis process  
473 which is a dynamic internalization way, and during which the nanoparticles uptake is  
474 carried out by endocytic vesicles. The second and less probable process is the  
475 internalization of individualized nanotubes by passive diffusion through the cellular  
476 membrane. The latter mechanism is observed when the endocytosis phenomenon is  
477 inhibited with amiloride. This molecule is also used by (Huerta-Garcia *et al.*, 2015) to  
478 illustrate the TiO<sub>2</sub> nanoparticles internalization by macropinocytosis for glial cells. It  
479 has been chosen because macropinocytosis is the main internalization pathway of

480 “large” objects (Benmerah and Lamaze, 2002). Moreover, this endocytic route has been  
481 previously emphasized for TiONts internalization in cardiomyocytes (Papa *et al.*, 2013).  
482 Since the nanotubes elaborated in this study are agglomerated with sizes higher than  
483 several hundreds of nanometers (see Figure 7), macropinocytosis seems to be relevant.  
484 Moreover, the presence of nanoparticles in contact with microglial cells may induce a  
485 change in mitochondria location and size which can be explained by changes in  
486 mitochondrial activity, however no change in cell size or morphology is observed.

487         Particles taken up by the cells ultimately fuse with lysosomes. Lysosomal  
488 membrane permeabilization (LMP) is one of the factors contributing to metal oxide  
489 nanoparticles toxicity (Sabella *et al.*, 2014). In this work TiONts-APTES nanoparticles  
490 do not induce LMP in BV-2 cells as it is indicated by the unaltered intensity of AO  
491 signal.

492         ROS production is an important factor contributing to nanoparticle mediated  
493 toxicity. In the present study, BV-2 cells exhibit dose dependent increase in superoxide  
494 anion production after 6 h which get normalized by 24 h. On the other hand, the level of  
495 peroxide radicals remains similar across all experiment groups for 6h which get  
496 elevated in a dose dependent manner for 24 h. This time-dependent increase in peroxide  
497 radicals may be due to the delayed induction of superoxide dismutase which carries out  
498 the enzymatic conversion of superoxide radicals. Microglia in response to toxic signals  
499 may undergo an oxidative burst. This can be monitored by the immediate rise in  
500 superoxide radicals which ultimately get converted into peroxide radicals (Long *et al.*,  
501 2006). The results obtained in this work are in concordance with the aforementioned  
502 findings.

503         BV-2 cells exposed to TiONts-APTES exhibit an increase in  $\Delta\Psi_m$  6 h after  
504 incubation. Hyperpolarization is an early and reversible event in immune cell activation

505 and apoptosis (Perl *et al.*, 2004). Oxidative burst and mitochondrial hyperpolarization  
506 point to the chance of TiONts-APTES mediated microglial activation in cells.  
507 Microglial activation is characterized by increased cell proliferation, altered  
508 morphology and increase in the cell size (Block and Hong, 2007). To examine  
509 microglial activation potential of TiONts-APTES, cell cycle analysis is carried out. No  
510 sign of cell cycle alterations is observed in TiONts-APTES exposed cells. Normal ‘S’  
511 phase in treated cells confirms the absence of increased DNA synthesis in BV-2 cells.  
512 On the other hand, cytotoxic agent 7-kc cholesterol induces a significant reduction in S  
513 and G2-M phases with a corresponding increase in Sub G1 (apoptotic) population. The  
514 morphological analysis further supports these findings where no change in morphology,  
515 cell size or cell density is observed in BV-2 cells exposed to TiONts-APTES suggesting  
516 their low cytotoxicity.

517 Nanoparticle-mediated cell death can occur *via* the two main types of cell death:  
518 apoptosis and necrosis (Hirsch *et al.*, 1998). To study the mechanism of TiONts-APTES  
519 mediated cell death, the PARP cleavage assay is carried out. PARP cleavage is a marker  
520 for apoptotic mode of cell death and is mediated by suicidal proteases like caspases and  
521 cathepsin (Boya and Kroemer, 2008). TiONts-APTES exposure induced PARP  
522 cleavage in BV-2 cells. Studies show that lysosomal proteases are probably involved in  
523 nanoparticles toxicity (Sruthi and Mohanan, 2016). To evaluate whether the PARP  
524 cleavage is influenced by the lysosomal proteases (cathepsin), cells are pre-treated with  
525 a cathepsin inhibitor Z-FA-FMK. In contrary to the expectation, the cathepsin inhibitor,  
526 which also induces PARP fragmentation, does not attenuate PARP cleavage in BV-2  
527 cells. The result of the PARP cleavage suggests a potential activation of an apoptotic  
528 mode of cell death in TiONts-APTES exposed BV-2 cells.

529           The analysis of plasma membrane integrity further confirms the apoptotic mode  
530 of cell death. The loss of plasma membrane integrity occurs in the late phase of  
531 apoptosis. Cells at the early stage of apoptosis possess intact membrane. Absence of PI  
532 positive cells in the population after 24 h of TiONts-APTES incubation further  
533 supported TiONts-APTES mediated apoptosis in BV-2 cells. Moreover, it suggests that  
534 BV-2 cells are probably at their early stage of apoptosis at 24 h of treatment.

### 535 **Conclusion**

536           Safety and toxicity are major concerns when it comes to biomedical applications  
537 of nanoparticles. In the present study, bio-interaction of APTES-functionalized TiONts  
538 were carried out using BV-2 microglial cells. Despite the changes in mitochondrial  
539 activity and ROS production, no morphological variation or plasma membrane  
540 permeability alteration was observed in cells exposed to TiONts-APTES. These results  
541 were confirmed by TEM images that showed a normal cellular morphology after  
542 TiONts-APTES uptake and changes in mitochondria location and size. The particles  
543 induced an initial increase in transmembrane mitochondrial potential ( $\Delta\Psi_m$ ) which  
544 reverted to normal state by 24 h. TiONts-APTES do not interfere with lysosomal  
545 membrane integrity or cell cycle. Caspase-dependent apoptosis probably mediates the  
546 toxicity. The time- and dose-dependent toxicity is obvious only at the highest  
547 concentration used (80  $\mu\text{g/mL}$ ). It is shown through TEM images that the main process  
548 of nanoparticles internalization in BV-2 cells is endocytosis. These cells do not show  
549 any sign of activation which is a major hurdle in drug delivery applications across brain.  
550 This study proves that APTES-functionalized TiONts are a potential candidate for drug  
551 delivery applications across the brain.

