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# Synergistic Phenomena between Iron-Doped ZnO Nanoparticles and Shock Waves Exploited against Pancreatic Cancer Cells

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therapy; pancreatic ductal adenocarcinoma.

## ABSTRACT

We propose the use of iron-doped zinc oxide nanoparticles (Fe:ZnO NPs) showing theranostic capabilities and being synergistically active against pancreatic ductal adenocarcinoma once combined with mechanical pressure waves, like shock waves. Fe:ZnO NPs are synthesized employing oleic acid as a capping agent and are functionalized with amino-propyl groups. We first report their superior characteristics with respect to undoped ZnO NPs in terms of magnetic

properties, colloidal stability, cytocompatibility and internalization into BxPC-3 pancreatic cancer cells in vitro. These Fe:ZnO NPs are also cytocompatible towards normal pancreatic cells. We then perform a synergistic cell treatment with both shock waves and Fe:ZnO NPs once internalized into cells. We also evaluate the contribution to the synergistic activity of the NPs located in the extracellular space. Results show that both NPs and shock waves, when administered separately, are safe to cells, while their combination provokes an enhanced cell death after 24 hours. Various mechanisms are then considered, like dissolution of NPs, production of free radicals and cell membrane disruption or permeation. It is understood so far that iron-doped ZnO NPs can degrade intracellularly into zinc cations, while the use of shock waves produce cell membrane permeabilization and possible rupture. In contrast, the production of reactive oxygen species is here ruled out. The provoked cell death can be recognized in both apoptotic and necrotic events. The proposed work is thus a first proof-of-concept study enabling promising future applications to deep-seated tumors like pancreatic cancer, which is still an unmet clinical need with a tremendous death rate.

## 1. Introduction

Theranostic nanosystems are acquiring more and more relevance in nanomedicine<sup>1</sup>. The potential advantages offered by the administration of a site-specific therapy while constantly monitoring the patient conditions are actually enormous in the context of cancer, as for all other diseases which are very difficult to treat<sup>2,3</sup>. Several prototypes of smart nano-sized particles able to perform both these tasks can be found in the literature<sup>4-6</sup>. One of the major trends includes the use of nanosystems incorporating imaging moieties and carrying antitumoral drugs<sup>7</sup>, such as polymeric

1 nanoparticles (NPs)<sup>8–11</sup>, liposomes<sup>12–14</sup> or mesoporous silica NPs<sup>15,16</sup>. However, a main drawback  
2 of these devices is the inability to precisely control their release location, leading to an unwanted  
3 leakage of their cargo before correctly reaching the tumor environment.

4 New approaches feature NPs which can be activated to perform the therapy only once they have  
5 reached the site of interest. Recent advancements are going toward smart nanomaterials able to  
6 react to different physical and chemical stimulations. Examples includes pH-sensitive materials  
7 capable of releasing anti-tumoral drugs only in the acidic tumor environment<sup>17,18</sup> or magnetic  
8 nanoparticles, like iron oxide ones<sup>19,20</sup>, which can be remotely directed and excited to induce  
9 hyperthermia once inside the cancer tissues<sup>21–23</sup>. Other classes of stimuli-responsive nanomaterials  
10 are NPs aimed at photothermal<sup>24–26</sup> (PTT) and photodynamic<sup>27</sup> (PDT) therapies. The underlying  
11 concept is to excite such nanoparticles with a light source, in order to induce toxic phenomena in  
12 nearby tissues. PTT consists in the excitation of optically responsive materials, i.e. metal  
13 nanoparticles, with an electromagnetic radiation, typically in the infrared region of the light  
14 spectrum. Once the light is absorbed, the excited material converts this energy into heat, which  
15 can ablate the tumor tissue and eventually cause cell death. Due to the electronic quantum  
16 confinement given by the nano-scale, gold nanoparticles result extremely suitable for this purpose,  
17 as widely demonstrated by the large amount of related works in the literature<sup>28–31</sup>. On the other  
18 hand, most of PDT approaches rely on the generation of reactive oxygen species (ROS) upon light  
19 irradiation. ROS production is expected to induce toxicity on the diseased cells<sup>32</sup>.  
20 Whatever the energy sources used or the mechanism involved, the advantage of these remotely-  
21 controlled NPs is their ability to activate a toxic phenomenon, provoking cell death exclusively on  
22 cancer cells and only once the therapeutic moiety has effectively reached its target<sup>33</sup>.

1 The use of remotely-controlled NP approaches can face some drawbacks too. Indeed, the visible  
2 light possesses a reduced tissue penetration, while UV radiations may damage healthy tissues with  
3 side effects potentially overwhelming the benefits of the therapy<sup>34</sup>. Alternate magnetic field, if too  
4 much intense, can provoke damages to normal tissues and create undesired eddy currents<sup>35</sup>.  
5 Tissue penetration becomes a very important parameter to consider when treating deep-seated  
6 tumors, like pancreas. Pancreatic cancer and in particular the most common form of this disease,  
7 namely pancreatic ductal adenocarcinoma (PDAC)<sup>36</sup>, is very difficult to treat due to the  
8 characteristics of its development, which provide it with resistance toward standard cancer  
9 treatments<sup>37,38</sup>. Therapeutic approaches like PDT could successfully address the difficult task of  
10 reducing tumor growth. However, the location of the pancreas inside the human body does not  
11 allow an effective stimulation with light. Different remote stimulations have been proposed as  
12 possible alternatives. An example is represented by the stimulation with periodic pressure waves,  
13 like ultrasounds (US) or shock waves, these last being sharp discontinuities, i.e. compressive and  
14 tensile waves, involving a sudden and strong change in pressure and density in a medium<sup>39,40</sup>.  
15 Indeed, US represents a valuable alternative to light because pressure waves can penetrate deeper  
16 in the organic tissues interposed between the stimulation source and the target, while preserving a  
17 good level of safety on normal tissues when low irradiation powers are employed. On the other  
18 hand, higher powers can be exploited for tumor thermoablation, like with focused ultrasound  
19 treatments<sup>41</sup>. Several molecules have been proposed as enhancers of this phenomenon, and are  
20 referred to as sonosensitizers, i.e. systems able to induce hyperthermia or ROS generation only  
21 upon US stimulation so as to localize the therapy performed on the system<sup>42</sup>. In this perspective,  
22 nanoparticles such as gold<sup>43-45</sup>, titanium oxide<sup>46,47</sup> and zinc oxide<sup>48-51</sup> have been suggested as  
23 sonosensitizing agents and have shown a great potential in this field. The superiority of NPs with

respect to organic sonosensitizer molecules appears clear and is at present extensively debated in the literature<sup>52</sup>. NPs can be better dispersed in biological water-based media than organic molecules and they can be biocompatible. NPs can accumulate in the tumor mass thanks to the enhanced permeation and retention (EPR) effect or, even better, thanks to the use of functional biomolecules at their surface for the active targeting of cancer cells<sup>53</sup>. Furthermore, NPs can be designed to act as theranostic tools and to be fully biodegradable at the end of their mission in the biological environment.

In this work we propose the use of biocompatible, colloidal, biostable and biodegradable NPs that display both imaging and therapeutic functionalities. We prove their effectiveness as sonosensitizing agents, since they reduce pancreatic cancer viability in vitro when coupled with a remote mechanical stimulation, i.e. shock waves. More specifically, the subject of this work is represented by iron-doped zinc oxide nanoparticles (Fe:ZnO NPs). They have been proved to possess good biocompatibility, magnetic properties and therefore magnetic resonance imaging (MRI) potentialities in previous studies<sup>54</sup>. The target of this first proof-of-concept test is a PDAC cell line (BxPC-3). Actually, PDAC can only be reached with a deep stimulation and displays innate and acquired drug resistance. It therefore urgently requires more effective therapeutic approaches, as the one here proposed. Fe:ZnO NPs are thus tested first in terms of their cytocompatibility, cellular uptake and NPs dissolution in cell culture media in comparison to undoped ZnO NPs. Control tests with normal pancreatic cells were also carried out. The obtained results aim to increase the understanding of the Fe:ZnO NPs fate inside the cell, when their administration is not coupled with a remote physical stimulation, and to evaluate their final and safe biodegradation.

1 The coupling of the NPs with a mechanical stimulation, i.e. pressure shock waves, is exploited to  
2 induce cell death only on demand, to achieve a remotely-controlled and safe therapy. Therefore,  
3 BxPC-3 viability is assessed with the coupled treatment and then the cell fate is observed to  
4 determine the cell death mechanism and other possible aspects that could be improved in view of  
5 a future clinical translation.

