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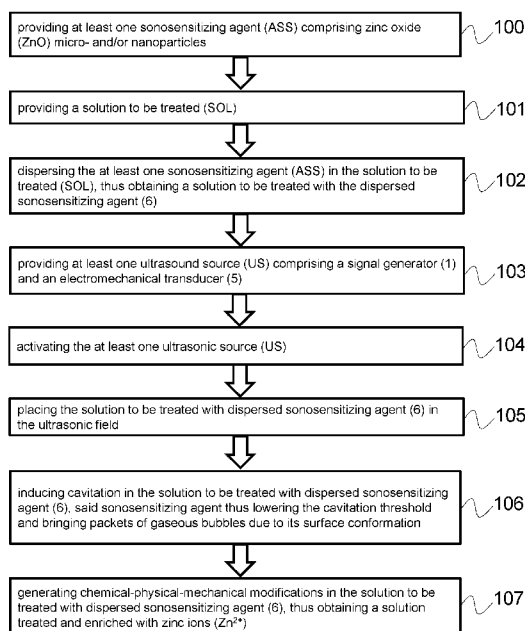


Fig. 1

(57) Abstract: The invention concerns a sonosensitizing agent (ASS) based on zinc oxide (ZnO) micro- and/ or nanoparticles activated by external pressure stimuli, specifically by ultrasounds (US). The invention also concerns a method of ultrasonic activation of a solution to be treated with dispersed sonosensitizing agent (6). The invention also concerns an ultrasonic activation system (10) of a solution to be treated with dispersed sonosensitizing agent (6) by ultrasounds (US). The invention finds preferred and advantageous applications as a contrast medium to induce targeted cytotoxicity in target tumour cells. The most advantageous implementation provides for the use of the invention described in the Italian patent application no. 102017000129243 filed on November 13th, 2017 and titled "Nanocostrutto biomimetico non immunogenico per la terapia antitumorale" and in the corresponding international application published with no. WO 2019/092550 A1 on May 16th, 2019.

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“Method of activation of a sonosensitizing agent”

DESCRIPTION

TECHNICAL FIELD

The present invention refers to the field of medicine and, in particular, to the treatment of tumours.

5 The present invention relates, in particular, to sonosensitizing agents and methods and systems thereof for the activation of such sonosensitizing agents.

The invention finds preferred and advantageous applications as a contrast medium and to induce targeted cytotoxicity in target tumour cells; in particular, the present invention is used as a contrast medium for ultrasounds.

10 The most advantageous implementation provides for the use of the invention described in the Italian patent application no. 102017000129243 filed on November 13th, 2017 and titled “Nanocostrutto biomimetico non immunogenico per la terapia antitumorale” and in the corresponding international application published with no. WO 2019/092550 A1 on May 16th, 2019.

STATE OF THE ART

15 Recent years have seen a remarkable progress in the anti-cancer field with significant improvements in the field of surgery, chemotherapy and radiotherapy, but especially with the introduction of immunotherapy in the treatment of lung, kidney, head and neck tumours, melanoma and Hodgkin's lymphoma.

20 However, all these progresses continue to be affected by problems related to individualisation of therapy and adverse reactions that can make the progress achieved sometimes useless in terms of effectiveness, if not harmful.

In fact, ideally, the anti-cancer therapy, should be selective, as well as effective, i.e. should only kill tumour cells, be personalized and free of any adverse effect to allow the anti-cancer treatment to exert all its effectiveness over time, without interruptions
25 or definitive suspension because of, for example, adverse events generated by the therapy itself.

This problem is constantly addressed both clinically and experimentally through

genetic and/or genomic, early diagnosis studies and the search for new antineoplastic methods capable, for example, to combine different approaches that, *per se* safe but ineffective, are able, if combined together, to exert an anti-tumour effect.

For this reason, innovative approaches in this field are attracting considerable interest from the scientific community for their application characteristics that could overcome some of the problems highlighted.

The first approach combines diagnosis with therapy and is called theranostics; it includes all those biomedical procedures in which the imaging techniques used in *in vivo* clinical diagnostics are made available for therapeutic treatments.

Since the response to pharmacological treatment is subjective, theranostic protocols form the basis of personalised medicine.

The development of these procedures requires the design of sophisticated high-sensitivity contrast agents capable of providing a visual aid to evaluate the biodistribution and therapeutic effect of a drug.

Examples of this first approach are described in the Italian patent application no. 102017000129243 filed on November 13th, 2017 and titled “Nanocostrutto biomimetico non immunogenico per la terapia antitumorale” by some of the present co-inventors and in the corresponding international application published with no. WO 2019/092550 A1 on May 16th, 2019.

The second approach is a newly developed anti-tumour treatment in which ultrasounds, both continuous and pulsed, are used to trigger the cytotoxic effect of chemical compounds, known as sonosensitizing agents, which otherwise would not be able to exert any cytotoxic effect on tumour cells.

This approach, called sonodynamic therapy (SDT) is able to solve some negative aspects intrinsic to conventional anti-tumour therapies, since both ultrasound and the sonosensitizing agent do not constitute *per se* any risk for the patient, while their combination ensures the effectiveness of the treatment.

Moreover, the focusing of the ultrasound through targeted directioning systems, accompanied by a selective release of the sonosensitizing agent solves a second problem always related to conventional anti-tumour therapies, namely the selectivity

of the cytotoxic action only on tumour cells.

From all this, it is easy to infer that much of the success of this innovative approach is related to the chemical-physical structure of the sonosensitizing agent, capable to exploit the non-thermal effects of both pulsed and continuous ultrasounds and its ability to focus mainly in tumour cells.

Currently, known sonosensitizing agents include a heterogeneous group of molecules and chemical compounds, often neither biocompatible nor biodegradable, whose effects, once subjected to ultrasound stimulation, result variable even applying the same experimental set-up; such sonosensitizing agents can differ significantly both in chemical structure and biological interaction as well as in anti-cancer effects.

This heterogeneity reflects the limited knowledge existing, at present, on the precise mechanism of action present in sonodynamic therapy where water pyrolysis, cavitation and sonoluminescence are proposed as possible mechanisms involved in this therapy.

However, although the mechanism of action remains debated, sonodynamic therapy has now achieved solid experimental evidences on the fact that the reactive oxygen species (ROS) are the final mediators through which the sonodynamic therapy is able to exercise its anti-tumour function.

Examples of this second approach are described in WO 2002/100478 A2 and in US 2015/328485 A1.

A solution capable of combining the two above-mentioned approaches through a set-up composed of pulsed or continuous ultrasounds and a nanoassembly based on zinc oxide (ZnO) capable of behaving, under controlled ultrasound conditions, both as a contrast agent for imaging and as a sonosensitizing agent capable of increasing the production of cavitation bubbles through ultrasound radiation, inducing mechanical damages and producing specific radical oxygen species capable of causing selective cell death would meet the need to diagnose and treat tumour cells therapeutically, overcoming the current conventional chemotherapy or radiotherapy approaches and the well-known problem of developing resistance to drugs or radiotherapy after the first treatments.

Currently, to the knowledge of the Applicant, there are no technologies able to combine ultrasound waves and a ZnO-based nanoassembly in the theranostic field, i.e. able to diagnose and treat tumour cells therapeutically.

Scientific publications are known in the state of the art:

- 5 - citation [1]: Mahdavi R. et al. "Enhancement of ultrasound-assisted degradation of Eosin in the presence of nanoparticles of ZnO as sonocatalyst" *ULTRASONIC SONOCHEMISTRY* 2018, vol. 51, pages 230-240;
- citation [2]: C. Lops, A. Ancona, K. Di Cesare, B. Dumontel, N. Garino, G. Canavese, S. Hernández, V. Cauda "Sonophotocatalytic degradation mechanisms of Rhodamine B dye via radicals generation by micro- and nano-particles of ZnO" *APPLIED CATALYSIS B: ENVIRONMENTAL*, 2019, vol. 243, pages 629-640; and
- 10 - citation [3]: V. Vighetto, A. Ancona, L. Racca, T. Limongi, A. Troia, G. Canavese, V. Cauda "The synergistic effect of nanocrystals combined with ultrasound in the generation of reactive oxygen species for biomedical applications" *FRONTIERS IN BIOENGINEERING AND BIOTECHNOLOGY - NANOBIOENGINEERING*, 2019, vol. 7, pages 374,

in which the generation of radical oxygen species using ultrasound-irradiated ZnO nano- or microparticles is documented.

However, here ZnO is referred to (in particular in citations [1] and [2]) as a sonocatalyst and in none of such scientific publications the anti-tumour application is shown, nor the other effects generated by ZnO subjected to ultrasounds, such as mechanical damages and damage to cell membrane or cell compartments, jet-streaming, release of zinc ions.

20

In addition, none of these scientific publications report the use of functionalized zinc-oxide surfaces.

25

Furthermore, the use of ZnO as a contrast agent for ultrasound images is not suggested.

It should be noted that the scientific publications referred to in the above-mentioned citations [2] and [3] are the result of the experimental work of the present co-inventors and are based on the Italian patent application no. 102018000009966 filed on 31st

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October 2018, which also forms the basis of the present international application.