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566

567 **Declaration of interest**

568 The authors report no conflicts of interest.

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700

701 **Tables**

702

	<b>Starting degradation temperature (°C)</b>	<b>Mass loss (%)</b>	<b>Degraded molecular weight (g.mol<sup>-1</sup>)</b>	<b>Molecule.nm<sup>-2</sup></b>
<b>TiONts</b>	175	2.8	18	(10.6 ± 1.5) OH, n=10
<b>TiONts-APTES</b>	160	10.3	58	(7.3 ± 0.5) APTES, n=7

703 Table 1. Results of mass loss and hydroxyl groups density and grafting ratio of bare  
 704 TiONts and TiONts-APTES respectively (n is the number of syntheses realized before  
 705 pooling the different batches, they allow to evaluate the reproducibility of our  
 706 syntheses).

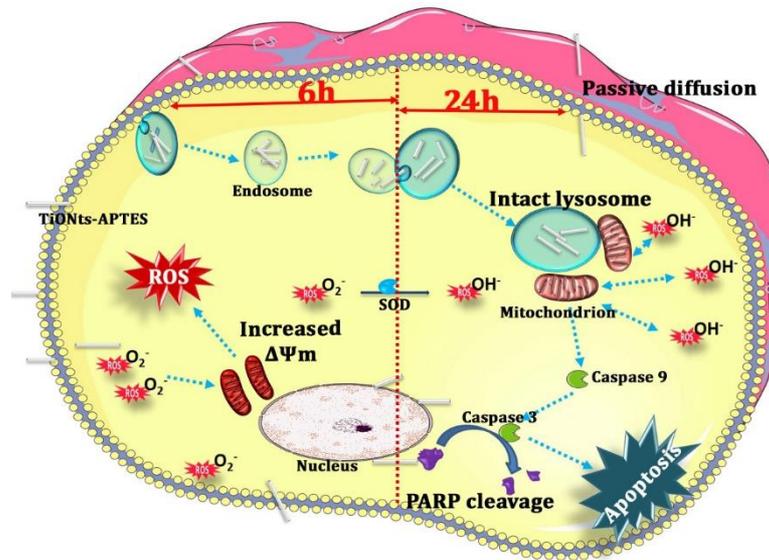
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<b>Atomic concentration (%)</b>	<b>C<sub>1s</sub></b>	<b>O<sub>1s</sub></b>	<b>Na<sub>KLL</sub></b>	<b>Ti<sub>2p</sub></b>	<b>N<sub>1s</sub></b>	<b>Si<sub>2p</sub></b>
<b>TiONts</b>	7.3	58.7	13.5	20.5	-	-
<b>% Elements TiONts / Ti</b>	0.3	2.9	0.7	1.0	-	-
<b>TiONts-APTES</b>	11.2	56.8	5.7	21.5	2.3	2.5
<b>% Elements TiONts-APTES / Ti</b>	0.5	2.6	0.3	1.0	0.1	0.1

708 Table 2. XPS elemental analyses and their corresponding ratio to titanium of bare  
 709 TiONts and TiONts-APTES.

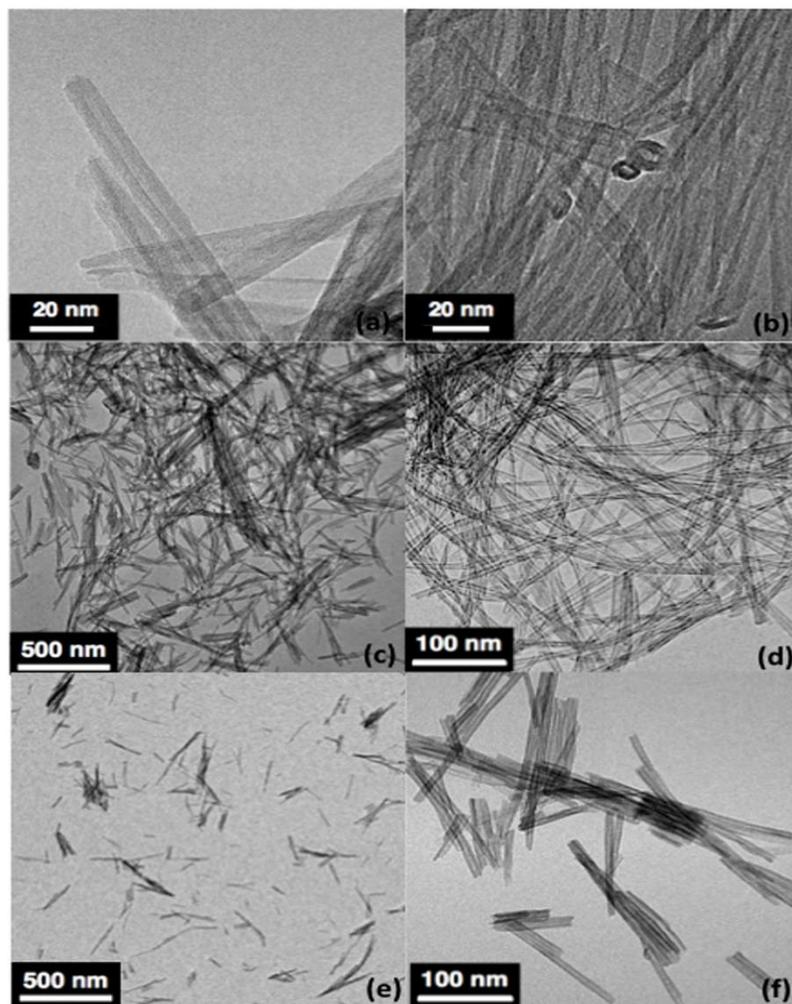
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711 **Figures**