## 6 7 2. Materials and methods

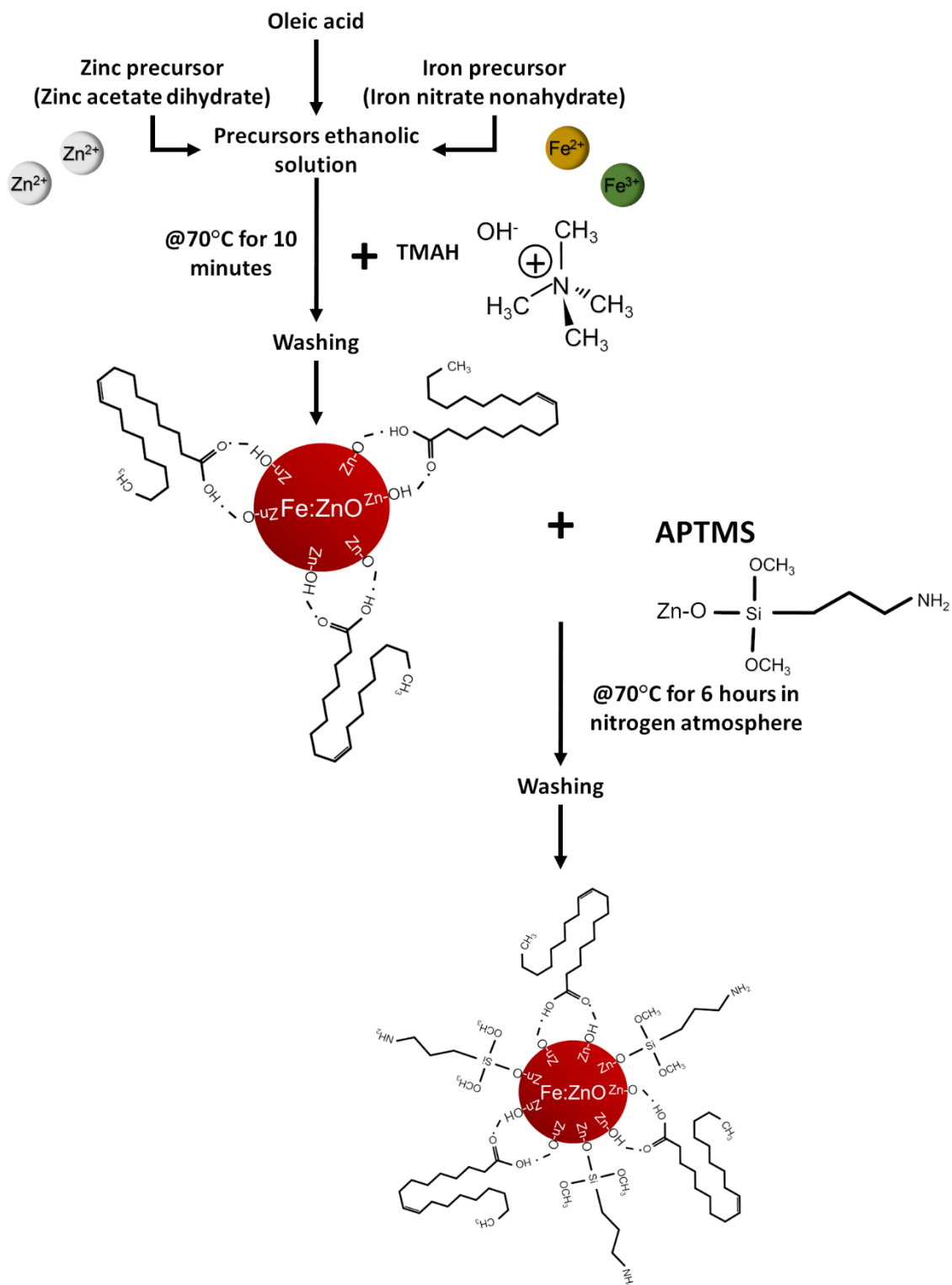
### 8 2.1. Synthesis of ZnO and Fe:ZnO NPs

9 Iron-doped ZnO nanoparticles (Fe:ZnO NPs) were synthesized with a wet chemical method  
10 previously developed in other works<sup>54</sup>. In particular, zinc acetate dihydrate (526 mg, 2.37 mmol,  
11 ACS Reagent, Sigma-Aldrich) and ferric nitrate nonahydrate (58 mg, 143  $\mu$ M, HiMedia) were  
12 weighted to obtain a molar ratio of 0.06 between Fe and Zn ions. The salts were dissolved in 40  
13 mL of ethanol (99%, Sigma-Aldrich) in a round bottom flask under moderate stirring.  
14 Additionally, 1 mL of bidistilled water (from a Direct Q3 system, Millipore, Burlington) and 140  
15  $\mu$ L of oleic acid ( $\geq 99\%$ , Sigma-Aldrich) were included in the solution to favor hydrolysis of the  
16 precursors and NPs stability, respectively. The flask was heated up to 70 °C in a silicon bath under  
17 refluxing condition to limit solvent evaporation.

18 To favor the hydrolysis of the zinc precursor, according to the mechanism previously reported  
19 <sup>55</sup>, 1.044 g of tetramethylammonium hydroxide (TMAH, 98.5%, Sigma-Aldrich) were dissolved  
20 in an ethanol:water solution (10 mL : 1.052 mL). Then, TMAH was rapidly added to the zinc and  
21 iron precursors' solution to provide the hydroxide required for the precursors' hydrolysis. After 10

1 minutes, the formation of Fe:ZnO NPs was completed and 40 mL of ice-cooled ethanol was added  
2 in the flask to stop the reaction. The whole dispersion was collected and centrifuged for 10 minutes  
3 at 8000 g. The supernatant was discarded, and the NPs pellet was redispersed in 40 mL of ethanol.  
4 This washing procedure was repeated three times, to obtain the final batch of Fe:ZnO NPs.  
5 A scheme of the synthetic and functionalization procedures is reported in Figure 1.  
6 The same procedure, without the inclusion of the iron precursor, was followed to synthesize  
7 undoped NPs, which were used as control.





**Figure 1.** Fe:ZnO NPs synthesis procedure scheme.

## 2.2. Amino-propyl functionalization

Undoped and Fe:ZnO NPs were also provided with an amino-propyl functionalization (Figure 1), as reported in other works<sup>55,56</sup>. The functionalization was performed on 40 mg of NPs (either undoped or iron-doped) dispersed in 16 mL of ethanol to obtain a concentration of 2.5 mg/mL and placed in a 25 mL round bottom flask. The solution was heated up to 70 °C under nitrogen refluxing condition. After 10 minutes of moderate stirring, 8.6 µL of 3-aminopropyltrimethoxysilane (APTMS, Sigma-Aldrich, corresponding to the 10% mol with respect to the ZnO amount) were added to the solution. The reaction was held for 6 hours. The NPs dispersion was finally collected and centrifuged at 14000 g for 20 minutes. The supernatant was discarded to remove the unreacted APTMS. Resuspension was performed with fresh ethanol and the washing steps were repeated twice, to obtain the batch that could be directly used in *in vitro* tests.

## 2.3. Physical and chemical characterization

### 2.3.1. Dynamic light scattering measurements

The prepared NPs were characterized from the physical and chemical standpoints. More in detail, Dynamic Light Scattering (DLS) measurements of the hydrodynamic size were performed with a Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK) both in water and in RPMI 1640 (cell culture medium, ATCC) supplemented with 10 %v of Fetal Bovine Serum (FBS, ATCC) and 100 µg/mL streptomycin and 100 units/mL penicillin (P/S, Sigma-Aldrich). For the measurement, 100 µg of NPs were centrifuged for 10 minutes and redispersed in 1 mL of the medium of choice (either water or cell culture medium) by the help of a sonication bath (in detail, 10 minutes of sonication at 40 kHz with a Branson 3800 CPXH, Branson Ultrasonics Corporation). All the measurements

were performed in triplicate. A similar sample preparation procedure was adopted to measure the NPs' Z-potential in water, with the same instrument employed for DLS measurements.

### 2.3.2. X-ray diffraction measurements

The crystallinity of the nanoparticles was investigated by means of X-ray diffraction analysis. Undoped and iron-doped NPs suspensions for a total amount of 1 mg of NPs were dropped onto a monocrystalline silicon substrate and let dry. The resulting film was analyzed by means of a Panalytical X'Pert diffractometer working in Bragg-Brentano mode (Cu-K $\alpha$  source,  $\lambda = 0.154$  nm, 30 mA and 40 kV). The crystallite size was evaluated exploiting the software Origin (OriginLab).

### 2.3.3. Electron microscopy

Electron microscopy was used to determine the morphology of the synthesized nanoparticles. For ZnO and Fe:ZnO NPs were deposited onto a silicon substrate, let dry and analyzed with a Field Emission Scanning Electron Microscopy (FESEM, SUPRA 40, Zeiss), also equipped with an Energy-Dispersive X-ray Spectroscopy (EDS) detector (x-act 10 mm<sup>2</sup> Silicon Drift Detector, Oxford Instruments) used for the elemental analysis and the determination of the amount of dopant element included in the nanocrystals.

Transmission Electron Microscopy (TEM) was also employed to characterize both ZnO and Fe:ZnO NPs. The samples were prepared by dispersing the NPs in water at a concentration of 25  $\mu\text{g/mL}$ . Then, 10  $\mu\text{L}$  of the solution were deposited onto a Lacey Carbon Support Film (300 mesh, Cu, Ted Pella Inc.) and let dry. The measurements were held with a Talos<sup>TM</sup> F200X G2 S(TEM) from Thermo Scientific at an operating voltage of 200 kV.

#### 2.3.4. ROS generation under shock wave stimulation analysis

Fe:ZnO NPs were investigated in terms of reactive oxygen species (ROS) generation under remote mechanical stimulation using Electron Paramagnetic Resonance (EPR) spectroscopy coupled with the spin-trapping technique. More in detail, 1.5  $\mu\text{g}$  of Fe:ZnO NPs were withdrawn from the ethanolic stock solution and dispersed in 90  $\mu\text{L}$  of water. The dispersion was placed in a 96-well plate for cell culture (TC-Treated, Corning). Then 10  $\mu\text{L}$  of a water solution of a spin trap (5,5-dimethyl-L-pyrroline-N-Oxide, DMPO, Sigma-Aldrich) at a concentration of 100 mM was added to the solution. The final concentration of Fe:ZnO NPs was 15  $\mu\text{g/mL}$  while the DMPO final concentration for each sample was 10 mM. The Fe:ZnO NPs containing well was stimulated from the bottom with a high-energy focalized shock wave (SW) device PW<sup>2</sup> (R. Wolf, ELvation Medical) at an energy flux density of 0.04  $\text{mJ/mm}^2$  (12.5 MPa as maximum pressure peak) and a number of shots equal to 500 with a frequency of 4 shots/s. The well and the transducer were acoustically coupled with a gel (Stosswellen Gel, ELvation Medical GmbH). After the stimulation, a small volume of the NPs dispersion was withdrawn by means of a quartz capillary and analyzed with an EMXNano X-Band spectrometer (Bruker, center field 3426 G, 10 scans, 60 s sweep time). The spectra were processed with Bruker Xenon software (Bruker).

#### 2.3.5. ZnO and Fe:ZnO dissolution in cell culture medium

The dissolution of Fe:ZnO NPs into zinc cations in cell culture medium at 37 °C was also evaluated. Fe:ZnO NPs at a concentration of 1  $\text{mg/mL}$  were dispersed in cell culture medium. Then, 70  $\mu\text{L}$  of this dispersion was placed in the cap of a 1.5 mL centrifuge tube, opportunely separated by a dialysis membrane (SnakeSkin dialysis Tubing 3.5K MWCO, 16 mm dry I.D. by Thermofisher Scientific) from the rest of the tube to avoid NPs leakages. Then, 630  $\mu\text{L}$  of cell

1 culture medium was added in the tube, in contact with the NPs solution through the dialysis  
2 membrane so as to bring the NPs concentration in the overall solution to 100 µg/mL and to allow  
3 only ions exchange. The tube was placed upside down in an orbital shaker at 37 °C for different  
4 incubation time steps (up to 72 h). At the end of each incubation time, the solution contained in  
5 the main tube (i.e. without NPs) was withdrawn and stored at 4 °C up to further analysis. The  
6 amount of zinc atoms (derived from dissolved zinc cations) was evaluated through Graphite  
7 Furnace Atomic Absorption Spectroscopy (GF-AAS) following EPA method 289.1.