Scientific publications are also known in the state of the art:

- citation [4]: Bogdan J. et al. "Nanoparticles of Titanium and Zinc Oxides as Novel Agents in Tumor Treatment: a Review" NANOSCALE RESEARCH LETTER, 2017, vol. 12, n. 1, pages 1-5; and

- citation [5]: G. Canavese, A. Ancona, L. Racca, M. Canta, B. Dumontel, F. Barbaresco, T. Limongi, V. Cauda "Nanoparticle-assisted ultrasound: A special focus on sonodynamic therapy against cancer" CHEMICAL ENGINEERING JOURNAL, 2018, vol. 340, pages 155-172,

in which the use of nanoparticles of various kinds - e.g. metallic, polymeric, oxide-metallic - is proposed for the generation of radical species by means of ultrasounds.

However, to support such generation, neither substantial data nor specific experimental evidences are reported for zinc oxide ZnO, other mechanisms or other effects generated by ZnO subjected to ultrasounds, such as mechanical damages and damages to cellular compartments or cell membrane, jet-streaming, zinc ion release, are not listed.

In addition, none of these scientific publications report the use of functionalized zinc-oxide surfaces.

Furthermore, the use of ZnO as a contrast agent for ultrasound imaging is not proposed.

It should be noted that the scientific publication mentioned in citation [5] is by some of the present co-inventors.

The present invention exploits the approaches of therapy and diagnosis outlined above and aims to meet the above-mentioned need to diagnose and treat tumour cells therapeutically.

In particular, the present invention aims to solve the technical problem of how to combine ultrasonic waves and a ZnO-based nanoassembly.

Furthermore, the present invention aims to solve the technical problem of how to improve the ZnO-based nanoassembly in terms of safety compared to both common sonosensitizing agents used in SDT and photosensitizing agents used in

photodynamic therapy (PDT).

Moreover, the present invention aims to solve the technical problem of how to avoid both the use of heat as the main means to kill cancer cells and the occurrence of coagulative necrosis, inevitable consequence of the destruction of tumour forms through high-intensity focused ultrasound (HIFU), by means of a thermoablation mechanism.

In addition, the present invention aims to solve the technical problem of how to avoid the use of light as an external agent capable of activating the sensitizing agent.

In the scientific publication - here identified as citation [6] - A. Ancona, B. Dumontel, N. Garino, B. Demarco, D. Chatzitheodoridou, W. Fazzini, H. Engelke, V. Cauda "Lipid-Coated Zinc Oxide Nanoparticles as Innovative ROS-Generators for Photodynamic Therapy in Cancer Cells" NANOMATERIALS, 2018, vol. 8 (143), pages 1-15, on the contrary, the use of ZnO nanoparticles, also functionalized on the surface with metal-organic and organic (phospholipids) agents for their activation by ultraviolet light (photodynamic therapy) is proposed, which has the disadvantage of being *per se* cytotoxic and little penetrating into the tissues (maximum 1 mm penetration depth).

In summary, therefore, up to the present time, to the knowledge of the Applicant, there aren't any known solutions which allow to meet the above detailed requirements.

Therefore, the Applicant, with the present invention, intends to remedy these lacks.

For this reason the present invention intends to protect a ZnO nanoassembly, suitably functionalized with specific and hydrophobing chemical agents to increase the sonosensitivity thereof, but also biocompatible and biodegradable, as a new sonosensitizing agent able to produce, in a controlled way according to the ultrasonic conditions used, toxic and harmful species to be applied in the medical and/or environmental field.

The documents are also known in the state of the art:

- citation [7]: B. Dumontel, M. Canta, H. Engelke, A. Chiodoni, L. Racca, A. Ancona, T. Limongi, G. Canavese, V. Cauda "Enhanced Biostability and Cellular Uptake of Zinc Oxide Nanocrystals Shielded with Phospholipid Bilayer" JOURNAL OF MATERIALS

CHEMISTRY B, 2017, vol. 5, pages 8799–8813; and

- citation [8]: the international application published under no. WO 2019/092550 A1 on 16th May 2019, already cited in the present description and in respect of which the present invention is proposed as a further development.

- 5 In such prior art documents, only the ZnO and its possible superficial functionalizations with alkyl-silane groups and double phospholipidic layers of synthesis or exosomal cellular derivation are dealt with, but neither ZnO as a sonosensitizing agent nor ultrasonic irradiation, nor the method, system or contrast medium thereof are mentioned.
- 10 In particular, the above citation [8] differs from the present invention in that:
- it describes not a single material, such as ZnO, but a multi-material nanoassembly, in which ZnO constitutes the core of the nanoassembly and is coated with a double phospholipidic layer and proteins derived from extracellular biovesicles, in particular derived from the same organism in which it will be reused for anti-cancer therapy; the aim
 - 15 is to reduce the probability of adverse immune reactions occurring once the nanoassembly is injected into the organism of the patient to be treated with anti-cancer therapy;
 - the nanoassembly is in turn equipped with targeting molecules, in particular proteins, such as monoclonal antibodies;
 - zinc oxide is conjugated with dyes or doped with other metallic elements to amplify
 - 20 the fluorescence emission radiation in the ultraviolet-visible spectrum down to infrared; therefore, it is used as an imaging medium but, unlike the present invention, the operating principle is based on excitation and emission of light radiation;
 - zinc oxide is doped with paramagnetic or diamagnetic elements suitable to be detected by means of a nuclear magnetic resonance scanner; therefore, it is used as an
 - 25 imaging medium but, unlike the present invention, the operating principle is based on magnetic resonance;
 - citation [8] also describes the method of coupling ZnO with extracellular vesicles;
 - the therapeutic activity described in the citation [8] is performed by spontaneous release of zinc ions and reactive radical oxygen species (ROS) in the absence of
 - 30 ultrasonic or luminous or other stimuli;

- thanks to the molecules present on the coating of the nanoassembly, the nanoassembly interacts ("cellular targeting") with the phospholipidic layer of the membrane of the tumour cells by delivering in a selective manner the zinc oxide nanocrystals therein.

5 Finally, the structural characteristics of the nanoassembly according to the present invention, when subjected to ultrasonic fields unsuitable for the production of harmful species, has proven to possess properties capable of increasing the contrast of the ultrasonic image, with a consequent increase in the diagnostic power of this important non-invasive imaging technique.

10 OBJECTS AND SUMMARY OF THE INVENTION/

The object of the present invention is to overcome the drawbacks of known art related to the use of traditional technologies, from chemotherapy and radiotherapy to more advanced techniques, such as theranostics, photodynamic therapy (PDT), sonodynamic therapy (SDT) and high intensity focused ultrasound therapy (HIFU).

15 More precisely, the object of the present invention is to overcome the drawbacks of the systems listed above, taken both individually and in combination.

In particular, the present invention aims to solve the problem of how to combine ultrasonic waves and a ZnO-based nanoassembly to treat tumour cells without causing damages, even serious, to tissues other than tumour tissues and adjacent thereto.

20 Such objects are reached with the system according to the present invention which, advantageously and thanks to the combination of a sonosensitizing agent comprising zinc oxide (ZnO) micro- and/or nanoparticles and a specific ultrasound set-up, allows to introduce in the current anti-tumour scenario a diagnostic, therapeutic, customizable and selective tool.

It is believed that the main innovation consists in the combination of metal oxide, in particular ZnO, micro/nanoassemblies and the ultrasound delivery system, capable of generating a series of highly synergistic effects capable of inducing the tumour cell death, as well as imaging to obtain a complete theranostic system.

30 Specifically, the above and other objects and advantages of the invention, as will

appear from the following description, are achieved with a method of ultrasonic activation using at least one sonosensitizing agent according to claim 1.

Preferred embodiments and variants of the method of ultrasonic activation according to the present invention form the object of the dependent claims 2 to 8.

5 Another aspect of the present invention relates to an ultrasonic activation system comprising at least one sonosensitizing agent and constitutes the object of claim 9.

Another aspect of the present invention relates to a sonosensitizing agent to be used in the ultrasonic activation method and constitutes the object of claim 11.

10 Another aspect of the present invention concerns a contrast medium for ultrasounds and constitutes the object of claim 16.

It is understood that all the appended claims form an integral part of the present description and that each of the technical characteristics claimed therein is possibly independent and can be used autonomously with respect to the other aspects of the invention.

15 It will be immediately apparent that countless modifications could be made to what described (for example related to shape, sizes, arrangements and parts with equivalent functionalities) without departing from the scope of protection of the invention as claimed in the appended claims.

Advantageously, the technical solution according to the present invention allows to

20 - improve the effects of organic molecules normally used as sonosensitizing agents or of other oxides, such as titania;

- generate extra- and intra-cellular ROS (in case of nanoparticles internalization), due to the semiconductor nature and cavitation of ultrasound in an aqueous environment;

25 - release Zn^{2+} ions, due to the chemical instability of the specific ZnO material in a biological environment at a weakly acidic pH such as that of tumour cells/masses and due to the interaction with ultrasounds in a defined frequency and intensity range;

- lower the cavitation threshold, due to the presence of micro- or nanoparticles in interaction with ultrasounds in a defined frequency and intensity range;

30 - generate jet-streaming effects resulting from the interaction of nanoparticles and/or ultrasound on the cell, which affect the integrity of the cell membrane;

- combine ultrasound imaging by effect of the joint presence of ultrasounds and micro/nanoparticles.