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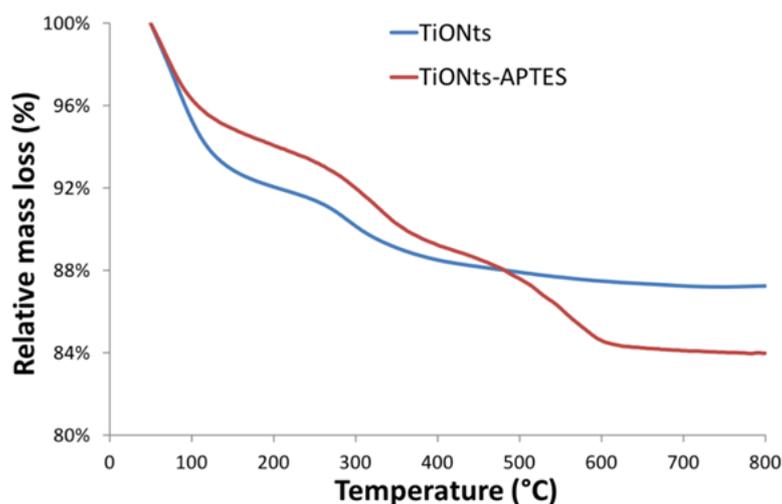
713 **Graphical abstract:** (3-aminopropyl) triethoxysilane (APTES) functionalized titanate  
714 nanotubes (named TiONts on the scheme) enter the BV-2 cell both *via* endocytosis and  
715 passive diffusion. Inside cell, TiONts-APTES are capable of inducing superoxide  
716 radicals which later get converted to peroxides by SOD activity. TiONts-APTES  
717 interact with mitochondria to induce a transient increase in  $\Delta\Psi_m$  which further gives  
718 way to mitochondria mediated ROS production. TiONts-APTES do not affect  
719 lysosomal integrity or cell cycle pattern. At high concentration TiONts-APTES  
720 probably induce caspase-mediated apoptosis in BV-2 cells leading to PARP cleavage.



721

722 Figure 1. TEM images highlight (a-b) the formation of bare TiONts and the evolution of  
723 TiONts dispersion (c-d) before and (e-f) after APTES grafting.

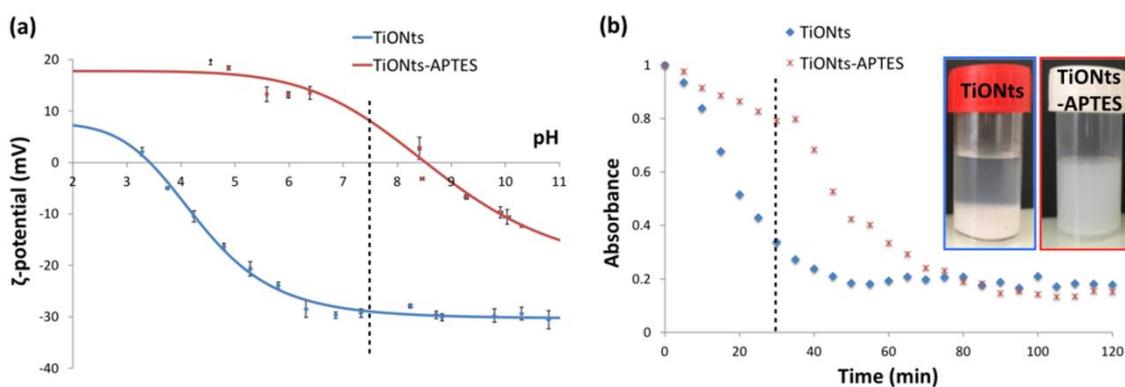
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725

726 Figure 2. TGA curves of bare TiONts and TiONts-APTES under air atmosphere.

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728

729 Figure 3. (a)  $\zeta$  potential curves of bare TiONts and TiONts-APTES (the vertical dashed

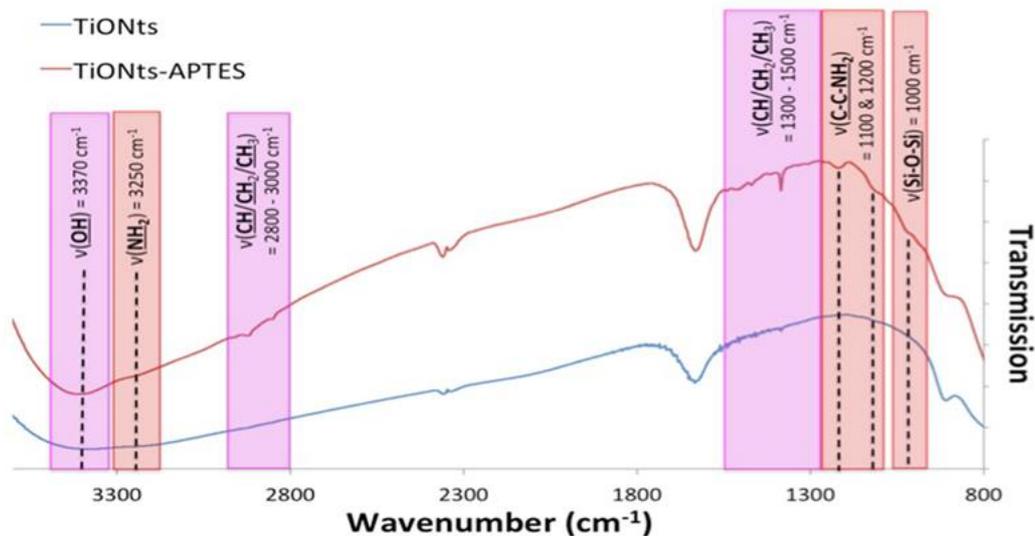
730 line corresponds to the physiological pH). (b) Colloidal stability of bare TiONts and

731 TiONts-APTES (PBS 0.1 M; pH 7.4) over 120 min following their absorbance

732 at 600 nm by turbidimetry. The pictures of TiONts (left) and TiONts-APTES (right)

733 suspensions in PBS (0.1 M; pH 7.4) correspond to the vertical dashed line at 30 min.