#### 8 2.4. Cell culture, viability and internalization assays

##### 9 2.4.1. Fe:ZnO and ZnO cytotoxicity

10 Fe:ZnO and ZnO NPs were tested in terms of cytotoxicity on a PDAC cell line, i.e. BxPC-3 (ATCC  
11 CRL-1687) and on the healthy counterpart (HPDE-H6c7, human pancreatic duct epithelial cells,  
12 Kerafast, Inc., Boston, MA). For daily cell culture, cells were cultured at 37 °C and 5% of CO<sub>2</sub> in  
13 RPMI 1640 medium (ATCC) supplemented with 10%v of previously heat-inactivated fetal bovine  
14 serum (FBS, ATCC), 100 µg/mL of streptomycin and 100 units/mL of penicillin (Sigma-Aldrich).  
15 Cytotoxicity tests were performed in 96-well plates (TC-treated, Corning) with the WST-1  
16 proliferation assay (Roche). More in detail, 2500 cells dispersed in 100 µL of cell culture medium  
17 were seeded in each well and let adhere on the bottom of the well. After 24 h of incubation, Fe:ZnO  
18 and ZnO NPs were directly taken from the ethanolic stock solution (2 mg/mL and 1 mg/mL for  
19 Fe:ZnO and ZnO NPs respectively) and dispersed at different concentrations in fresh culture  
20 medium (10, 15, 20 and 25 µg/mL of NPs). The NPs dispersions were then administered to cells  
21 by simple cell culture medium substitution.

The cytotoxicity was evaluated at different times of incubation (24, 48 and 72 h). In particular, 10  $\mu$ L of WST-1 reagent were added to each well 2 hours before the expiration of the incubation time step in question. A plate reader (Multiskan GO microplate spectrophotometer, Thermo-Fisher Scientific) was exploited for the measurements. More in detail, the absorbance of the samples was evaluated at 450 nm ( $A_{450}$ ), while the one at 620 nm ( $A_{620}$ ) was used as reference. For all the samples, the absorbance value obtained by the cell culture medium with no cultured cells ( $BK_{450}$ - $BK_{620}$ ) was subtracted from the actual measure; the resulting value was then divided by the absorbance of the control cells ( $CT_{450}$ - $CT_{620}$ ), in order to obtain a percentage value, as reported in other works<sup>46,50</sup>. More explicitly, the formula leading to the cell viability percentage evaluation ( $C_{\%}$ ) is:

$$C_{\%} = 100 \cdot \frac{(A_{450} - A_{620}) - (BK_{450} - BK_{620})}{(CT_{450} - CT_{620}) - (BK_{450} - BK_{620})}$$

#### 2.4.2. Fe:ZnO and ZnO internalization in the BxPC-3 cell line

The internalization of Fe:ZnO and ZnO NPs in BxPC-3 cells was evaluated through flow cytometry. To do so,  $3 \times 10^5$  cells were seeded in a 24-well plate (TC treated, Thermo Fisher) with 500  $\mu$ L of cell culture medium. After 24 h from cell plating, the medium was replaced with cell culture medium containing NPs (10  $\mu$ g/mL for pure ZnO NPs, 10 and 15  $\mu$ g/mL for Fe:ZnO NPs). The NPs had been previously labelled with ATTO647-NHS ester, as described in a previous work<sup>57</sup>, and their uptake by BxPC-3 cell line was evaluated after 5 and 24 h. The aim was to determine the timing at which the majority of BxPC-3 was able to internalize the administered NPs, to maximize the effects of further treatments. At the end of the incubation time, the cells were washed twice with PBS to remove non-internalized NPs and then detached through trypsinization. Once collected, cells were centrifuged (130 g for 5 minutes) and resuspended in 300  $\mu$ L of PBS.

The cell suspension was analyzed through a Guava EasyCyte 6-2L flow cytometer (Merk Millipore), following the same procedure already described elsewhere<sup>50</sup>. The analysis of the results was performed with InCyte Software (Merk Millipore).

All biological tests were performed at least in triplicates and ANOVA tests were performed with the software Origin (OriginLab).

The internalization of Fe:ZnO NPs inside BxPC-3 cells was also analyzed through spinning-disk confocal fluorescence microscopy (Ti2 Nikon equipped with Crest Large FOV laser and 60x PlanAPO objective, NA = 1.40) to locate the position of the NPs inside the cell. To do so,  $1 \times 10^4$  cells were seeded into 8-well chamber slides (Nunc Lab-Tek II CC2 Chamber Slide System, Thermo Fisher Scientific) with 250  $\mu$ L of complete cell culture medium. ATTO647-NHS labelled Fe:ZnO NPs were then administered to cells (15  $\mu$ g/mL) 24 h from seeding. 24 h later, cells were fixed by replacing cell culture medium with 150  $\mu$ L of Image-IT fixative solution (Thermo Fisher). After 10 minutes at room temperature, cells were washed twice with phosphate-buffered saline (PBS) solution and their membranes were stained by incubating them with 250  $\mu$ L of PBS containing Wheat Germ Agglutinin conjugated with an Alexa Fluor 488 dye (WGA-488, Thermo Fisher) at a concentration of 2.5  $\mu$ g/mL for 10 minutes in normal cell culture conditions. After this time, cells were washed twice with PBS and then Hoechst (Thermo Fisher), at a concentration of 0.3  $\mu$ g/mL in PBS, was administered to cells for nuclei staining. After 5 minutes at 37°C, cells were washed twice with PBS and Live Cell Imaging (LCI, Molecular Probes) solution was finally added. The samples were immediately analyzed following staining.

## 2.5. Shock wave treatment

The behavior of BxPC-3 cells subjected to mechanical stimulation coupled with the administration of the safe dose of Fe:ZnO NPs was analyzed. Cells were seeded in a 96-well plate adopting the same procedure previously described, following a specific plate layout designed to avoid cross-mechanical stimulation, i.e. leaving at least one well without cells between two nearby samples.

Cells were treated with the highest safe dose of Fe:ZnO NPs (15  $\mu\text{g/mL}$ ) and, after 24 h from NPs administration, they were mechanically stimulated with the PW<sup>2</sup> shock waves generator. The same power conditions and stimulation times exploited during ROS generation analysis were here employed (0.04  $\text{mJ/mm}^2$ , 500 shots, 4 shots/s). In particular, three main stimulation typologies were set. In the first one, cells were only treated with the shock waves, in absence of NPs, to assess the toxicity of the SW treatment itself. The second typology consisted in the stimulation of cells that were first treated with Fe:ZnO NPs and then deprived of non-internalized NPs by means of a double washing with PBS and its replacement with freshly-prepared cell culture medium just before the SW treatment (Fe:ZnO Int). Finally, the third typology considered the stimulation of cells that were treated with Fe:ZnO and mechanically stimulated after 24 h from the NPs administration without any washing steps (Fe:ZnO Int+Ext). This last sample aimed at the exploitation of both intracellular and extracellular NPs for cells killing.

BxPC-3 cell viability was assessed with the WST-1 assay 24 h after the mechanical stimulation and compared to that of untreated cells, set as 100 % of viability.

## 2.6. NPs dissolution test and cell death mechanisms assays

### 2.6.1. Free zinc cations detection in cells without mechanical stimulation

An evaluation of the free zinc cations, due to intracellularly dissolved NPs after their internalization and in absence of any mechanical treatment, was initially performed. Cells were



1 plated in chamber slides, treated with Fe:ZnO NPs and fixed (see details above). The intracellular  
2 free zinc cations were labelled through FluoZin-3 AM (Thermo Fisher) probe: cells were washed  
3 twice with PBS and a 1  $\mu$ M solution of FluoZin-3 AM fluorescent dye in PBS was administered.  
4 After 30 minutes of incubation at 37 °C, the excess of dye was removed by two washing steps with  
5 PBS. Then, cells lysosomes were labelled by substituting the PBS with a 1  $\mu$ M dye solution  
6 (LysoTracker Red DND-99, Thermo Fisher) in PBS and incubating the cells for 30 minutes at  
7 room temperature. After two further washing steps, cell membranes and cell nuclei were stained  
8 with WGA-647 (Thermo Fisher) and Hoechst, respectively, following the same procedure reported  
9 above. Images were collected with the spinning disk confocal microscope, keeping exposure times  
10 and laser powers constant among the different samples. They were then post-processed in the same  
11 way, to allow a direct comparison between the samples in terms of free zinc intensity fluorescence  
12 too.

#### 13 2.6.2. Effect of shock wave mechanical stimulation on BxPC-3 cells fate

14 To unravel the mechanism of cell death under SW stimulation, cells were plated in clear 96-well  
15 plates following the same protocol exploited for cytotoxicity assays. Cells were then treated with  
16 Fe:ZnO NPs and shock waves. Free zinc cations were labelled again with FluoZin3-AM, while the  
17 cell membrane integrity was assessed with Propidium Iodine (PI, ThermoFisher) using 100  $\mu$ L of  
18 1  $\mu$ M of PI solution in cell culture medium for 5 minutes at 37 °C. Then, cells were washed three  
19 times with PBS. The PBS was finally substituted with Live Cell Imaging solution and cells were  
20 imaged with a wide-field inverted fluorescence microscope (Eclipse TiE from Nikon) equipped  
21 with 40 $\times$  objective (NA = 0.60).

#### 22 1.1.1. Kinetics of BxPC-3 cells necrosis and apoptosis

The kinetics of both cell necrosis and apoptosis was evaluated through the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega) coupled with the microplate reader Glomax (Promega). More in detail, BxPC-3 cells were plated in a black 96-well plate with clear bottom (Corning) and treated with the same protocol exploited for SW treatments in the previous tests. Just before the SW treatment, 100  $\mu$ L of the 2x Detection Reagent freshly prepared in RPMI 1640 according to the manufacturer indications was added to all the samples. A first measurement was performed before SW treatments. Then, the measurements were repeated immediately after the SW treatment and after 30 minutes, 1 h, 2 h, 4 h, 6 h and 24 h from the treatment. Both the fluorescence and the luminescence signals retrieved from the measurements were analyzed, as recommended by the manufacturer. In particular, the obtained data were processed as fold induction of the signal obtained from the cells without any NPs and those subject to SW treatment, according to the formula:  $\text{SampleSignal}/\text{ControlSignal}$ .