More advantageously, in the most advantageous implementation that provides for the use of the invention described in the Italian patent application no. 102017000129243 filed on November 13th, 2017 and titled "Nanocostrutto biomimetico non immunogenico per la terapia antitumorale" and in the corresponding international application published with no. WO 2019/092550 A1 on May 16th, 2019, the technical solution according to the present invention allows to

- use autologous exosomes (extracellular biovesicles having a nanometric size) as a biomimetic and non-immunogenic coating of zinc oxide nanocrystals to create a hybrid and multifunctional nanoassembly for anti-cancer therapy;
- create a stable and colloiddally dispersed nanoassembly (having sizes smaller than 150-200 nm) in physiological media;
- use the ability to target by means of monoclonal antibodies attached to the outer surface of the exosomal coating to selectively deliver zinc oxide nanocrystals to the target cells;
- use zinc oxide nanocrystals as imaging agents in resolute optical microscopy.

Further advantageous characteristics of the invention will become more apparent from the following description of preferred, but not exclusive, embodiments, provided purely by way of example and not of limitation.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described hereinafter by way of some preferred embodiments, provided by way of example and not of limitation, with reference to the accompanying drawings. These drawings illustrate different aspects and examples of the present invention and, where appropriate, similar structures, components, materials and/or elements in different figures are denoted by similar reference numerals.

FIG. 1 is a block diagram illustrating the method of ultrasonic activation of a sonosensitizing agent according to the present invention;

FIG. 2 is a schematic representation of the ultrasonic activation system of a

sonosensitizing agent according to the present invention;

FIG. 3A is a transmission electron microscope (TEM) image of ZnO nanoparticles functionalized with aminopropyl groups of about 20 nm in diameter and wurtzite crystalline phase;

5 FIG. 3B is an X-ray diffraction pattern for ZnO nanoparticles functionalized with aminopropyl groups of about 20 nm in diameter and wurtzite crystalline phase;

FIG. 4A shows EPR spectra (obtained from EMXnano X-band, Bruker) representative of distilled water only and ZnO-NH₂ nanoparticles in aqueous solution (200 µg/mL) exposed to ultrasound with characteristics: 1 MHz, 0.7 MPa (peak rarefactional pressure, PRF), 10% DC, 1 Hz PRF, for 5 minutes;

10 FIG. 4B is a graph showing the concentration of the DMPO-OH spin adducts obtained from water and ZnO-NH₂ nanoparticles in water under the same ultrasonic conditions of FIG. 4A (1 MHz, 5 minutes, 10% DC, 1 Hz PRF) at different PRF pressures, i.e. using different ultrasonic powers; the concentration of DMPO-OH has been quantified by fitting the EPR spectra with the SpinFit software (Bruker);

FIG. 5 is a graph showing the concentration of the DMPO-OH spin adducts obtained from water and ZnO-NH₂ nanoparticles in water exposed to ultrasounds with characteristics: 1 MHz, 0.7 MPa (peak rarefactional pressure, PRF), 10% DC, 1 Hz PRF, for several time; the concentration of DMPO-OH was quantified by fitting the EPR spectra with the SpinFit software (Bruker);

20 FIG. 6 shows the graphs of acoustic emission of water and nanoparticles of ZnO-NH₂ (at a concentration of 200 µg/mL) in aqueous solution, exposed to continuous ultrasounds at 985 KHz frequency and using different voltages applied to the transducer to generate different PRF pressures; the measurements are taken by means of a hydrophone;

FIG. 7 is a graph showing the average intensities calculated at different acoustic pressures and, therefore, voltages applied to a piezoelectric transducer to generate ultrasounds in the presence of pure water and ZnO-NH₂ nanoparticles (concentration of 200 µg/mL); error bars = 1 SD, N=3;

30 FIG. 8 is a graph showing the evaluation of the linear dependence of ultrasonic

scattering depending on the different concentration of ZnO-NH₂ nanoparticles;

FIG. 9 is a bar graph showing the average intensity calculated by ultrasound videos at two different acoustic pressures of activation of ZnO nanoparticles in the presence or absence of ethanol and at various concentrations of the latter;

5 FIGs. 10A and 10B are representative images recorded during ultrasonic irradiation of pure water and ZnO-NH₂ nanoparticles in water (concentration of 200 µg/ml);

FIGs. 11A, 11B and 11C are graphs showing the cell viability at 24, 48 and 72 hours of prostate cancer cells (DU-145) treated with different concentrations of ZnO nanoparticles (5 to 40 µg/mL) and in the absence of ultrasound; IC₅₀ values, i.e. related

10 to the inhibitory concentration of ZnO nanoparticles (ZnO NPs) that induce cell population death of at least 50%, are calculated by means of a interpolation curve;

FIGs. 12A and 12B are bar graphs showing the viability of prostate cancer cells (DU-145) treated for 24 hours with a concentration of 10 µg/mL of ZnO nanoparticles (ZnO NPs) and then subjected to ultrasounds at 1 MHz frequency for three minutes at a

15 voltage applied to the unfocused piezoelectric transducer of 240 mVpp (in the case of ultrasounds named US1, FIG. 12A) or 360 mVpp (in the case of ultrasounds named US2, FIG. 12B); the cells treated in this way are replated and recultivated for 24, 48 and 72 hours for subsequent viability tests.

DETAILED DESCRIPTION OF THE INVENTION

20 The present international application is based on the Italian patent application no. 102018000009966 filed on 31st October 2018.

While the invention is susceptible to various modifications and alternative constructions, some preferred embodiments are shown in the drawings and will be described in detail hereinbelow.

25 It should be understood, however, that there is no intention to limit the invention to the specific embodiments illustrated, but, on the contrary, the invention is intended to cover all modifications, alternative constructions, and equivalents which fall within the scope of the invention as defined in the claims.

In the following description, therefore, the use of “for example”, “etc.”, “or”, “either”

30 indicates not exclusive alternatives without any limitation, unless otherwise indicated;

the use of “also” means “including, but not limited to” unless otherwise indicated; the use of “includes/comprises” means “includes/comprises but not limited to” unless otherwise indicated.

The present invention is based on the innovative concept of combining a sonosensitizing agent including micro- and/or nanoparticles of zinc oxide (ZnO) and a specific ultrasonic set-up.

In particular, the present invention consists of an innovative system for anti-cancer therapy, which can also be associated with methods of imaging of the tumour mass and targeted directioning systems, thus constituted:

- one or multiple semiconductor metal oxide crystals;
- an instrumental set-up able to deliver ultrasounds with a very precise spectrum of frequencies and intensities and that can induce, through cavitation of the solution in which the above-mentioned metal oxide is immersed, the development of cytotoxic effects, for example highly-oxidizing radical species and other species toxic to tumour cells.

Synthetically, the present invention makes it possible to combine in a single nanometric assembly many smart and biomimetic as well as completely innovative functions; the nanoassembly, suitably functionalized with organic agents and associated with targeting agents (such as monoclonal antibodies) is highly dispersed colloidally in various biological fluids, such as saline buffer solutions, cell culture medium, blood or plasma; the nanoassembly, remaining nanometric and not aggregating, always exposes optimally the monoclonal antibodies on the outermost surface, ensuring the rapid reaching of the target cells and its internalization; the selectivity and further non-immunogenicity of the nanoassembly avoid exposure to the treatment of healthy cells and tissues such as, for example, many of the cells involved in the immune response and organs most commonly subject to drug poisoning or suffering caused by chemotherapy and/or radiotherapy. Once the correct time of internalization in the cells has been calibrated, the nanoassembly is activated remotely/externally by the ultrasound source; in the absence of the nanoassemblies mentioned above, the ultrasound delivery is not toxic for the cells that are exposed

thereto; on the contrary, the development of radical species occurs only thanks to the co-presence of ultrasounds and nanoassemblies; the latter, if internalized into the cell, generate the radical species that lead to the cell death, verified by means of suitable cyto-/genotoxicity tests.

- 5 The high flexibility and modularity of the present nanoassembly allow the application thereof for various types of tumour pathologies, ranging from solid tumours to blood tumours, subject to the use of the appropriate monoclonal antibody or, more generally, of the appropriate targeting agent that ensures the selective directioning.

In the present description, the term “nanoassembly” means a system consisting of one
10 or more metallic oxide crystals, in particular zinc oxide responsive to the action of ultrasounds.

In the present description, the term “nanoparticle” means a material having dimensions ranging from 1 to 500 nm, crystalline or amorphous, spherical in shape or with sharp edges or irregular in shape, of any material, unless specifically indicated of
15 metal oxide, and in particular zinc oxide.

In the present description, the term “surface functionalization” means the presence on the surface of micro- and nanoparticles of organic chemical groups (polymers such as polyethylene glycol, lipids such as lipid double layers of synthetic origin (liposomes) or exosomal vesicles, proteins such as preferably peptides and antibodies, nucleic
20 acids such as preferably aptameres), metal-organic acids such as preferably alkyl-silanes; such functionalizing agents are preferably of partially hydrophobic nature, so as to better immobilise bubbles of gas dissolved in solution and capable of cavitating during ultrasonic irradiation; the functionalizing agents are bound to the surface of the particle by means of covalent, electrostatic, ionic, Van der Waals', coordination,
25 hydrogen bonds.