734



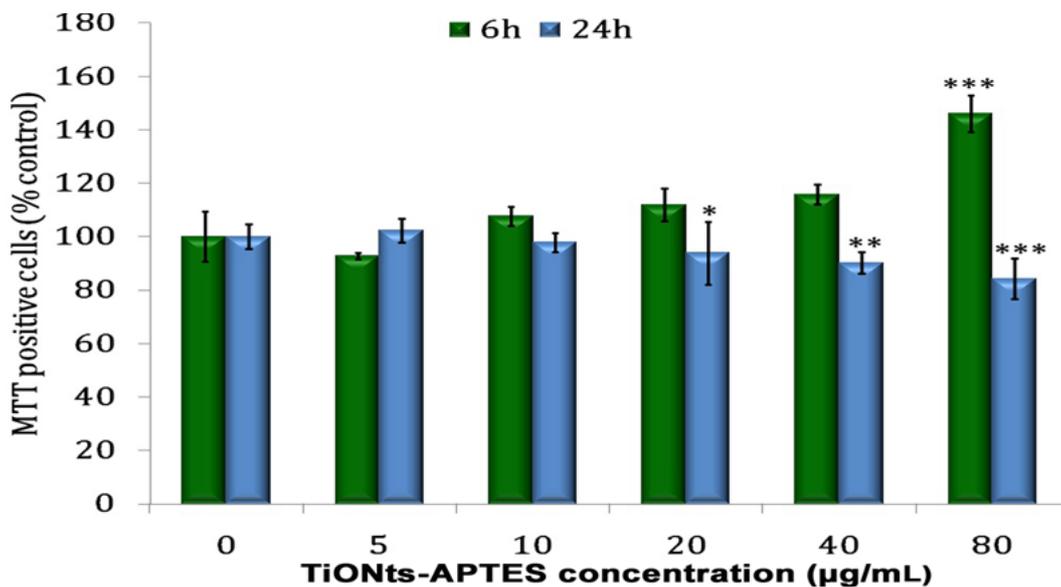
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736 Figure 4. FT-IR spectra of bare TiONts and TiONts-APTES. Red vibrational bands

737

highlight the presence of APTES on the surface of TiONts.

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740 Figure 5. (a) Dose response and cytotoxicity in BV-2 cells exposed to TiONts-APTES

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for 6 and 24 h indicated by percentage MTT positive cells. Data represents mean  $\pm$  SD

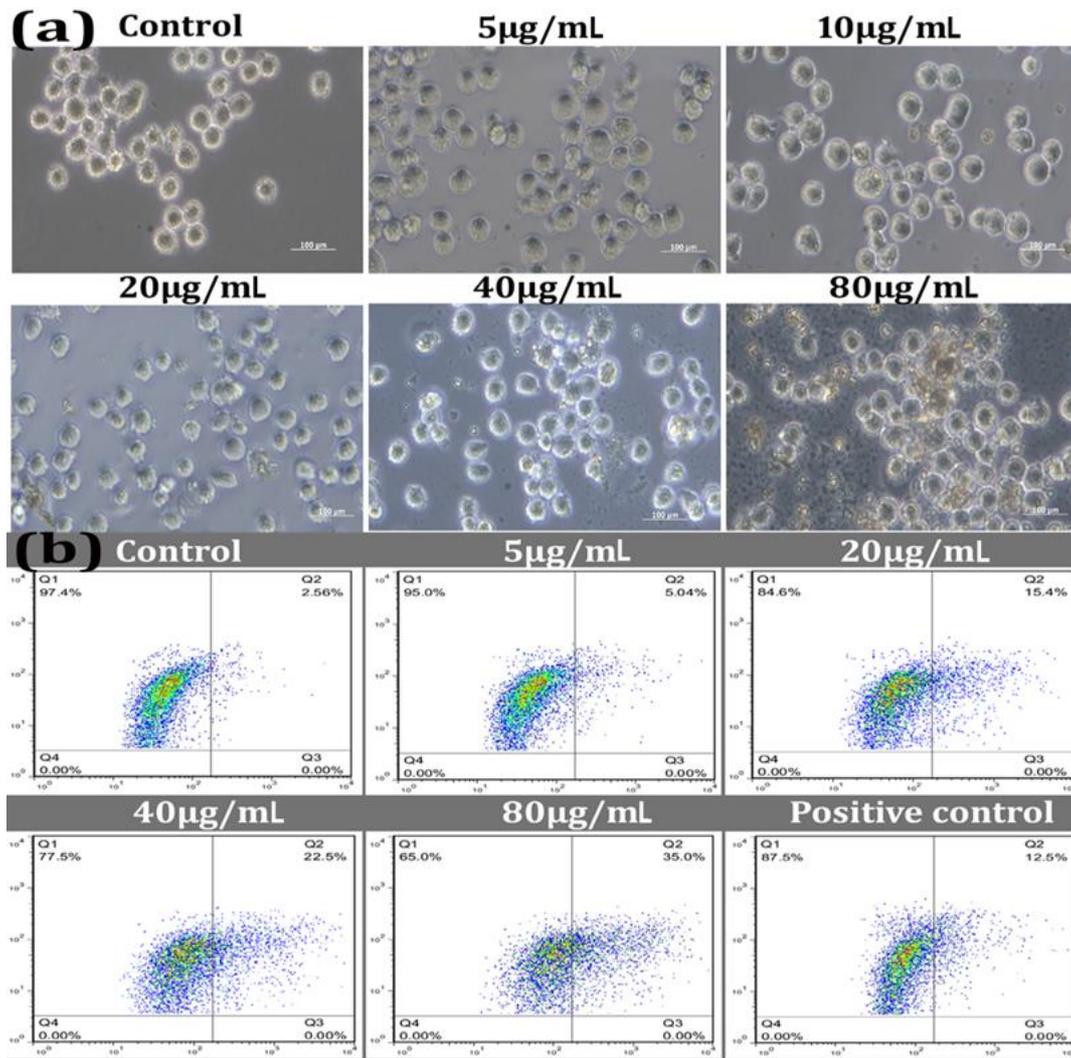
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of three independent experiments. Asterisks denote statistically significant differences