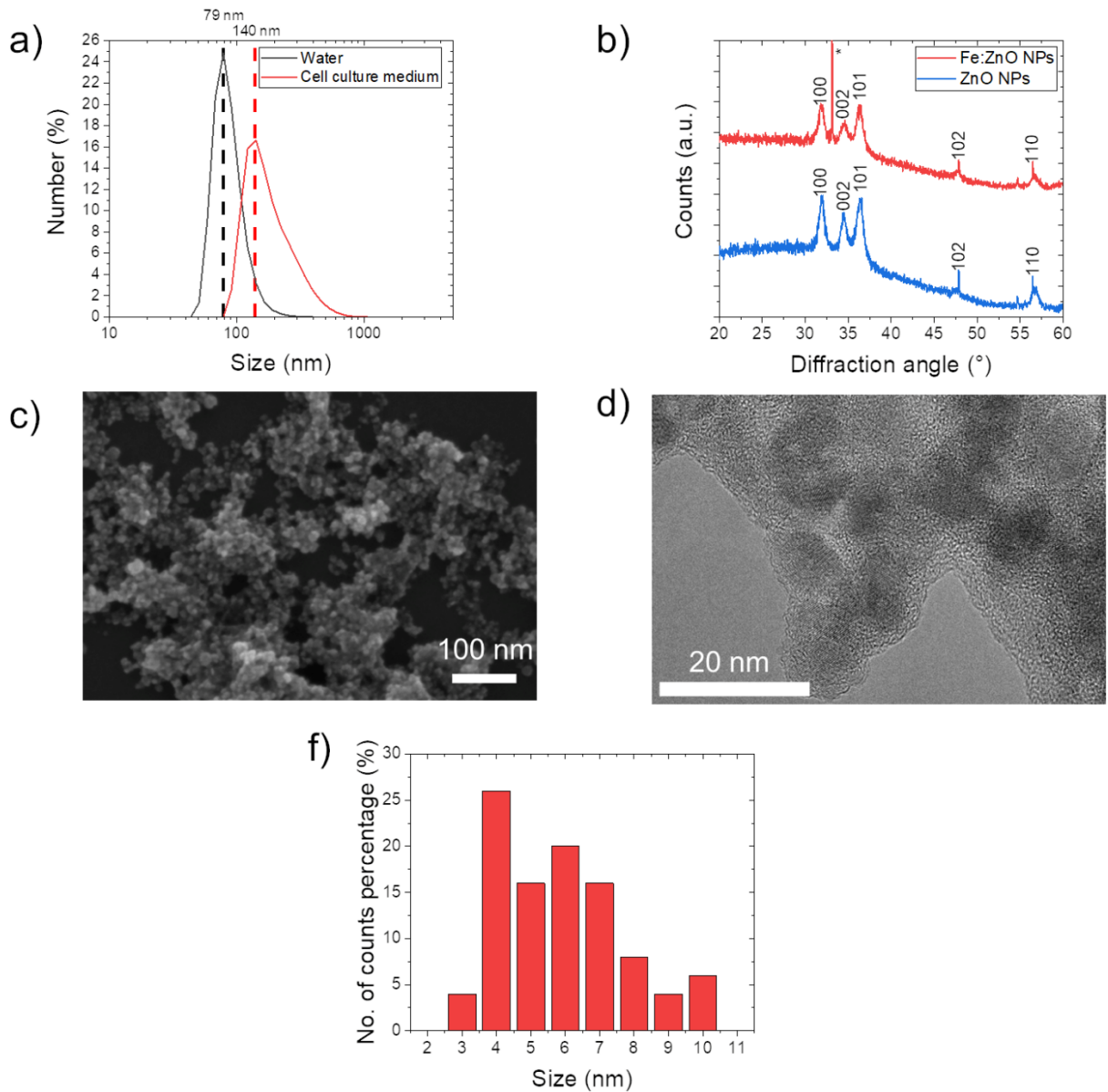
## 2. Results and discussion

### 2.1. Physical and chemical characterization

Fe:ZnO NPs were characterized in terms of crystalline structure, morphology and hydrodynamic behavior in both water and cell culture medium (Figure 2) and compared to their undoped counterparts ZnO NPs (Figure S1 in the Supporting Information, S.I.). Fe:ZnO NPs present an average hydrodynamic diameter weighted on the scattered light intensity of 136.6 nm with a polydispersity index (PDI) of 0.138, indicating a monodisperse population<sup>55</sup>. A higher diameter is observed when NPs are immersed in the cell culture medium. Indeed, the hydrodynamic diameter observed with the DLS is 249.6 nm. The reason of this behavior may be attributed to a larger degree of aggregation of the NPs in a more complex medium than pure water, caused by the

1 interaction of the Fe:ZnO NPs with proteins, amino acids and biomolecules in general<sup>56</sup>. The PDI  
2 in cell culture medium is higher than that obtained in water (PDI of 0.264 and 0.110, respectively),  
3 indicating a higher polydispersity which is however still acceptable to guarantee a homogeneous  
4 NPs solution to be administered to the target cells.

5 A similar trend is observed for the undoped ZnO NPs, which present a hydrodynamic diameter of  
6 116.2 nm in water and 386.6 nm in cell culture medium, with a PDI of 0.148 and 0.394,  
7 respectively (Figure S1 of the S.I.). When measured in water, the size of undoped ZnO results  
8 lower than that of the iron-doped NPs. As previously stated, instead, the NPs behaviour in cell  
9 culture medium may be influenced by the interaction with various biomolecules and therefore  
10 results in a lower stability. In particular, undoped NPs possess both a higher PDI and  
11 hydrodynamic diameter with respect to the Fe:ZnO ones. This difference in stability is further  
12 corroborated by the Z-potential measured for the two typologies of NPs:  $25.9 \pm 0.9$  mV for Fe:ZnO  
13 NPs and  $22.7 \pm 0.9$  mV for ZnO NPs. The higher and positive Z-potential presented by the doped  
14 NPs is very likely increasing the coulombic repulsion among the nanoparticles, enhancing in turn  
15 their colloidal stability in solution.



**Figure 2.** Fe:ZnO NPs characterization: a) hydrodynamic diameter distribution in bidistilled water and cell culture medium obtained by DLS measurements; b) XRD pattern (\* refers to peaks belonging to the silicon substrate); c) representative FESEM image, d) representative TEM image and f) histogram of TEM size of Fe:ZnO NPs.

The crystalline structure of Fe:ZnO and pure ZnO NPs was investigated through XRD analyses. In both cases, the XRD patterns (Figure 2b for Fe:ZnO and Figure S1b for ZnO NPs) present the

1 typical diffraction peaks ascribable to the wurtzitic crystal structure of ZnO (JCPDS-ICDD, card  
2 No. 89-1397). Either way, the peaks are quite broadened, having a crystallite dimension of 10 nm  
3 and 11 nm, estimated for the 100 plane by means of the Debye-Sherrer formula<sup>58</sup>, for Fe:ZnO and  
4 ZnO NPs, respectively. The results also show no major differences in the crystalline structure  
5 caused by the introduction of defects sites due to iron doping. Thus, the Fe:ZnO pattern confirms  
6 the absence of any peaks related to secondary phases of possible other iron compounds.

7 The crystallite dimensions calculated through the XRD analysis are in agreement with electron  
8 microscopy images of Figure 2c and 2d. The morphology assumed by Fe:ZnO NPs is spherical,  
9 with a diameter ranging from 4 to 10 nm calculated by FESEM and TEM (as depicted by the  
10 histogram in Figure 2f). This size range corresponds to the crystallite dimension obtained from  
11 XRD and allows the assumption of monocrystalline nanoparticles. This is also confirmed by the  
12 TEM image (Figure 2d), in which some of the nanocrystals can be recognized. The discrepancies  
13 between the diameter found by means of electron microscopy and DLS measurements should be  
14 attributed to a certain level of aggregation that the NPs may display in the liquid media; moreover,  
15 the two instruments measure different parameters, namely the actual and the hydrodynamic  
16 diameter of the nanoparticles. In this case as well, from the morphological point of view, no visible  
17 difference between doped and undoped NPs can be appreciated.

18 To confirm the correct inclusion of iron in the ZnO NPs, EDS was performed. The results, reported  
19 in Table S1 of the S.I., show that the atomic ratio between iron and zinc atoms inside ZnO crystals  
20 is 0.0507. This result is fairly in agreement with the precursor ratio exploited during the synthesis  
21 (0.06) and confirms the inclusion of the doping elements inside the crystalline structure, in  
22 accordance with both the results obtained by XRD analyses and a previous study concerning iron  
23 doped NPs, synthesized by some of our group<sup>54</sup>. In the work in question, it was also demonstrated

that iron assumes both the  $\text{Fe}^{+2}$  and  $\text{Fe}^{+3}$  states, with a slight preponderance of  $\text{Fe}^{3+}$  ions for the NPs doped with a 6 at% of iron with respect to zinc.

Moreover, the inclusion of iron inside the Fe:ZnO NPs crystal gathers novel magnetic responsiveness with respect to the pure ZnO NPs counterparts (as reported in Figure S2 of SI) in terms of maximum magnetization, with consequent potentialities in magnetic resonance imaging (MRI). This result is in agreement to what was found in the literature, where this magnetic behavior is attributed to the introduction of magnetic atoms (Fe) in the ZnO crystal<sup>54,59–61</sup>.