In the present description, the term “ultrasounds” means a pressure wave, characterized by compression and decompression peaks, defined by a certain frequency and intensity, which is transmitted in a liquid/gel medium, typically aqueous.

In the present description, the term “sonosensitizing agent ASS” means a molecular agent or an organic material or, preferably, an inorganic material, preferably a metal oxide, preferably consisting of zinc oxide, preferably micro- or nanostructured or with surface nanoroughness, preferably with semiconductor properties, preferably with surface functionalized by hydrophobing organic chemical groups, capable of producing various effects when subjected to ultrasonic irradiation, including: production of radical oxygen species, lowering of the cavitation threshold in the ultrasonic transmission liquid medium, release of ions, preferably of Zn^{2+} ions, mechanical effects such as generation of jet-streaming effects or damages to cellular components.

Another aspect of the invention is constituted by a sonosensitizing agent ASS comprising zinc oxide ZnO micro- and/or nanoparticles.

Preferably, the zinc oxide ZnO micro- and/or nanoparticles are nanostructured.

More preferentially, the zinc oxide ZnO micro- and/or nanoparticles have surfaces functionalized with organic, metallo-organic, metallic groups or coordinating agents, or are coated with mono- or multilayers of organic agents; the coordinating agents can be alkyl-silanes, molecules with carboxylic or amine groups, metal-conjugates, etc.; the organic agents can be lipids, proteins, polymers, etc. In order to increase their non-immunogenicity in a biological environment, they can be preferably functionalized with organic agents such as polyethylene glycol polymers or lipid double layers, synthetic (liposomes) or, as in the case of exosomal vesicles, derived from cell culture supernatant or directly isolated from biological fluids of various kinds. In order to increase their selective directioning towards cells of a specific tumour line, micro- and/or nanoparticles can be equipped with proteins or nucleic acids, such as preferably peptides and monoclonal antibodies.

Zinc oxide (ZnO) micro- and/or nanoparticles have sizes ranging from 5 nm to 100,000 nm; preferably, zinc oxide (ZnO) microparticles have sizes ranging from 500 nm to 10,000 nm and zinc oxide (ZnO) nanoparticles have sizes ranging from 1 nm to 500 nm.

In greater detail, the sonosensitizing agent ASS comprises:

- a micro- and/or nanoassembly consisting of one or more crystals of semiconductor metal oxide, in particular zinc oxide ZnO, having a size from about 10 nm to 20,000 nm (or 20 μ m), with a poly- or monocrystalline structure, in the case of ZnO with a wurtzitic poly- or monocrystalline phase and any morphology (spherical, elongated, hexagonal section, spiked, porous or dense particle);

- zinc oxide micro- and/or nanocrystals are prepared by chemical synthesis, e.g. by wet-chemical hydrothermal or microwave-assisted synthesis;

- micro- and/or nanoassemblies, in the absence of external stimuli, are safe for the organism and non-cytotoxic below certain threshold doses; they do not carry chemotherapy drugs, unless properly functionalized;

- metallic oxide, in particular zinc oxide micro- or nanoassemblies can be functionalized with chemical, organic, metallorganic, polymeric and/or lipidic molecules, proteins, peptides and/or targeting agents to be more biocompatible, directable and/or non-immunogenic;

- metallic oxide, in particular zinc oxide micro- or nanoassemblies may be activated by an external stimulus of ultrasonic pressure waves that allow the excitation of the material and induce it to lower the cavitation threshold and produce highly cytotoxic species, including also reactive oxygen species (ROS) species.

With reference to FIG. 1, a method of ultrasonic activation constitutes another independent aspect and can be used autonomously with respect to the other aspects of the invention, and comprises the following steps:

- providing at least one sonosensitizing agent ASS as described above (step 100);
- providing a solution to be treated SOL (step 101);
- dispersing the at least one sonosensitizing agent ASS in the solution to be treated SOL, thus obtaining a solution to be treated with dispersed sonosensitizing agent 6 (step 102);
- providing at least one ultrasound source US comprising a signal generator 1 and an electromechanical transducer 5 (step 103);

- activating the at least one ultrasound source US (step 104);
- placing the solution to be treated with dispersed sonosensitizing agent 6 in the ultrasonic field (step 105);
- inducing cavitation in the solution to be treated with dispersed sonosensitizing agent 6, said sonosensitizing agent ASS thus lowering the cavitation threshold and bringing packets of gas bubbles due to its surface conformation (step 106); and
- generating chemical-physical-mechanical modifications in the solution to be treated with dispersed sonosensitizing agent 6, thus obtaining a solution treated and enriched with zinc ions Zn^{2+} (step 107).

Preferably, the solution to be treated SOL is an aqueous solution.

Preferably, ultrasounds have a frequency ranging from 20 kHz to 100 MHz and a power ranging from $0.01 \text{ W} \cdot \text{cm}^{-2}$ to $25,000 \text{ W} \cdot \text{cm}^{-2}$; more preferably, ultrasounds have a frequency ranging from 40 kHz to 10 MHz and a power ranging from $0.1 \text{ W} \cdot \text{cm}^{-2}$ to $10 \text{ W} \cdot \text{cm}^{-2}$.

Preferably, the total activation time, possibly provided in several steps or cycles, ranges from 1 second to 12 hours; more preferably, the total activation time, possibly provided in several steps or cycles, ranges from 5 seconds to 30 minutes.

Preferably, the chemical-physical-mechanical modifications generated in the solution to be treated with dispersed sonosensitizing agent 6 are chosen from: production of reactive oxygen species and free radicals, production of Zn^{2+} ions, and/or adiabatic increase of temperature and/or mechanical damages to organelles and subcellular compartments, genetic material, double phospholipidic layers, comprising cell membranes.

More preferentially, chemical-physical-mechanical modifications also occur on the sonosensitizing agent ASS that generated them and include mechanical, piezoelectric, pyroelectric and triboelectric effects.

According to the method of the present invention, once the internalisation times of the metal oxide nanoassemblies in the cells have been calibrated, they are activated remotely/externally by the ultrasound source; in the absence of the

micro/nanoassemblies mentioned above, the ultrasound delivery is not toxic to the cells that are exposed thereto.

With reference to FIG. 2, which illustrates a scheme of the possible set-up for ultrasound delivery on a biological sample containing cells and micro/nanoassemblies, constitutes another independent aspect and usable independently from the other aspects of the invention, an ultrasonic activation system comprising:

- at least one solution to be treated with dispersed sonosensitizing agent comprising a sonosensitizing agent ASS as described above, dispersed in a solution to be treated SOL; and
- at least one ultrasound source US comprising a signal generator 1 and an electromechanical transducer 5; and
- at least an ultrasonic transfer medium M.

The at least one ultrasound source US comprising a signal generator 1 may be a wave generator to program the excitation signal, or a device or part thereof capable of generating an oscillatory signal.

The at least one electromechanical transducer 5 is either electromagnetic or piezoelectric, or a combination of both; it may be a set of piezoelectric disc-based ultrasonic transducers with a delivery frequency in the recognised range for sonodynamic therapy (50 kHz to 2 MHz) compatible with the transfer medium.

The transfer medium is usually a fluid, such as ultrasound gel, phantom materials, hydrogel, or a suitable reservoir to confine the ultrasound delivery.

Preferably, the at least one ultrasound source US further comprises:

- a signal amplifier 2;
- an oscilloscope 3; and
- a terminator resistive load 4.

The optional signal amplifier or power amplifier 2 is suitable for high frequencies, typically between 10 kHz and 12 MHz.

The optional terminator resistive load 4 is a protection terminator with resistive load.

Optionally and additionally, the system 10 may also comprise:

- a thermocouple or infrared thermal camera to control the temperature of the water bath equipped with an acquisition system;
- sterile plastic tubes or plates (single- or multiwell) with a volume ranging from 100 μ L to 5 mL to accommodate the tumour cells to be treated;
- 5 - a hydrophone or ultrasound system to measure the presence of cavitation in the aqueous system and the focus where the maximum pressure point of the ultrasonic waves generated by transducer 5 is concentrated.

Regarding the system 10 used for the generation and delivery of ultrasounds, it is specified that:

- 10 - ultrasounds that are delivered in a fluid medium in various forms, mainly using common ultrasonic baths with frequencies between 40 and 60 kHz and powers in the range 0.3 - 3 W/cm² or immersion probes of piezoelectric material for the generation of ultrasounds in *ad-hoc* set-ups with frequencies 100 kHz up to 3 MHz and similar power range of the previous one (between 0.3 and 5
- 15 W/cm²), or electromechanical devices to be coupled to suitable transfer medium;
- pressure waves are able to generate cavitation bubbles of the gases naturally present in the liquid medium in which they are delivered; such bubbles, in the implosion step, locally produce high temperatures (estimated > 5,000 K),
- 20 pressures (estimated > 20 MPa) and sonochemical reactions in the surrounding liquid medium and on the surface of the micro/nanoassemblies immersed in the fluid medium;
- pressure waves can penetrate deeply into tissues;
- it is necessary to operate with ultrasounds in frequency and intensity ranges
- 25 that do not induce toxicity in the cells;
- also, it is possible to combine ultrasound imaging (with a suitable detection system) by effect of the joint presence of ultrasounds and micro/nanoparticles;
- with the latter option, the system as a whole (ultrasonic set-up and micro/nanoassemblies) constitutes an innovative theranostic system, i.e.
- 30 suitable to carry out therapy and diagnosis/imaging at the same time.