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compared to the control (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

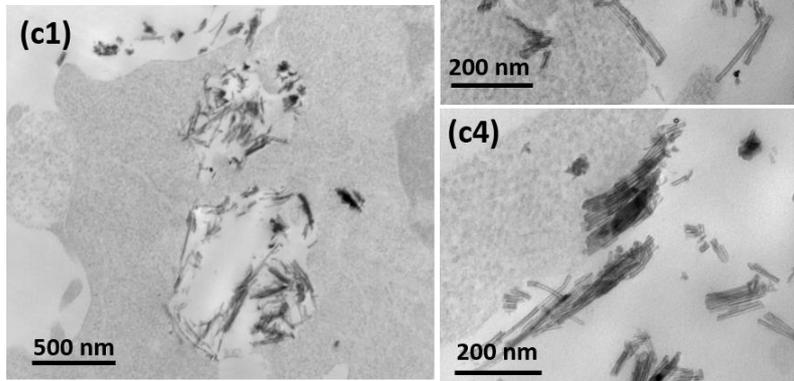
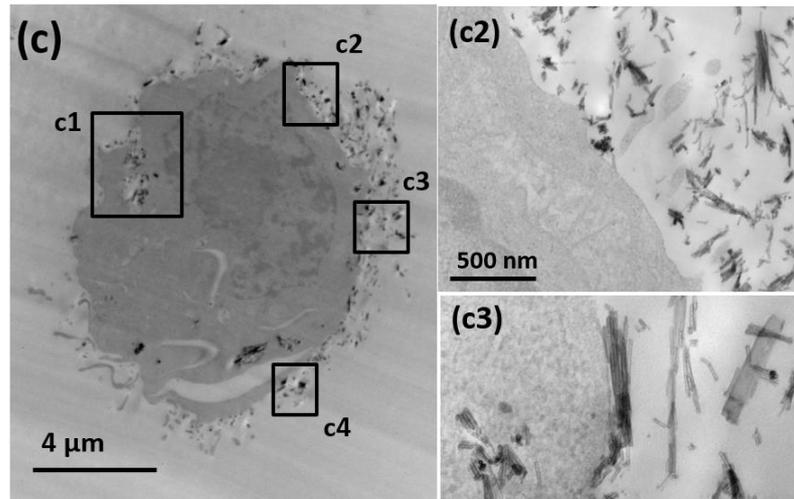
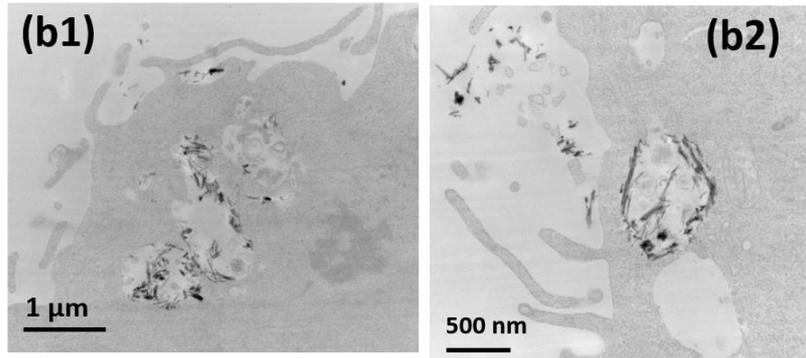
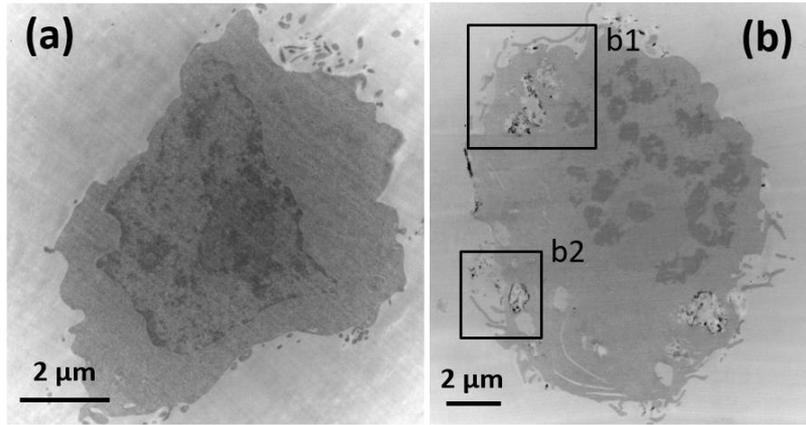
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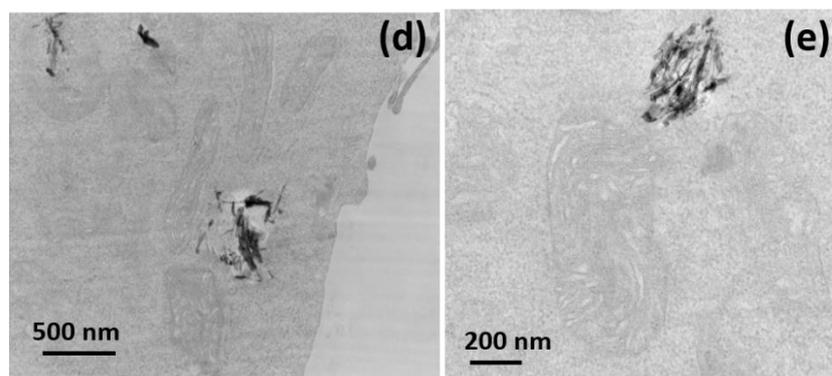
747 Figure 6. (a) Cell morphology analysis of BV-2 cells exposed to TiONts-APTES for  
 748 24 h. Scale bar represents 100  $\mu$ m. As the highest concentration used the nanoparticles  
 749 sediments could be observed. (b) Cellular uptake of TiONts-APTES by BV-2 cells:  
 750 cells were exposed to TiONts-APTES for 6 h and the increase in side scatter (SSC) with  
 751 respect to forward scatter (FSC) was measured. 7-ketocholesterol-treated cells were  
 752 used as positive control. All the cellular debris were gated out to represent the data.