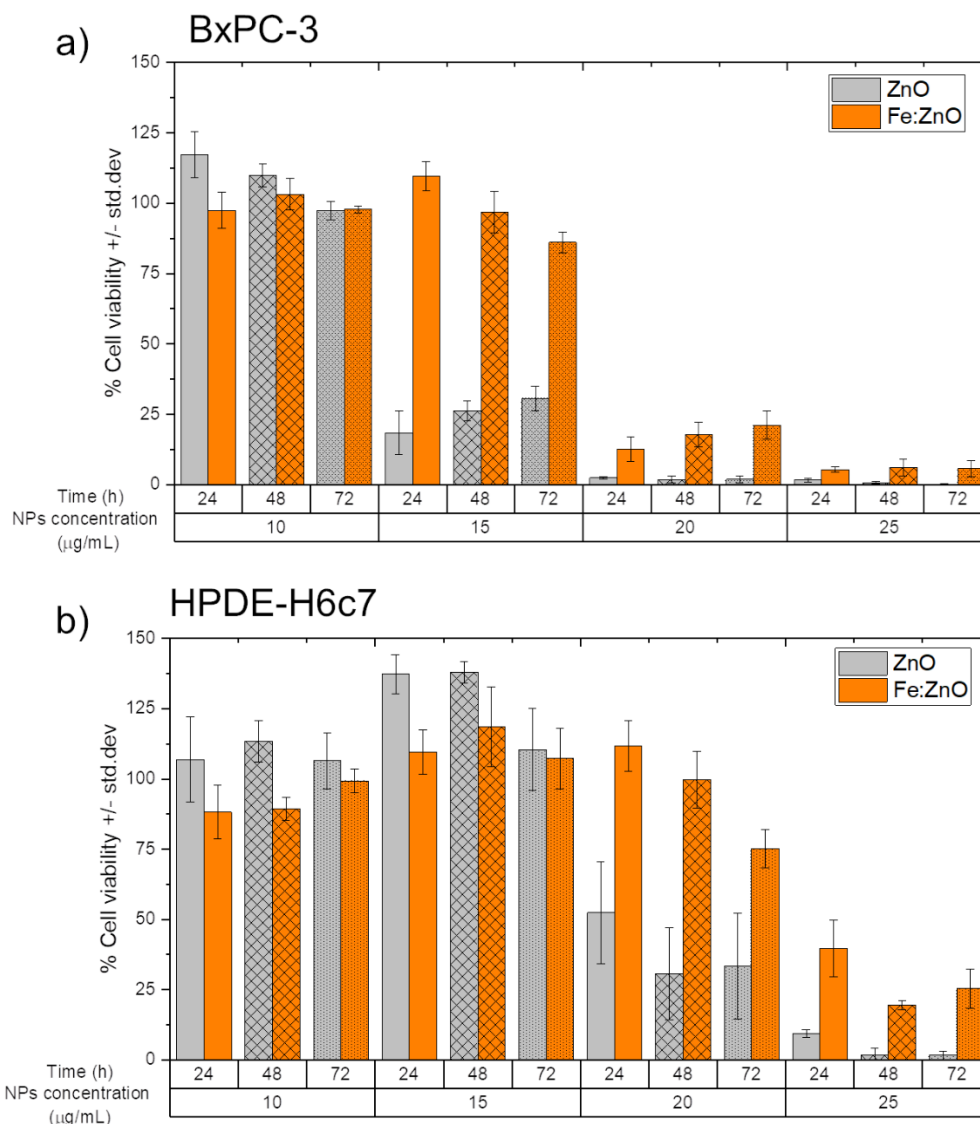
## 2.2. Cytotoxicity of pure and iron-doped ZnO NPs

Fe:ZnO and ZnO NPs were administered to BxPC-3 cells in order to assess their cytotoxicity and to understand which dose can be exploited for further treatments with acoustic stimulation. As a general trend, both NPs types show a dose-dependent cytotoxicity which does not vary sensibly depending on the incubation time, as shown in Figure 3a. In particular, the undoped ZnO NPs are safe up to 10  $\mu\text{g/mL}$  for all the considered incubation times in the present study. In contrast, the toxicity abruptly increases already at 15  $\mu\text{g/mL}$ , with cell viability close to 20 % compared to the control samples. Moreover, a slight viability increase in time can be observed, with values always below 40 %.

A similar trend is observed for Fe:ZnO NPs, however, the highest safe dose is extended up to 15  $\mu\text{g/mL}$  for the timesteps considered in the present study. The dose-dependent toxicity of ZnO is already well known in the related literature<sup>57,62,63</sup> and has been mainly attributed to two mechanisms: ZnO dissolution into  $\text{Zn}^{2+}$  ions, able to disrupt the cell homeostasis leading to cell death, and the oxidative stress generated by the nanoparticles due to ROS production. Iron doping has been demonstrated to reduce the dissolution of ZnO nanoparticles, providing them with a

1 higher stability in aqueous media<sup>64,65</sup>. This translates into a higher safety of the nanoparticles,  
2 which has also been evidenced in this work when Fe:ZnO NPs are compared to their undoped  
3 counterparts. A lower toxicity also opens the possibility of administering higher doses of NPs, that  
4 are therefore more likely to induce toxic phenomena under external stimulation (i.e., shock waves).

5 In this particular case, the highest safe dose, defined as the amount of NPs that can be administered  
6 without significantly damaging cells prior external stimulation, is increased by 50 % thanks to the  
7 iron doping (i.e., from 10  $\mu\text{g/mL}$  to 15  $\mu\text{g/mL}$ ). Furthermore, the iron-doped ZnO NPs show  
8 magnetic properties, not present in the undoped counterpart, as reported in previous works<sup>59,60,66</sup>.  
9 This is also true for the herein analyzed Fe:ZnO NPs, whose maximum magnetization is higher  
10 than the one measured for pure ZnO NPs (Figure S2). Such magnetic property is exploitable to  
11 employ them as contrast agents under MRI, yet leading to an intrinsically theranostic nanoparticle.  
12 For all these reasons, only iron-doped nanoparticles will be investigated henceforth when the  
13 mechanical stimulation is considered.



1

2 **Figure 3.** ZnO and Fe:ZnO NPs cytotoxicity on a) BxPC-3 and b) HPDE-H6c7 cells at different

3 incubation times from NPs administration, calculated from the results of the WST-1 assay. The

4 value reported by the bars represents the mean  $\pm$  std.dev percentage of  $n \geq 3$  measurements with

5 respect to control cells. The statistical analysis results are reported in the SI.

6 Despite a proper targeting mechanism should be implemented on the NP surface, cytotoxicity tests

7 on non-cancerous cells demonstrated an intrinsic selectivity of the NPs toward cancer cell killing.

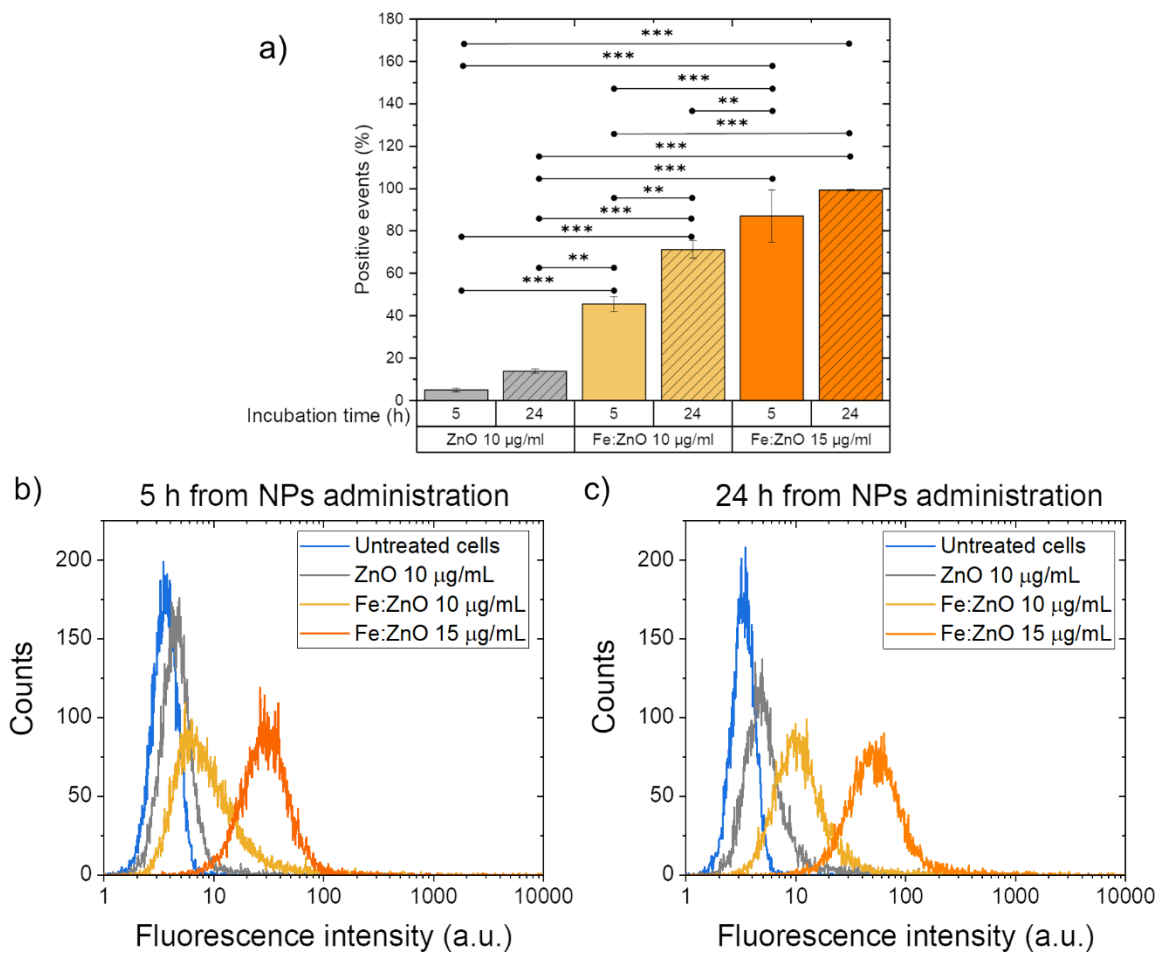


Figure 3b reports the results of the cytotoxicity experiments held on normal cells (HPDE-H6c7 cells). As it is clearly visible, with respect to what was found for cancerous cells, a higher dose of both ZnO and Fe:ZnO NPs can be administered to normal cells without affecting normal cell viability. Moreover, Fe:ZnO NPs are confirmed to be less toxic than ZnO NPs for normal cells too, further justifying their use during future treatments.

### 2.3. Fe:ZnO NPs internalization

ZnO and Fe:ZnO NPs were also investigated from the internalization standpoint. The ability of the BxPC-3 cell line to internalize the administered NPs was analyzed with cytofluorimetry and fluorescence microscopy.