System 10 as a whole, i.e. ultrasonic set-up and micro/nanoassemblies, is capable of generating a number of highly synergistic effects to induce the tumour cell death; these effects comprise:

- generating extra- and intracellular ROS (in case of internalization of metal oxide nanoassemblies, microassemblies are hardly internalized); without wanting to be bound by this theory, it is believed that the generation of extra- and intracellular ROS occurs according to the following mechanism: due to the semiconductor nature of the metallic oxide micro- and/or nanoassemblies and also due to the effect of ultrasonic cavitation alone in an aqueous environment, which itself generates ROS from water, therefore, not necessarily bound to metallic oxide or any other organic molecule of sonosensitizing agent (see FIGS. 4A, 4B, and 5);
- lowering of cavitation threshold, due to the presence of micro- or nanoparticles in interaction with ultrasounds in a defined frequency and intensity range (see FIGS. 6, 7, 8 and 9);
- releasing Zn^{2+} ions, due to the chemical instability of the specific ZnO material in a biological environment at a weakly acidic pH such as that of tumour cells/masses;
- generating jet-streaming effects, again as a result of the interaction of ultrasounds alone on the cell and of ultrasounds and micro/nanoassemblies near or inside the cell, which affect the integrity of the cell membrane.

A contrast medium for ultrasound comprising zinc oxide ZnO micro- and/or nanoparticles constitutes a further independent aspect usable autonomously with respect to the other aspects of the invention.

Preferably, the concentration of zinc oxide ZnO micro- and/or nanoparticles ranges from 0.1 $\mu\text{g/mL}$ to 10 mg/mL ; more preferably, the concentration of zinc oxide ZnO micro- and/or nanoparticles ranges from 1 $\mu\text{g/mL}$ to 500 $\mu\text{g/mL}$.

Preferably, zinc oxide ZnO micro- and/or nanoparticles have sizes ranging from 5 nm to 100,000 nm; more preferably microparticles have sizes ranging from 500 nm to 20,000 nm and nanoparticles have sizes ranging from 1 nm to 100 nm.

The present invention is described below in greater detail with reference to the following experimental data, which are meant as illustrative, but not limiting, of the

present invention.

Experimentally, a polymeric vessel having a volume from a few mL up to about 20 litres is used for the confinement of the ultrasonic transfer medium equipped with a positioning system adjustable in the three axes x, y, z, housing the ultrasound emitting probe(s).

Characterizations on micro/nanoassemblies

The characterizations of the prepared materials (metal oxides and in particular ZnO) are carried out by means of several characterization techniques, including: field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM), X-ray diffraction, dynamic light scattering (DLS) and Z-potential measurement, nanoparticle tracking analysis (NTA), infrared, ultraviolet and fluorescence spectroscopy, high resolution fluorescence microscopy.

With reference to FIG. 3A, it can be observed a transmission electron microscope (TEM) image for functionalized ZnO nanoparticles of about 20 nm in diameter and wurtzite crystalline phase; as it can be seen, the nanoparticles have homogeneous sizes and mainly spherical shape with some facets and an average size of 20 nm.

With reference to FIG. 3B, it can be observed a X-ray diffraction pattern for functionalized ZnO nanoparticles of about 20 nm in diameter and wurtzite crystalline phase; as it can be seen there are several diffraction peaks indexed as (100), (002), (101), (102), (110) and all corresponding to the wurtzite crystalline phase, typical of zinc oxide.

Characterizations on piezoelectric transducers (in a vessel with about 20L of water)

The following steps are carried out

- measurement of the pressure profile as a function of the distance from the surface of the piezoelectric transducer to determine the focal plane;
- measurement of the planar pressure map (on the plane of maximum intensity) to verify the shape and symmetry of the sound radiation;
- measurements with frequency variation to determine the frequency of the transducer resonance and if it deviates from the stated nominal one;
- measurement of bandwidth: evaluation of the amount of deviation from the

previously identified resonance frequency of the piezoelectric transducer, while still obtaining a substantial effect on the biological sample.

Characterizations on the ultrasound delivery set-up

The following steps are carried out

- 5 - fluid seal and electrical insulation of the vessel and the components immersed therein;
- verification of absence of secondary sound wave reflections in the implemented set-up;
- characterization of the thermal effect induced on the sample according to
- 10 different excitation factors: frequency, waveform, duty cycle, amplification level, sonication duration, total water volume.

Characterization of the overall system (ultrasonic set-up and micro/nanoassemblies)

The detection of acoustic cavitation in water containing ZnO is carried out by means of an instrument known as cavitometer, i.e. a hydrophone capable of detecting the

15 noise produced by the implosion of cavitation bubbles, appropriately connected to an amplification, transduction and data acquisition system.

The detection of radical oxidizing species generated by metal oxide, in particular ZnO, is carried out through a technique known as electronic paramagnetic resonance (EPR) associated with molecules able to capture and stabilize radicals over time (chemical

20 trap for electronic spins or spin trap) in order to estimate the ability to generate highly oxidizing radical species and therefore toxic to tumour cells; the micro/nanostructured sample is immersed in water or buffered saline solutions or cell culture media to promote internalization (for a time ranging from 2 to 24 hours) into tumour cells.

25 In particular:

- verification of the presence of radical species produced by the nanoassembly in the absence of tumour cells by means of spin-trapping assisted EPR technique (FIG. 4A);
- verification of the presence of radical species produced by the nanoassembly
- 30 in the presence of tumour cells by means of spin-trapping assisted EPR

technique specific for intracellular radicals;

- measurement of the type of radical species produced by means of spin-trapping assisted EPR technique (FIG. 4A);

- semi-quantitative analysis of radical species as a function of different excitation factors (frequency, waveform, duty cycle, amplification level, sonication time, total water volume), spin-trapping assisted EPR technique (FIGs. 4B, and 5);

- broadband measurement of the acoustic cavitation generated by ultrasounds on nanoparticles as a function of the pressure profile (FIG. 6); the acoustic cavitation is facilitated by the presence of ZnO micro- and/or nanoparticles; the measurement is carried out by means of a needle hydrophone immersed in the wells containing the solutions, and the samples are subjected to ultrasounds in continuous mode; the signals in the time domain are transformed into the frequency domain by the Fourier Transform, obtaining the frequency spectra of FIG. 6; for ZnO-NH₂ nanoparticle samples, the broadband acoustic emission appears at lower applied acoustic pressures and at higher intensities than water alone; this suggests that the beginning of inertial cavitation is favoured by the presence of nanoparticles; such result is believed to be related to the pressure needed to expand the surface gas nanobubbles up to a critical radius, capable of generating inertial cavitation;

- measurement of the acoustic cavitation generated by ZnO-NH₂ nanoparticles by means of the phenomenon of acoustic scattering (FIGs. 7 and 8); the presence of cavitation bubbles induces a phenomenon of acoustic scattering and radiates sound waves during the bubble collapse, which can be detected by an image ultrasound scanner; the sample (1 mL of pure water or ZnO-NH₂ nanoparticles in water) is irradiated by focused ultrasounds (with negative acoustic pressure peaks ranging from 0.5 to 2 MPa, 10 msec packet pulses with a repetition frequency of 10 Hz) into a tube, and the generated cavitation bubbles are detected by a phased array scanning probe ultrasound scanner operating at 10 MHz in B-mode;

- evaluation of the actual presence of acoustic cavitation generated by ZnO-NH₂ nanoparticles; ZnO-NH₂ nanoparticles in aqueous solution are treated at different concentrations (FIG. 9); in addition, ZnO-NH₂ nanoparticles in aqueous solution (200 µg/ml concentration) are treated in the presence of ethanol (EtOH) at two different concentrations: ethanol should destroy the gas nanobubbles on the surface of the nanoparticles by wetting this surface; in fact, following the addition of ethanol, the ultrasound scattering and, therefore, the bubble cavitation are drastically reduced; these data suggest that acoustic cavitation is facilitated by the presence of ultrasound-irradiated ZnO-NH₂ nanoparticles and that it is due to the periodic oscillation and subsequent implosion of surface nanobubbles trapped on the surface of such nanoparticles.

With reference to FIGs. 10A and 10B, it is observed that acoustic scattering is generated only in the presence of ZnO-NH₂ nanoparticles during ultrasonic irradiation.

Biological characterization of the overall system (ultrasonic set-up + micro/nanoassemblies)

The following steps were carried out

- viability tests on tumour cells (based on Trypan-Blue colorimetric, WST-1 and cytofluorimetry tests) treated using ultrasonic set-up + micro/nanoassemblies at different concentrations;

- control tests: these are cell viability tests with ultrasounds delivered on cells, but in the absence of micro/nanoassemblies, using the same operational parameters, as well as cell viability tests on tumour cells exposed to different concentrations of metal oxide, in particular ZnO micro/nanoassemblies, to measure the intrinsic toxicity thereof.