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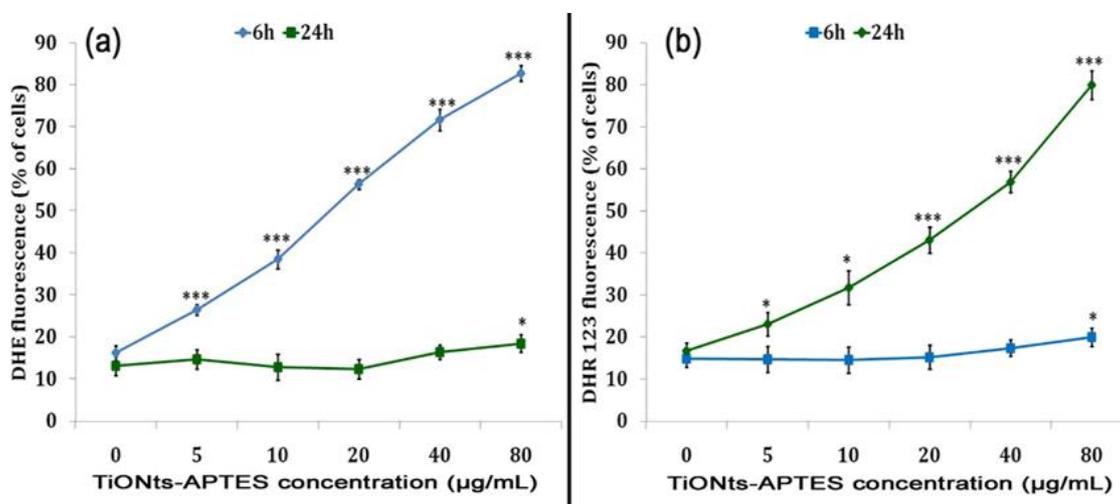
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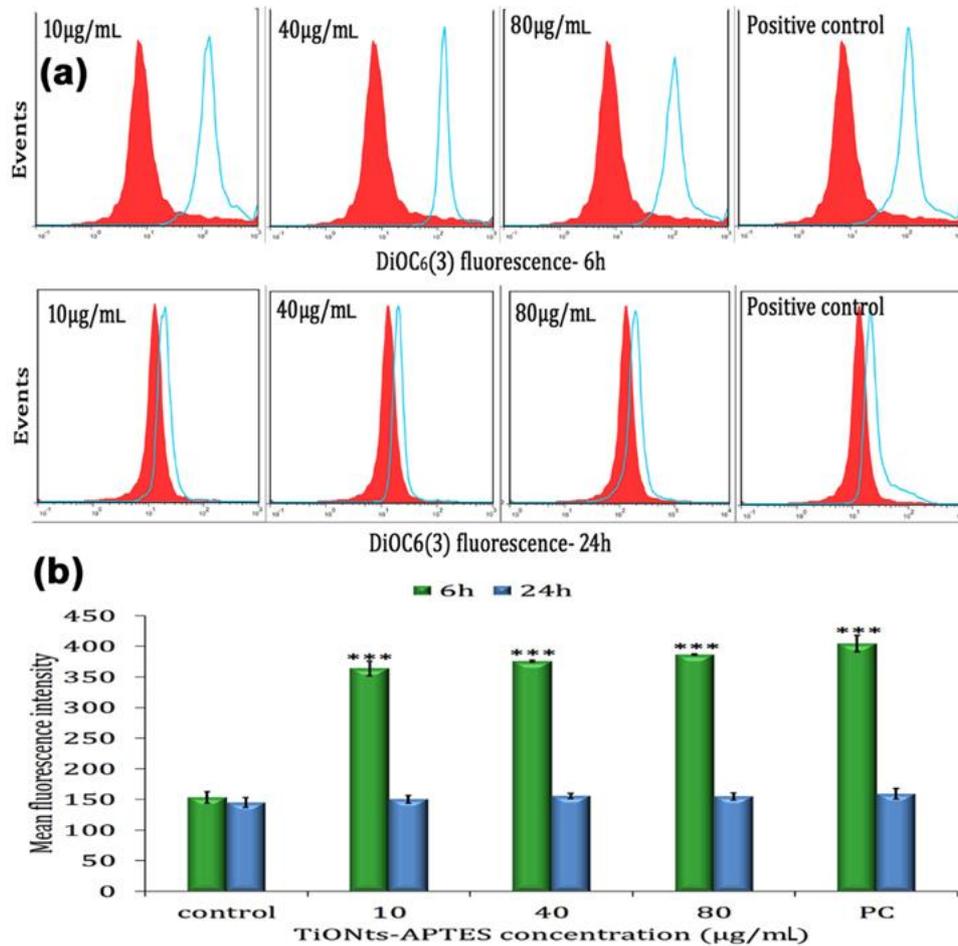
757 Figure 7 : TEM images of (a) BV-2 control cells, (b,b1,b2) BV-2 cells treated with  
 758 20  $\mu\text{g/mL}$  of TiONts-APTES without amiloride (c,c1,c2,c3,c4) BV-2 cells treated with  
 759 20  $\mu\text{g/mL}$  of TiONts-APTES and 100 $\mu\text{M}$  of amiloride, incubated for 24 h (d)  
 760 endocytosis vesicle surrounded by four mitochondria (e) endocytosis vesicle in contact  
 761 with mitochondria.