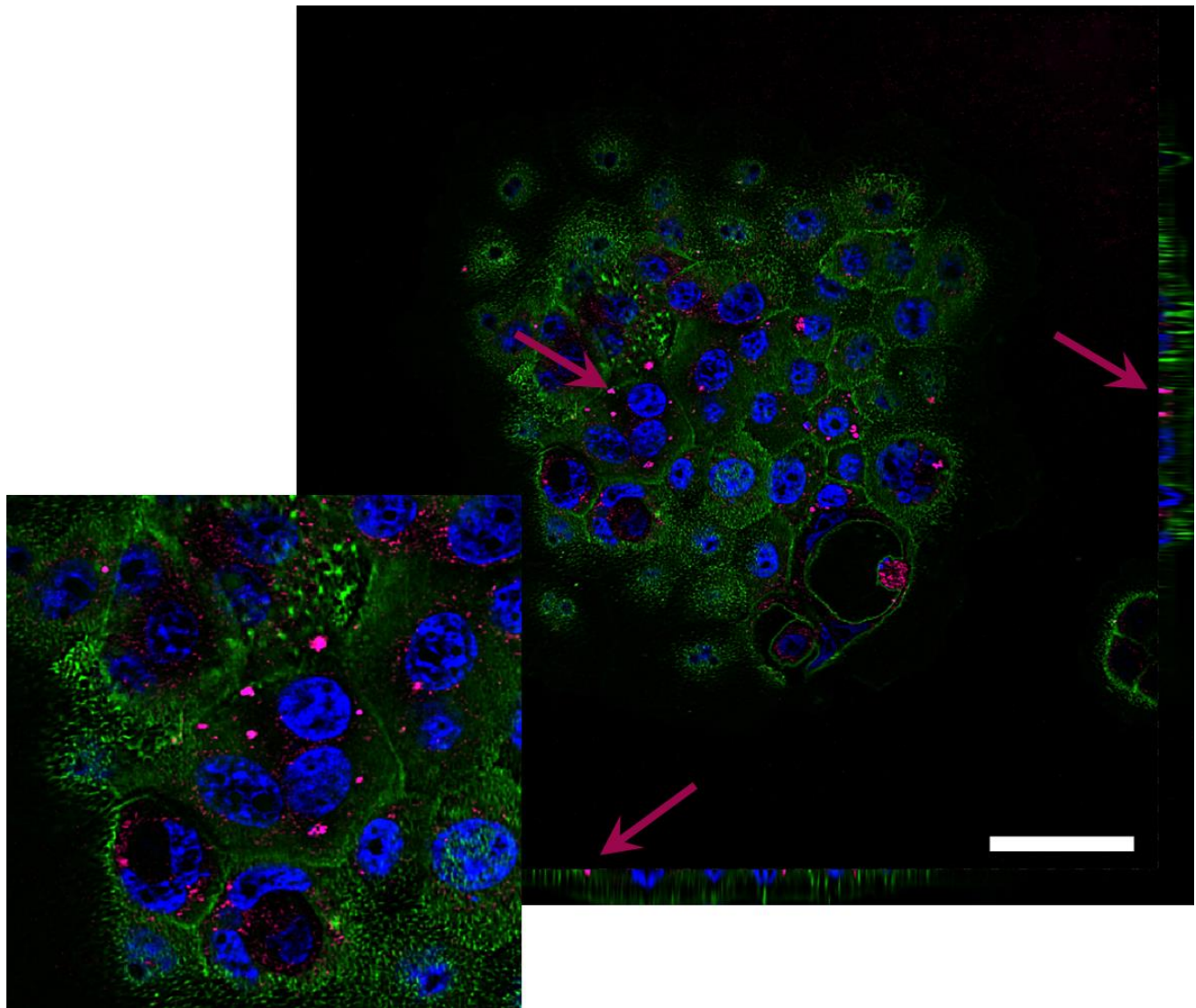
In particular, cytofluorimetric assays (Figure 4) indicate that a higher percentage of cells (positive events) is able to internalize (or adsorb at their surface) the Fe:ZnO NPs when compared to pure ZnO NPs. This behavior was already reported in similar conditions by our group<sup>54</sup>. It has been also reported in the literature that a high positive Z-potential can favor NPs internalization into cells, which typically possess a negatively charged cell membrane<sup>67–69</sup>. Here, the higher Z-potential of iron-doped nanoparticles, together with the larger exploitable dose due to their increased safety, may explain the superior internalization in cells with respect to the undoped ZnO.



**Figure 4.** a) BxPC-3 cells measured as positive events due to the internalization or immobilization at the outer cell membrane of ZnO and Fe:ZnO NPs. Data are reported at different incubation times from NPs administration, calculated through cytofluorimetric assays. The values reported by the bars represent the mean  $\pm$  std.dev. percentage of  $n = 3$  measurements with respect to control cells. The comparisons between the different treatments were performed using two-ways ANOVA. \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ . Representative histograms of the fluorescence intensity of cells measured through the cytofluorimetric assays exploited to evaluate the NPs internalization at b) 5 h and c) 24 h from NPs administration.

1 From the results of the cytofluorimetric analysis it can be observed that the percentage of positive  
2 events, namely cells with an increased fluorescence, improves with time and dose. In particular,  
3 the percentage of cells with a higher fluorescence signal than the control cells approaches 100 %  
4 after 24 h from 15  $\mu\text{g/mL}$  Fe:ZnO NPs administration, indicating that all the cells exhibit NPs on  
5 their cell membrane or inside of them. This result justifies and supports the use of these NPs dose  
6 and incubation time for further mechanical stimulation with shock waves in cells.

7 Spinning disk confocal fluorescence microscopy was exploited to localize Fe:ZnO NPs inside  
8 the cells. Figure 5 reports a representative image of BxPC-3 cells incubated with 15  $\mu\text{g/mL}$  of  
9 Fe:ZnO NPs. Despite the nanosized nature of the NPs, which dramatically reduces the possibility  
10 to clearly resolve them, it is still possible to locate the presence of some aggregates inside the cells,  
11 thus obtaining information about the NPs intracellular location. In particular, the majority of the  
12 NPs can be spotted inside the cell membrane but outside the cell nucleus, suggesting that the  
13 internalization process allows the permeation of the nanoparticles only inside the cytoplasm.



**Figure 5.** Representative image of Fe:ZnO NPs internalized in BxPC-3 pancreatic cancer cells after 24 h of incubation from NPs administration. Cell membranes (green), nuclei (blue) and Fe:ZnO NPs (purple) are evidenced in the image. Scalebar is 50  $\mu$ m.

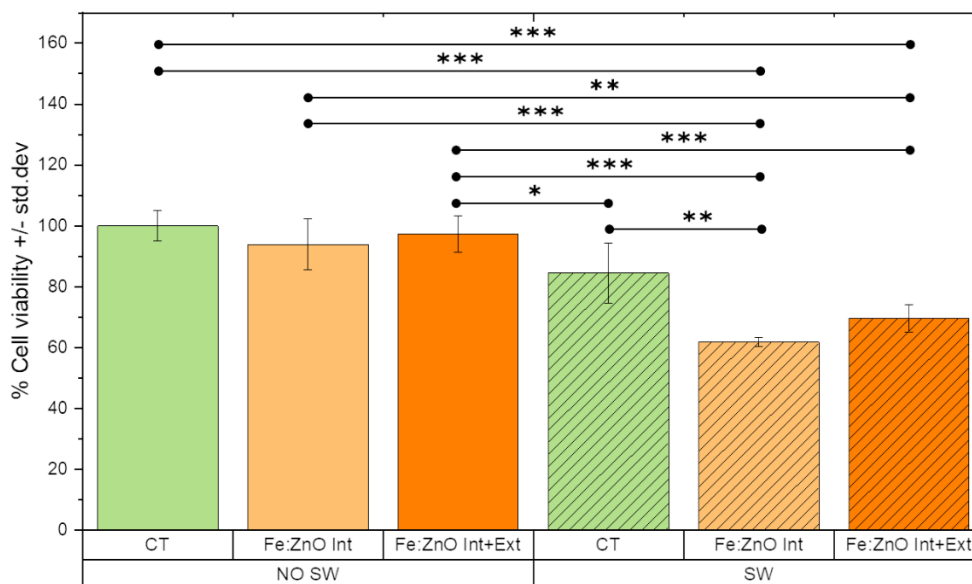
#### 2.4. Effects of shock waves coupled with Fe:ZnO NPs on pancreatic cancer cells

The final aim of the proposed NPs is to provide a tool that can be externally activated by means of a remote mechanical stimulation, to induce cell death in a diseased tissue. Pancreatic cancer cells were exploited as a tumor model for this purpose. Indeed, the highest safe dose of Fe:ZnO NPs

1 was administered to cells and, after 24 h of incubation, the cells were stimulated with SW in two  
2 different conditions: in the presence of NPs internalized in the cells and in the extracellular space,  
3 i.e. in cell culture medium (Int+Ext NPs) or with internalized NPs alone (Int NPs).

4 The cell viability was evaluated after 24 h from NPs incubation and mechanical stimulation (Figure  
5 6). As expected from the cytotoxicity tests, Fe:ZnO NPs are safe when no SWs are co-  
6 administered. Furthermore, there is no difference in viability between the cells where only  
7 internalized NPs are considered (Fe:ZnO Int) and those where both internalized and non-  
8 internalized NPs were retained (Fe:ZnO Int+Ext). Also, the mechanical stimulation with SW does  
9 not significantly affect cell viability per se, i.e. when NPs are not administered to cells.

10 Remarkably, a statistically higher toxicity with respect to the control samples is achieved when  
11 the two treatments (i.e., NPs and SW administration) are combined together. In particular, the  
12 viability of cells treated with Fe:ZnO Int and SW is  $61.8 \pm 1.5$  % with respect to the untreated  
13 cells; furthermore, a statistically relevant difference between this synergistic treatment and either  
14 the single NPs administration or the solely SW stimulation is evidenced. When the cells are treated  
15 with SW and Fe:ZnO Int+Ext, the viability is  $69.6 \pm 4.5$  % with respect to the control. The  
16 difference among the two types of NPs treatments is not statistically significant in terms of cell  
17 viability. This result suggests that most of the toxic effects could be attributed to the internalized  
18 NPs rather than to the extracellular ones. However, to determine the actual mechanism involved  
19 in cell death, further studies are required, as reported below.