The tumour cells, kept in cell culture flasks, are incubated with a solution of nanoparticles diluted in the culture medium at various concentrations (typically 5 to 40 µg/mL) for 24 hours. Also, controls with cells incubated with nanoparticles and not treated with ultrasounds, and cells treated with US and not incubated with nanoparticles as well as controls with cells without any kind of treatment have been arranged. Subsequently, the cells are separated by trypsinization, counted and

resuspended in buffers (e.g. phosphate buffered saline or cell culture medium) at concentrations ranging from 15×10^5 to 50×10^5 cells/ml for ultrasound treatment.

After 24, 48 and 72 hours after treatment, viability is measured by means of the WST-1 colorimetric assay, based on the ability of the mitochondrial enzyme succinate tetrazolium reductase to convert the WST-1 tetrazolium salt into a formazan salt. This enzyme is active only in viable cells; thus, the intensity of staining is directly proportional to the number of viable cells present in the culture under study.

In FIGs. 11A, 11B and 11C, cell viability graphs are reported at 24, 48 and 72 hours for DU-145 tumour cells treated with various concentrations of ZnO nanoparticles (5 to 40 $\mu\text{g}/\text{mL}$). The values of IC_{50} , i.e. the inhibitory concentration of ZnO nanoparticles that cause a cell population death of at least 50%, are calculated using an interpolation curve. The effective concentration is shown to be 24.47 $\mu\text{g}/\text{mL}$ after 24 hours (FIG. 11A), 24.88 $\mu\text{g}/\text{mL}$ after 48 hours (FIG. 11B), and 26.09 $\mu\text{g}/\text{mL}$ after 72 hours (FIG. 11C). Upon the IC_{50} values obtained, experiments to treat tumour cells in synergy with ultrasounds are provided.

In FIGs. 12A and 12B report bar graphs showing the viability of prostate tumour cells (DU-145) treated with a concentration of 10 $\mu\text{g}/\text{mL}$ for 24 hours of ZnO nanoparticles (ZnO NPs) and then subjected to ultrasounds at 1 MHz frequency for three minutes at a voltage applied to the unfocused piezoelectric transducer of 240 mVpp (in the case of ultrasounds named US1, FIG. 12A) or 360 mVpp (in the case of ultrasounds named US2, FIG. 12B) and stored in culture for 24, 48 and 72 hours. The viability bars, always evaluated with the WST-1 method, are reported for untreated control DU-145 tumour cells, DU-145 cells treated only with ultrasounds (both US1 and US2) and cells treated only with nanoparticles at the concentration of 10 $\mu\text{g}/\text{mL}$. The results show a higher mortality of tumour cells treated with the combination of ultrasounds (in both US1 and US2 modes) and ZnO nanoparticles.

Use of micro- and nanoassemblies irradiated by ultrasounds as an ecogenic contrast medium

Under the same conditions as for FIG. 7, the acoustic scattering of ZnO nanoparticles in an aqueous medium is detected, able to act as a contrast agent for ultrasound

imaging.

FIGS. 10A and 10B are representative images recorded during ultrasonic irradiation of pure water and ZnO-NH₂ nanoparticles in water (200 µg/ml concentration); the white spots in the dashed region indicate the acoustic reflection generated by bubble cavitation induced by nanoparticles in water; the lateral white bars at the edge of the image are due to the acoustic reflection of the walls of the vessel containing the solutions to be analyzed.

The measurement conditions are the same as those used to obtain FIG. 7 with, in particular, peak rarefactional pressure = 1.6 MPa, PRF = 10 Hz, pulse length = 10 msec.

As aforesaid, in the most advantageous implementation provides for the use of the invention described in the Italian patent application no. 102017000129243 filed on November 13th, 2017 and titled “Nanocostrutto biomimetico non immunogenico per la terapia antitumorale” and in the corresponding international application published with no. WO 2019/092550 A1 on May 16th, 2019; adopting such technical solution the following additional features are obtained in addition to what aforesaid:

- a nanometric-sized assembly consisting of spherical metal oxide (zinc oxide, ZnO) nanoparticles coated with a lipid double layer deriving from extracellular vesicles (e.g., exosomes) isolated from cell culture media or directly from patients' biological fluids;

- such nanoassembly (ZnO + exosome) results to be non-immunogenic, biocompatible, well dispersible in biological fluids (therefore, suitable for an easy and rapid internalization in target tumour cells), moreover it can be further decorated with antibodies for a more specific and selective action towards the target tumour cells, saving healthy cells;

- such nanoassembly (ZnO + exosome) is internalized into the target tumour cell;

- the zinc oxide nanocrystals, which constitute the core of said nanoassembly, in a biological environment are able to dissolve and release zinc ions Zn²⁺, which above certain values, if intracellular, are toxic to the cell;

- zinc oxide nanocrystals suitably excited by light sources with wavelengths in the ultraviolet region are able to re-emit light in the green region (showing an emission

centred at about 500-600 nm in wavelength), thus in the visible spectrum;

- the nanoassembly as a whole (ZnO + exosome) can therefore be used as a theranostic instrument, i.e. it can perform both therapy and imaging of the region of interest at the same time;

- 5 - the nanoassembly as a whole (ZnO + exosome) undergoes final biodegradation at the end of the expected activity, avoiding accumulation in other organs.

From the description above reported, it is therefore apparent how the present invention allows to achieve the intended objects.

- 10 Therefore, it is apparent to a person skilled in the art that it is possible to make modifications and further variants to the solution described with reference to the accompanying figures, without departing from the teaching of the present invention and from the scope of protection, as defined by the appended claims.

CLAIMS

1. An ultrasonic activation method comprising the following steps:
 - providing at least one sonosensitizing agent (ASS) comprising zinc oxide (ZnO) micro- and/or nanoparticles (step 100);
 - 5 - providing a solution to be treated (SOL) (step 101);
 - dispersing the at least one sonosensitizing agent (ASS) in the solution to be treated (SOL), thus obtaining a solution to be treated with dispersed sonosensitizing agent (6) (step 102);
 - 10 - providing at least one ultrasound source (US) comprising a signal generator (1) and an electromechanical transducer (5) (step 103);
 - activating the at least one ultrasound source (US) (step 104);
 - placing the solution to be treated with dispersed sonosensitizing agent (6) in the ultrasonic field (step 105);
 - 15 - inducing cavitation in the solution to be treated with dispersed sonosensitizing agent (6), said sonosensitizing agent thus lowering the cavitation threshold and bringing packet of gas bubbles due to its surface conformation (step 106); and
 - generating chemical-physical-mechanical modifications in the solution to be treated with dispersed sonosensitizing agent (6), thus obtaining a solution treated and enriched with zinc ions (Zn^{2+}) (step 107).
- 20 2. The ultrasonic activation method according to claim 1, wherein the solution to be treated (SOL) is an aqueous solution.
3. The ultrasonic activation method according to claim 1 or 2, wherein the ultrasounds have a frequency ranging from 20 kHz to 100 MHz and a power ranging from $0.01 \text{ W} \cdot \text{cm}^{-2}$ to $25,000 \text{ W} \cdot \text{cm}^{-2}$.
- 25 4. The ultrasonic activation method according to claim 3, wherein the ultrasounds have a frequency ranging from 40 kHz to 10 MHz and a power ranging from $0.1 \text{ W} \cdot \text{cm}^{-2}$ to $10 \text{ W} \cdot \text{cm}^{-2}$.
5. The ultrasonic activation method according to any claim 1 to 4, wherein the total activation time, in case provided in several steps or cycles, ranges from 1 second to
30 12 hours.

6. The ultrasonic activation method according to claim 5, wherein the total activation time, in case provided in several steps or cycles, ranges from 5 seconds to 30 minutes.
7. The ultrasonic activation method according to any claim 1 to 6, wherein the
5 chemical-physical-mechanical modifications generated in the solution to be treated with dispersed sonosensitizing agent (6) are the production of reactive oxygen and free radical species and/or adiabatic increasing of temperature and/or mechanical damages to phospholipid double layers comprising cell membranes and/or cellular organelles and/or ion release.
- 10 8. The ultrasonic activation method according to any claim 1 to 7, wherein the chemical-physical-mechanical modifications also occur on the sonosensitizing agent (ASS) that generated them, and include mechanical, piezoelectric, pyroelectric and triboelectric effects.
9. An ultrasonic activation system (10) comprising:
15
 - at least one solution to be treated with dispersed sonosensitizing agent (6) comprising a sonosensitizing agent (ASS) comprising zinc oxide (ZnO) micro- and/or nanoparticles dispersed in a solution to be treated (SOL);
 - at least one ultrasound source (US) comprising a signal generator (1) and an electromechanical transducer (5); and
 - 20 - at least one ultrasonic transfer medium (M).
10. The ultrasonic activation system (10) according to claim 9, wherein the at least one ultrasonic source (US) also comprises:
 - a signal amplifier (2);
 - an oscilloscope (3); and
 - 25 - a terminator resistive load (4).
11. A sonosensitizing agent (ASS) comprising micro- and/or nanoparticles of zinc oxide (ZnO) to be used in the ultrasonic activation method according to any claims 1 to 8.
12. The sonosensitizing agent (ASS) according to claim 11, wherein the micro- and/or
30 nanoparticles of zinc oxide (ZnO) are nanostructured.