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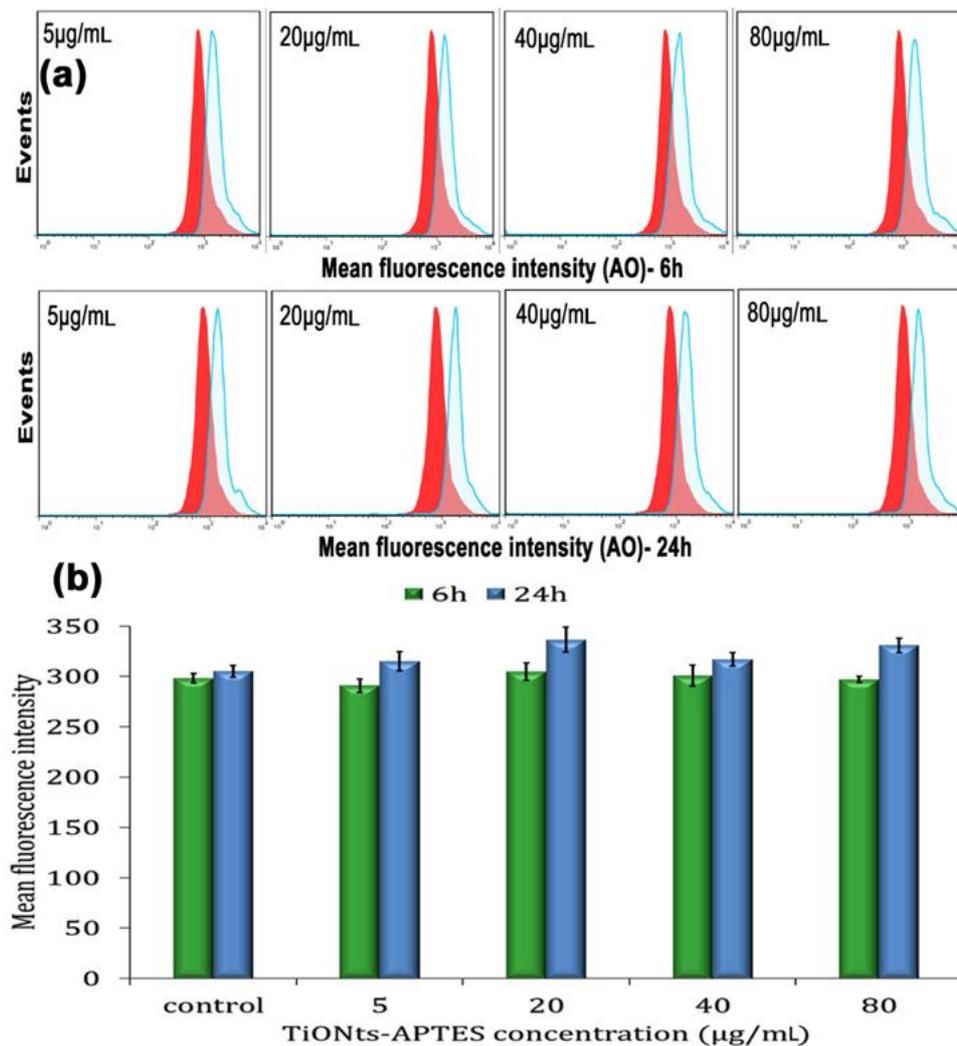
764 Figure 8. ROS generation in BV-2 cells exposed to TiONts-APTES for 6 and 24 h. (a)  
 765 Superoxide radical formation (b), hydrogen peroxide radical formation. Data represents  
 766 mean  $\pm$ SD of three independent experiments. Asterisks denote statistically significant  
 767 differences compared to control (\* p<0.05, \*\* p<0.01 and \*\*\* p<0.001).



769

770 Figure 9. (a) Transmembrane mitochondrial potential in BV-2 cells exposed to TiONts-  
 771 APTES at 6 and 24 h. Cells incubated with 4% paraformaldehyde were used as positive  
 772 control (PC). Red solid peaks represent DiOC<sub>6</sub>(3) intensity of untreated control while  
 773 blue tinted peaks represent treated groups. The gated population includes both dead and  
 774 viable cells. (b) Flow cytometric quantification of transmembrane mitochondrial  
 775 potential ( $\Delta\Psi_m$ ). Data shown represent mean  $\pm$  SD of three independent experiments.  
 776 Asterisks denote statistically significant differences compared to control (\*\*\*) p value  
 777 <0.001).

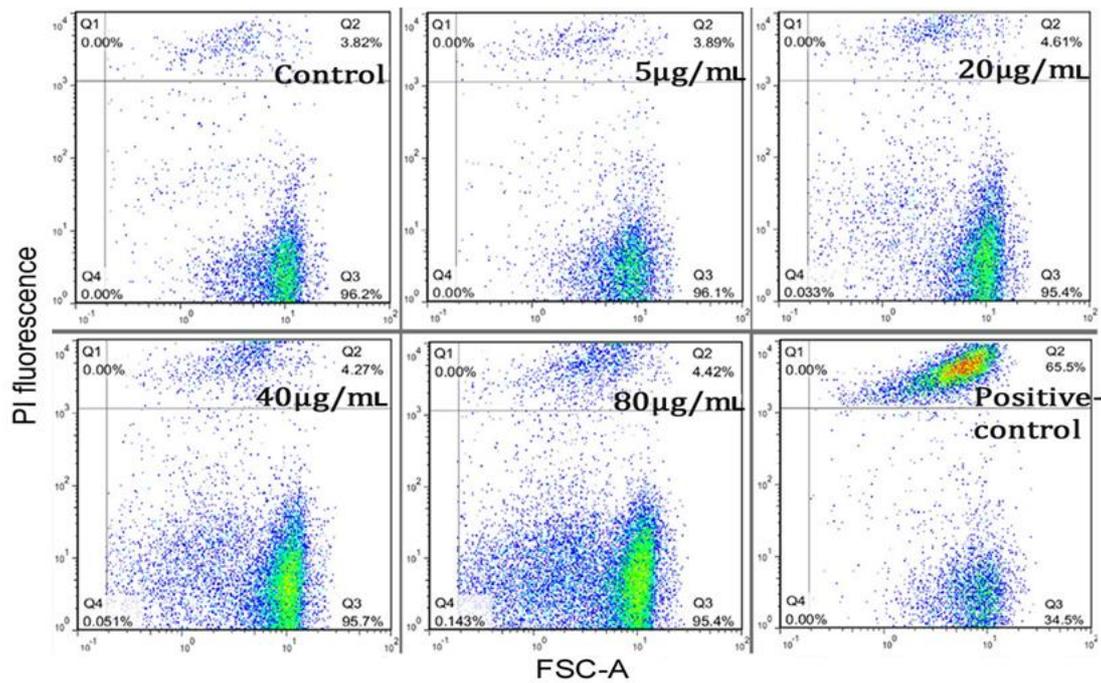
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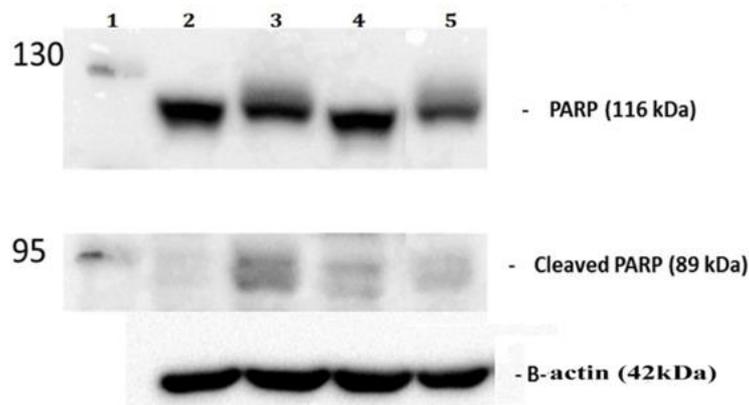
780 Figure 10. (a) Lysosomal membrane integrity of BV-2 cells exposed to TiONts-APTES  
 781 for 6 and 24 h. The red solid peaks denote AO intensity in control population while blue  
 782 tinted peaks represent treatment groups. The gated population includes both viable and  
 783 dead cells. (b) Flow cytometric quantification of the lysosomal membrane integrity.  
 784 Data shown represent mean  $\pm$  SD of three independent experiments.