**Figure 6.** Cell viability percentages, with respect to untreated BxPC-3 cells, in the presence of Fe:ZnO NPs and SW stimulation. The graph shows the difference between Shock Wave-treated (SW in the graph) and untreated (NO SW in the graph) cells and the difference among control cells (CT), cells treated with only internalized NPs (Fe:ZnO Int in the graph) and with NPs internalized in cells and in the extracellular space (Fe:ZnO Int+Ext in the graph). The values reported by the bars represent the mean  $\pm$  std.dev percentage of  $n \geq 3$  measurements with respect to control cells. The comparisons between the different treatments were performed using three-ways ANOVA. \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ .

## 2.5. Toxicity mechanisms

There are typically three toxicity mechanisms that could be involved in cell death due to the presence of both ZnO NPs and shock waves: (i) ROS generation and consequent oxidative stress; (ii) ZnO dissolution in Zn ions; (iii) mechanical cell membrane disruption due to the mechanical

1 wave and the presence of NPs. Here we have investigated all of them to unravel the mechanism of  
2 toxicity due to the combination of NPs and mechanical pressure waves, in the present case SW.

3 A first analysis was performed to assess the extent of ROS generation contributing to this  
4 phenomenon. Ultrasound stimulation has already been reported to induce a high ROS generation  
5 due to gas bubble cavitation trapped on the ZnO surface<sup>49</sup>. However, to our knowledge, the effect  
6 of repeated shock waves has never been analyzed in these terms. EPR spectroscopy was thus  
7 exploited: Fe:ZnO NPs were mechanically stimulated in water and the amount of generated ROS  
8 was analyzed. The results of this measurements (Figure S3 in the S.I.) clearly indicate the absence  
9 of any signal related to the existence of hydroxyl and alkyl radicals, suggesting that none of these  
10 types of ROS are generated due to the SW stimulation, either alone or in combination with NPs.  
11 A possible explanation for this phenomenon might be that the excitation frequency of SW is not  
12 suitable to establish any cavitation phenomena. An alternative explanation can also be that the  
13 DMPO trap is not sensitive to the specific radicals formed in response to this stimulation.  
14 Therefore, ROS generation, if potentially present, may not be provoked by the combination of SW  
15 and NPs, and thus it is excluded from being the main responsible for the observed cell death.

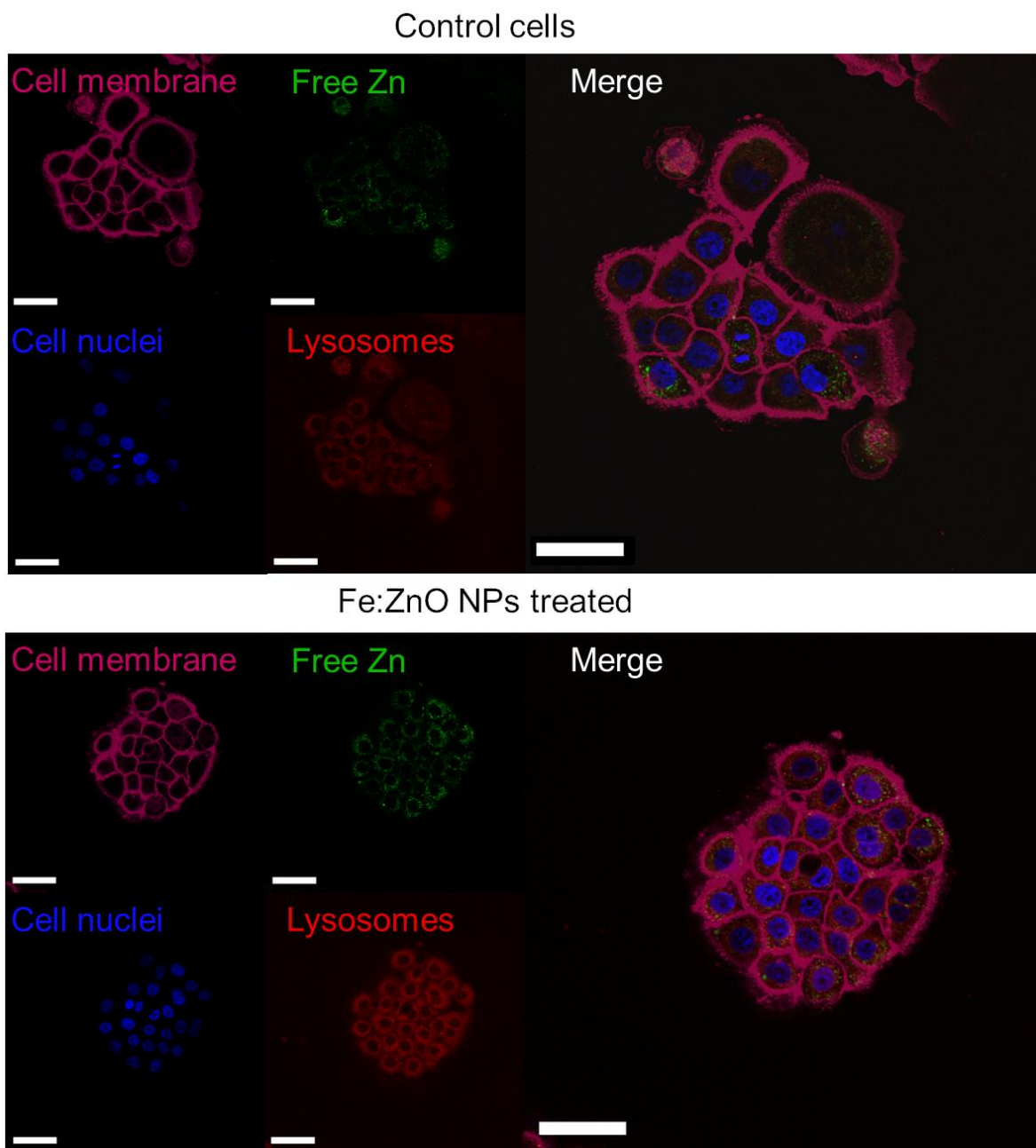
16 A second analysis was performed to evaluate the dissolution of the Fe:ZnO NPs inside cell culture  
17 medium, first in absence of SW and then with their mechanical stimulation in the presence of  
18 living cells.

19 Preliminarily, the Zn ions dissolved in RPMI 1640 were measured at different times of incubation  
20 at 37 °C, in absence of cells. The results (Figure S4 in the S.I.) suggest that the NPs dissolution is  
21 almost immediate and approximately corresponding to 60 %, with no relevant differences between  
22 the various incubation times. It is therefore assumed that an equilibrium is probably reached very  
23 soon in these conditions.

1 Fe:ZnO NPs dissolution and the intracellular permeation of the resulting  $\text{Zn}^{2+}$  ions were  
2 also evaluated through fluorescence microscopy assays. In particular, a fluorescent dye was used  
3 to label free zinc inside the cells and another fluorescent dye was exploited to label lysosomes and  
4 to determine the location of the retained zinc inside the cell. Figure 7 shows a representative image  
5 of a control group of BxPC-3 cells without any NPs treatment, in comparison with Fe:ZnO NPs  
6 treated cells. As it can be noticed, the control group already presents a fluorescence signal in the  
7 green channel, indicating that  $\text{Zn}^{2+}$  ions are physiologically present inside the cells. This is  
8 expected from previous literature evidence, being  $\text{Zn}^{2+}$  ions important in a series of physiological  
9 signalling pathways<sup>70–72</sup>, and therefore this fluorescence signal should be considered as a basal  
10 level.

11 When cells are treated with Fe:ZnO NPs, the obtained fluorescence signal corresponding to zinc  
12 ions is brighter than the control one. It is clear that  $\text{Zn}^{2+}$  ions are released by the nanoparticles and  
13 permeate the cells, probably affecting the intracellular homeostasis equilibrium and, in case of an  
14 excessive dose, eventually inducing cell death. Another aspect, emerged from this analysis, is the  
15 increase in intensity of the signal related to the lysosomes. Indeed, when cells are treated with NPs,  
16 the lysosomes are present in a larger number than in the control cells. If the NPs are trapped in an  
17 acid environment as the one distinctive of lysosomes<sup>73–75</sup> they might dissolve because of the well-  
18 known instability of ZnO in a low pH solution<sup>56,76</sup>. This phenomenon can explain the partial co-  
19 localization of the lysosome tracker with the zinc related signal. After NPs dissolution, it is very  
20 likely that  $\text{Zn}^{2+}$  ions diffuse throughout the cell, altering the cell homeostasis and to some extent  
21 explaining the partial absence of colocalization between the  $\text{Zn}^{2+}$  related signal and the lysosomes'  
22 one.





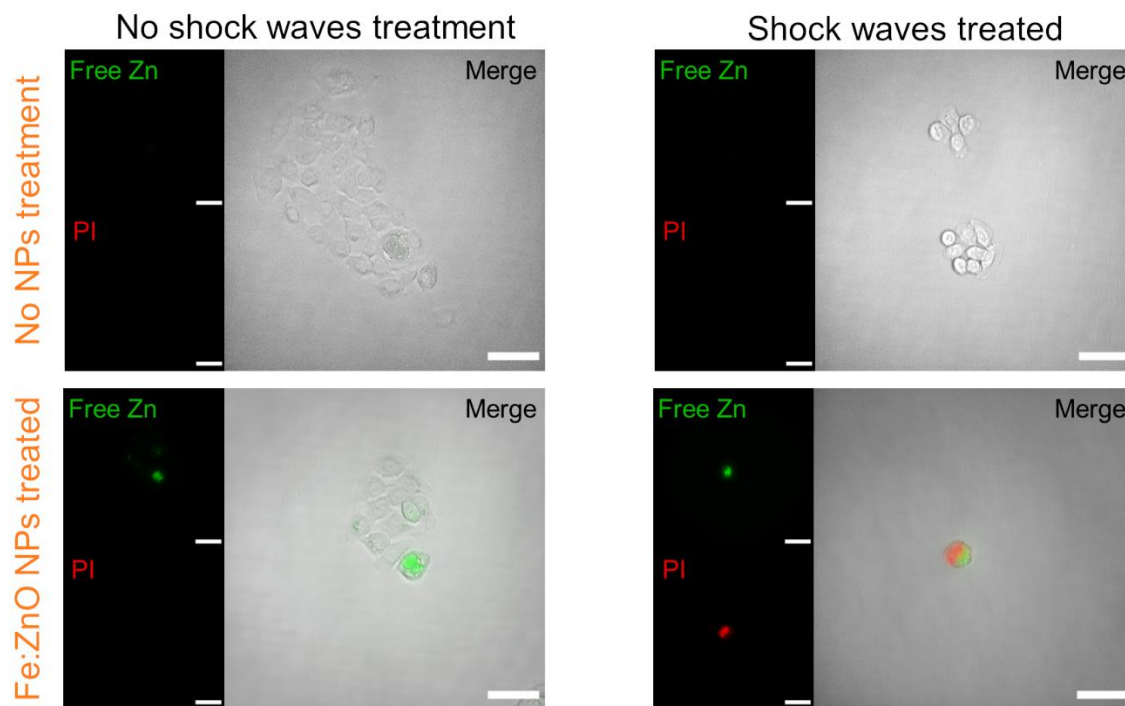
**Figure 7.** Representative fluorescence microscopy images of BxPC-3 cells treated with Fe:ZnO NPs and control ones. In the images, membranes (purple), nuclei (blue), lysosomes (red) and free zinc inside the cells (green) are referred. Scalebar is 50  $\mu\text{m}$ .