13. The sonosensitizing agent (ASS) according to claim 11 or 12, wherein the micro- and/or nanoparticles of zinc oxide (ZnO) have surfaces functionalized with organic, metallo-organic, metallic groups or coordinating agents, or have surfaces coated with mono- or multilayer of organic agents.
- 5 14. The sonosensitizing agent (ASS) according to any of the previous claims 11 to 13, wherein the micro- and/or nanoparticles of zinc oxide (ZnO) have sizes ranging from 5 nm to 100,000 nm.
15. The sonosensitizing agent (ASS) according to claim 14, wherein microparticles of zinc oxide (ZnO) have sizes ranging from 500 nm to 20,000 nm and nanoparticles
10 of zinc oxide (ZnO) have sizes ranging from 1 nm to 500 nm.
16. A contrast medium for ultrasound (20) comprising micro- and/or nanoparticles of zinc oxide (ZnO) to activate by means of the ultrasonic activation method according to any claims 1 to 8.
17. The contrast medium for ultrasound (20) according to claim 16, wherein the
15 concentration of micro- and/or nanoparticles of zinc oxide (ZnO) ranges from 0.1 µg/mL to 10 mg/mL.
18. The contrast medium for ultrasound (20) according to claim 17, wherein the concentration of micro- and/or nanoparticles of zinc oxide (ZnO) ranges from 1 µg/mL to 500 µg/mL.
- 20 19. The contrast medium for ultrasound (20) according to any claim 16 to 18, wherein the micro- and/or nanoparticles of zinc oxide (ZnO) have sizes ranging from 5 nm to 100,000 nm.
20. The contrast medium for ultrasound (20) according to claim 19, wherein the nanoparticles of zinc oxide (ZnO) have sizes ranging from 1 nm to 500 nm.
- 25 21. The contrast medium for ultrasound (20) according to claim 16, wherein the nanoparticles of zinc oxide (ZnO) have surfaces functionalized with organic, metallo-organic, metallic groups or coordinating agents, or have surfaces coated with mono- or multilayer of organic agents.