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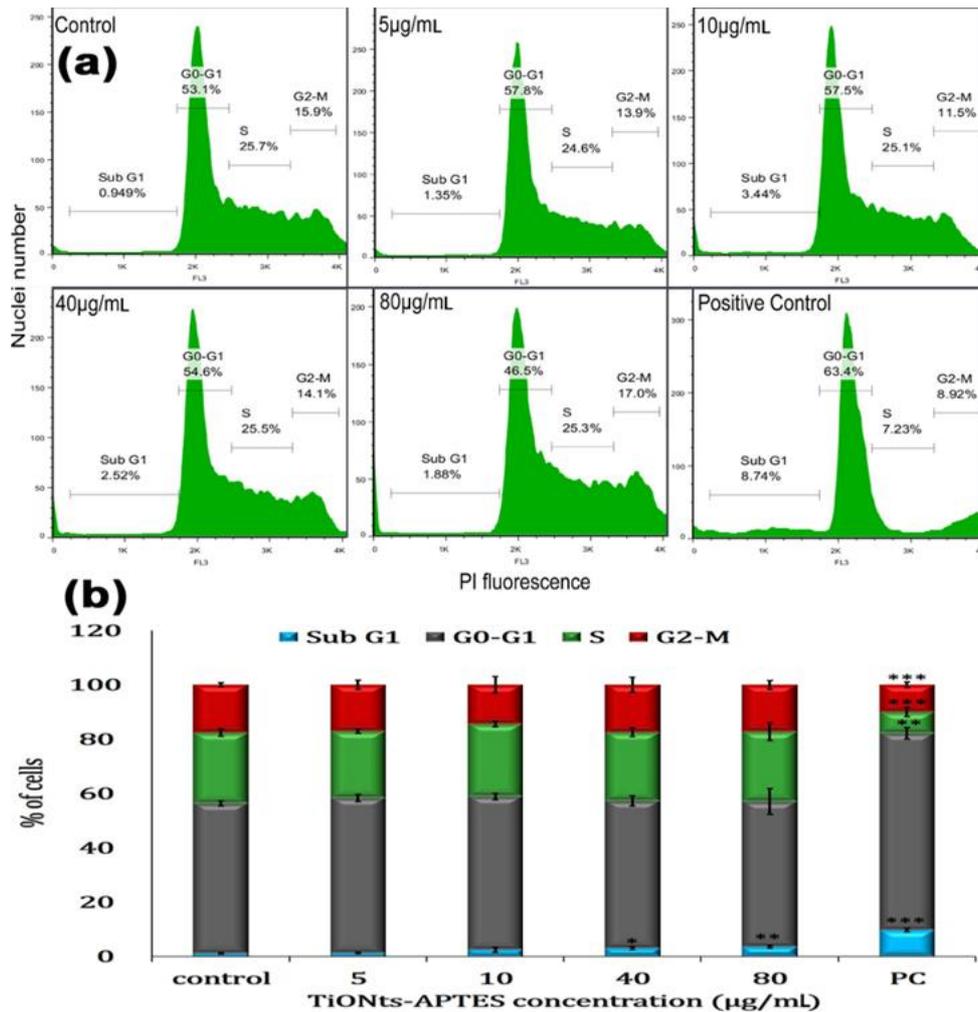
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787 Figure 11. Plasma membrane integrity in BV-2 cells exposed to TiONts-APTES for 24  
 788 h. 7-ketocholesterol treated cells were used as positive control. The gating was done  
 789 with respect to PI fluorescence of positive control. Gated population includes both live  
 790 and dead cells. n = 3.



791

792 Figure 12. PARP cleavage assay in BV-2 cells exposed to TiONts-APTES for 24 h.  
 793 Lane 1: protein marker, Lane 2: control cells, Lane 3: control cells pre-treated with Z-  
 794 Fa-FMK, lane 4: TiONts-APTES (80 μg/mL), lane 5: 80 μg/mL TiONts-APTES pre-  
 795 treated with Z-Fa-FMK.



796

797 Figure 13. (a) Cell cycle analysis of BV-2 cells exposed to TiONts-APTES for 24 h. (b)  
 798 quantification of the cell cycle analysis. 7-ketocholesterol-treated cells were used as  
 799 positive control (PC). All the cellular debris has been gated out to represent the data.  
 800 Data shown represent mean  $\pm$  SD of three independent experiments. Asterisks denote  
 801 statistically significant differences compared to control (\* p value <0.05, \*\* p value <  
 802 0.01 and \*\*\* p value <0.001).

803