1 Further information about the cells' fate after the synergistic treatments can also be obtained by  
2 staining cells with both the zinc ions probe and propidium iodide (PI) under fluorescence  
3 microscopy (Figure 8). The PI is a cell impermeant dye, able to emit fluorescence when bound to  
4 the DNA. Therefore, it is detectable only when the cell membrane is compromised, allowing the  
5 dye to permeate it.

6 As expected, when no treatments are administered to cells, the signals related to both  $Zn^{2+}$  ions  
7 and cell membrane disruption are completely absent. Cells are well adherent to the substrate and  
8 viable. The treatment with Fe:ZnO NPs induces an increase in free Zn ions inside the cells, as  
9 already noticed in the previous microscopy analyses, but no changes in viability and in cell  
10 morphology are observed in this case. The PI signal is completely absent, indicating that the cell  
11 membrane is intact, and that cells are not severely suffering for the treatment.

12 When only the SW are administered to the cells, PI and Zn ions probe signals are also absent.  
13 However, the morphology of cells seems slightly rounder than the one observed in the control  
14 samples. Anyway, the absence of PI signal indicates that the cell membrane is still intact and  
15 therefore cells could be still considered viable.

16 When both treatments are applied to the cells, the situation dramatically changes. First, it becomes  
17 very difficult to find a sufficient number of cells in the field of view. This is due to the pronounced  
18 cell death, which causes their removal during the washing steps performed during the staining  
19 procedure. The few remaining cells present both the PI and Zn ions related signals. It is therefore  
20 possible to suppose that in this case cells are suffering for the treatment. Probably, the Zn ions  
21 released by the Fe:ZnO NPs weaken the cell, which in turn becomes more susceptible to the  
22 mechanical stimulation. The final result is cell membrane disruption and the resulting cell  
23 detachment, which turns to cell death shortly afterwards.



**Figure 8.** Representative microscopy images of BxPC-3 cells treated with shock waves and Fe:ZnO NPs. The images highlight the phenomena occurring after the treatment with Fe:ZnO NPs and the shock waves in terms of free zinc present in the cells (green) and membrane integrity (red, PI). Only when both the treatments are applied to cells the two signals are clearly visible. Scalebar is 50  $\mu\text{m}$ .

A qualitative kinetic analysis of cell death was carried out throughout 24 h of incubation starting from the SW stimulation, evaluating two signals, i.e. luminescence and fluorescence. The results are reported in Figure 9. The luminescence signal is related to the exposure of phosphatidylserine outside the cell membrane, which is reported in the literature to indicate apoptosis<sup>77,78</sup>. Conversely, the fluoresce signal is associated to the secondary necrosis, being it based on a cell-impermeant profluorescent DNA dye which can emit a fluorescence only when cell membrane is broken<sup>50</sup>. This assay allows us to determine the cells' death mechanism by the detection of one, both or none

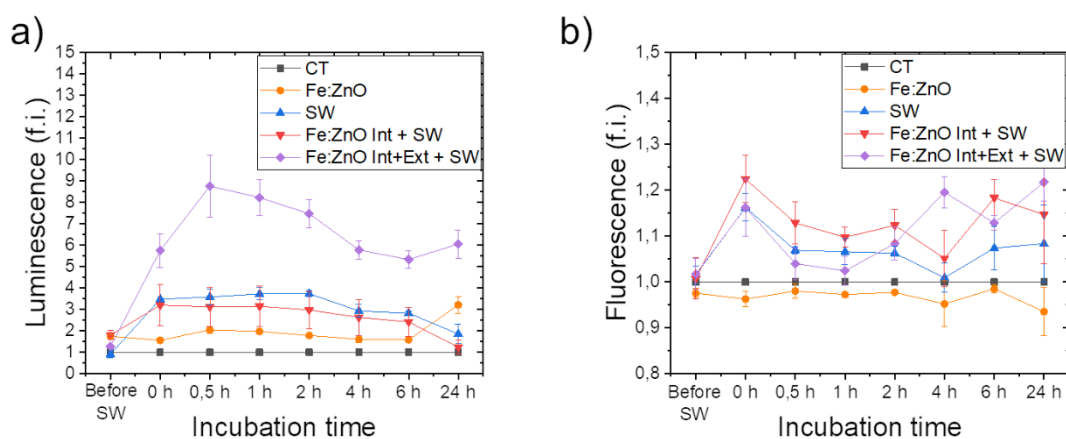
of the above-mentioned signals. In the case under analysis in Figure 9, the signals are all referred to the untreated cells' one. As shown, the luminescence signals of cells treated with only Fe:ZnO NPs (orange curves in Figure 9) are slightly higher than the control one. This indicates that cells are not particularly suffering for the NPs administration, being the signal almost constant all along the measurement time. However, the fluorescence signal is lower than the control for all the considered time steps, excluding the occurrence of necrosis during the incubation time. It is therefore fair to suppose that the cell death mechanism in response to the iron-doped ZnO NPs administration is apoptosis. When cells are treated with SWs alone (blue curves in Figure 9), the situation is slightly different. Indeed, as soon as the SW treatment is completed, both fluorescence and luminescence signals increase with respect to the basal one. This means that there are cells that are going toward alternative forms of cell death, e.g. necrosis, immediately during the treatment. Anyway, despite the luminescence signal remains constant during the 24 hours of analysis, the fluorescence signal initially decreases and raises again only in the last hours of incubation. The reason of this behavior may be attributed to the occurrence of both apoptosis and necrosis at the beginning. Then, the early apoptotic cells undergo late apoptosis, with the consequent secondary increase of the fluorescence signal.

A similar behavior is found for the cells treated with both SW and internalized Fe:ZnO NPs (red curve), which display the increase of both luminescence and fluorescence signals with respect to the basal one. In particular, a slightly enhanced fluorescence signal is reported, qualitatively indicating a higher level of secondary necrosis.

More relevant differences can be found when cells are treated with both SW and Fe:ZnO NPs (both extracellular and intracellular ones), as shown by the violet curves. Indeed, as in the case of SW alone, there is an increase of the luminescence signal just after the SW treatment. However, the

increase is notably higher and continues over the first 30 minutes. The signal decreases over time but still indicates a considerable level of apoptotic events. The fluorescence signal is visible from the very beginning and does not underlie differences with respect to the cells treated with SW alone or with SW in the presence of internalized NPs.

In summary, these analyses allow us to hypothesize that a combination of both apoptosis and necrosis mechanisms is occurring in the first 24 hours of NPs incubation after the SW treatment.



**Figure 9.** Cell death kinetics after SW treatment, obtained through the apoptosis-necrosis assay. In panel (a), the luminescence is reported as fold induction (f.i.) of the control sample, while panel (b) reports the fluorescence as fold induction of the respective control. Cells belonging to the control sample (CT) were treated neither with Fe:ZnO NPs nor SW. BxPC-3 cells were analyzed when incubated with Fe:ZnO NPs (Fe:ZnO sample) without any further treatment, without NPs but treated with SW (SW), treated with SW after a washing aimed at removing the non-internalized NPs that were administered 24 h before (Fe:ZnO Int +SW) and treated with SW after 24 h from Fe:ZnO NPs administration.

Combining all the collected data, it can be assumed that cells die within the first 24 hours due to the synergistic combination of SW and Fe:ZnO NPs. The most important outlined damages could be attributed to cell membrane rupture and ZnO dissolution into cytotoxic Zn cations, whose presence seems to lead to a conspicuous level of cell death.

### 3. Conclusions

In the present paper we report on the use of biocompatible and biodegradable iron-doped ZnO nanoparticles having bioimaging capabilities as magnetic NPs and a pronounced cytotoxic effect when combined with mechanical pressure waves, i.e. shock waves. The iron-doped NPs show superior features than the undoped ones, having better cytocompatibility and higher internalization rate than the pristine ZnO NPs. They were both tested in an in-vitro model of PDAC and on the healthy counterpart. These features allow their use in a synergistic treatment with SW for an innovative stimuli-responsive therapy. From the cytotoxicity results and the related cell death mechanism investigations, the intracellular release of  $\text{Zn}^{2+}$  ions and the permeabilization of cell membrane are both considered responsible to induce cell death. In contrast, no ROS generation is observed. Ad hoc cell death analyses further allow us to point out the pronounced apoptotic and necrotic mechanisms of BxPC-3 cell death, once these cells are exposed to the combination of Fe:ZnO NPs and shock waves for 24 hours.

The obtained proof-of-concept results pave the way for a deep future study towards clinical translation. The advantages of the proposed synergistic activity rely on the use of potentially theranostic NPs having magnetic properties and of an on-demand therapeutic activity once activated by shock waves. In addition, SW allow the ease treatment of deep-seated tumor masses, like pancreatic cancer, opening new perspectives in its therapeutic approach while monitoring the state of the disease. The NPs here presented still lack of a proper targeting system toward cancer

cells despite a good selectivity in killing cancer cells rather than normal cells. Further improvements are therefore envisioned to render the proposed NPs even more biomimetic with an organic coating and able to actively target the diseased tissue and its microenvironment, with the final aim of overcoming the high interstitial pressure of this specific tumor mass.

## ASSOCIATED CONTENT

### **Supporting Information.**

Supporting information (PDF) containing information about: the chemical characterization of ZnO NPs, the magnetic properties of Fe:ZnO and ZnO NPs, the generation of ROS in water after shock wave stimulation and an evaluation of the dissolution of Fe:ZnO NPs in cell culture medium. The following files are available free of charge.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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