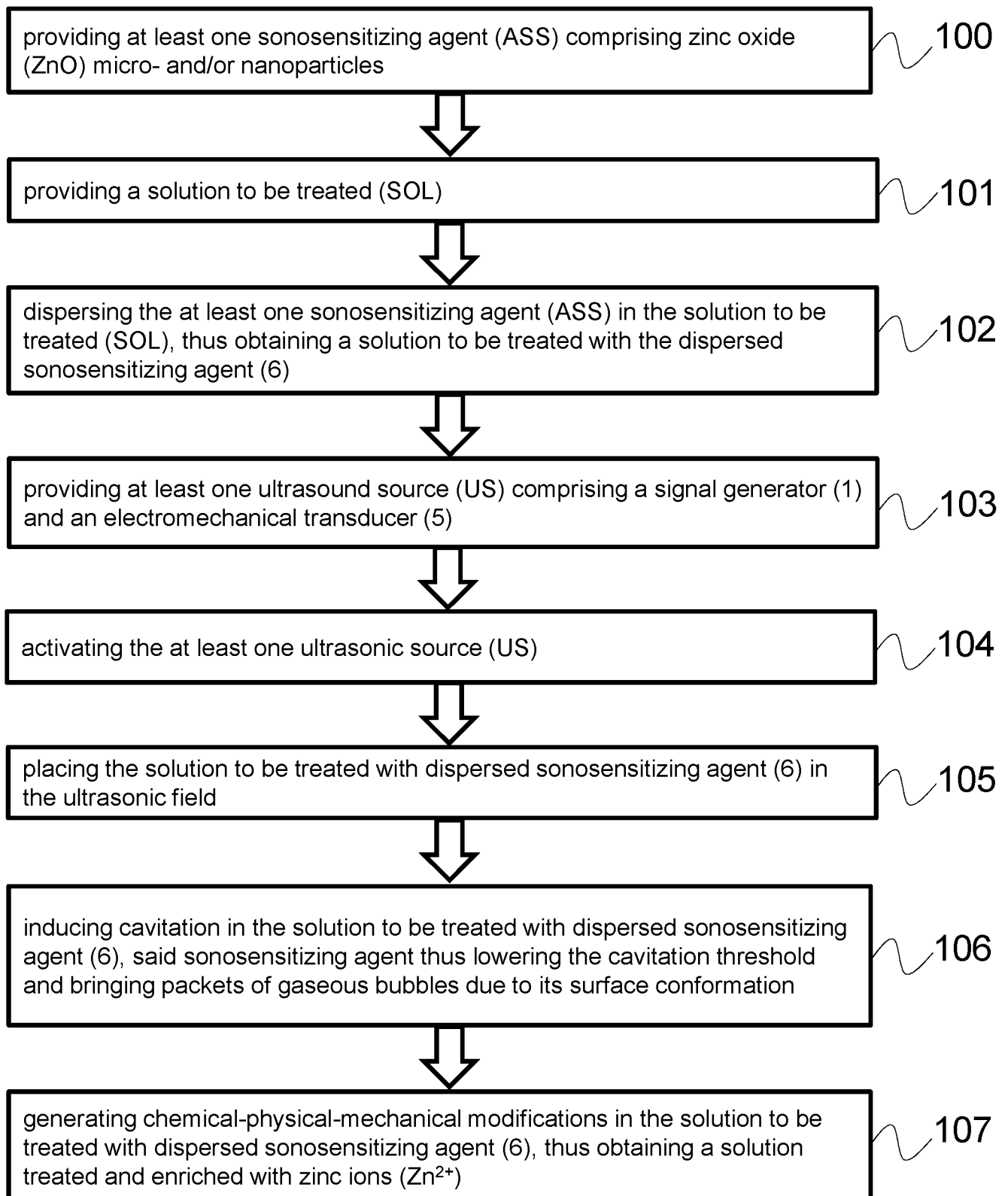


Fig. 1

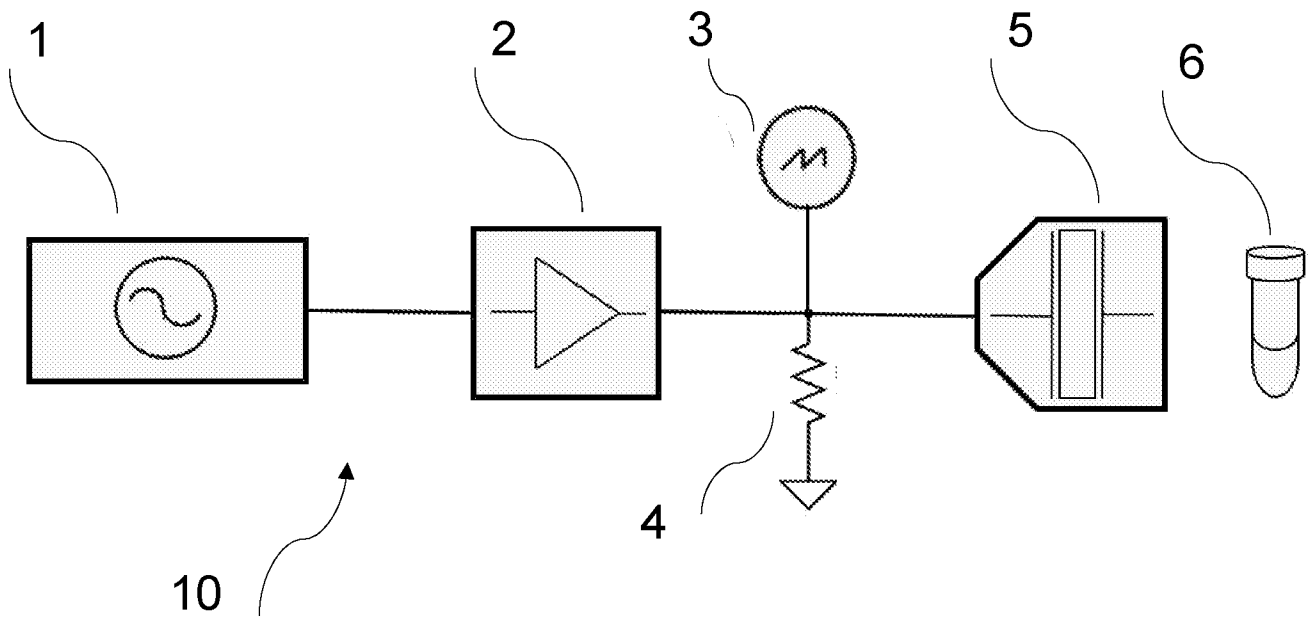


Fig. 2

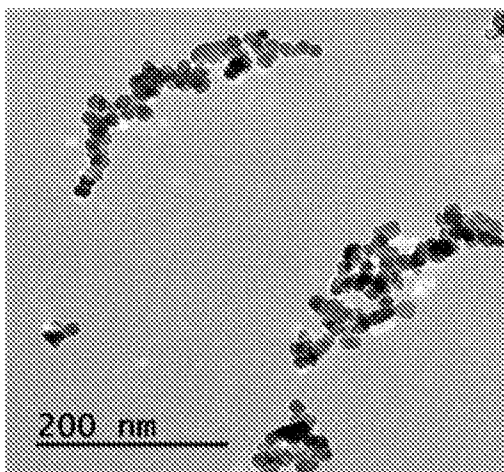


Fig. 3A

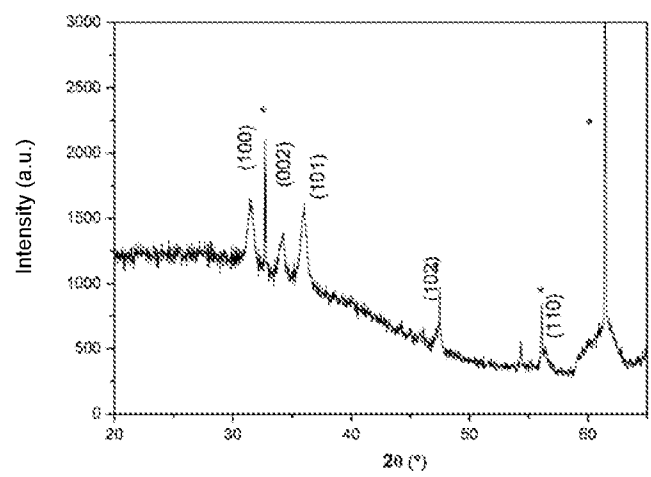


Fig. 3B

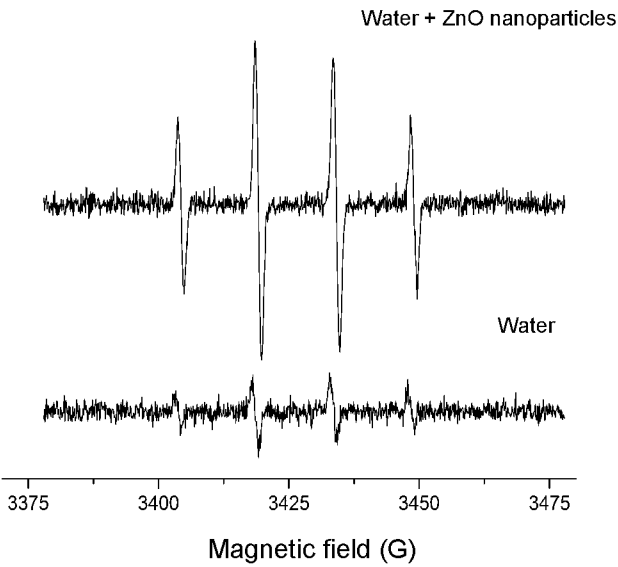


Fig. 4A

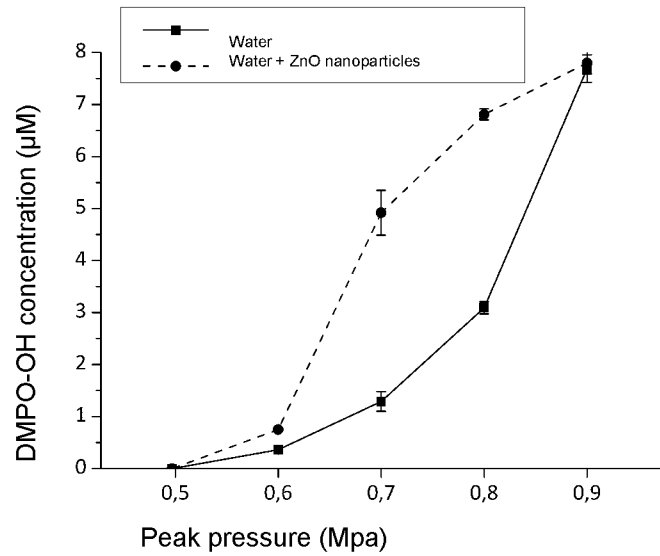


Fig. 4B

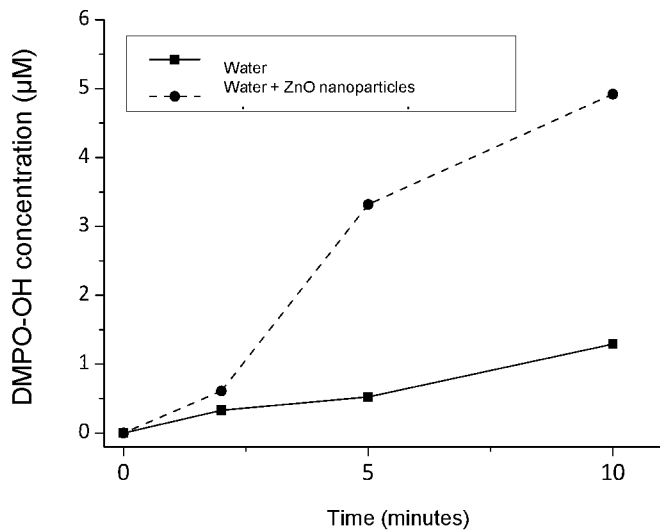


Fig. 5

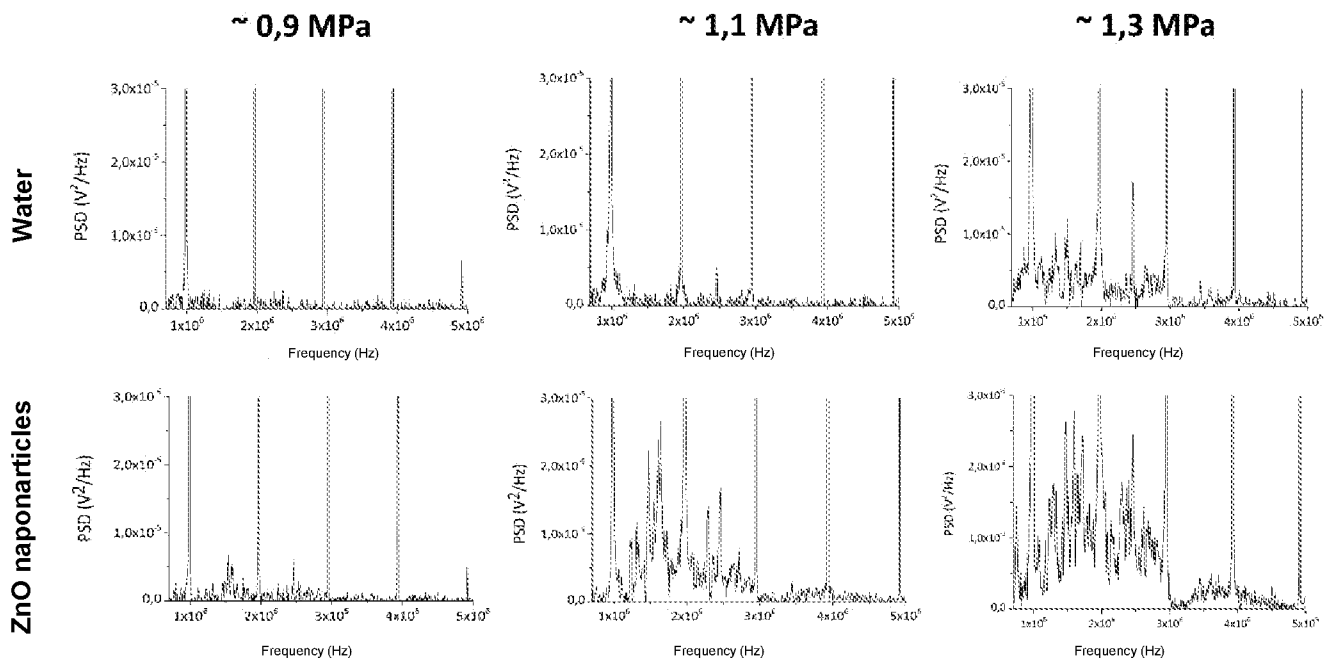


Fig. 6

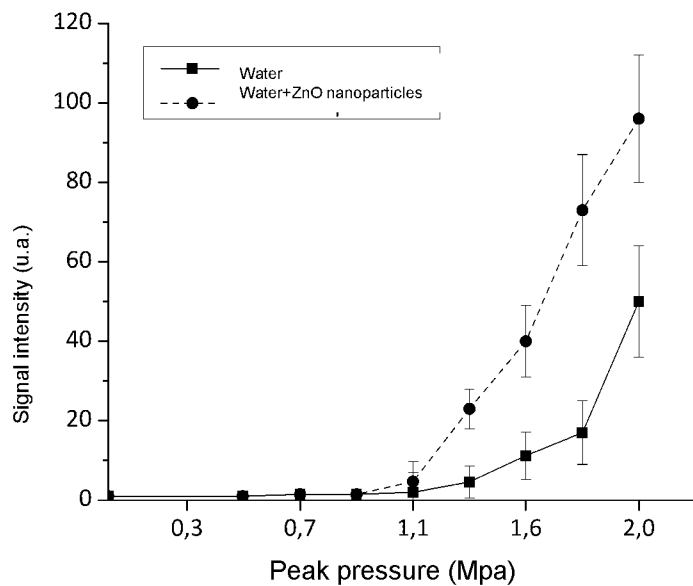


Fig. 7

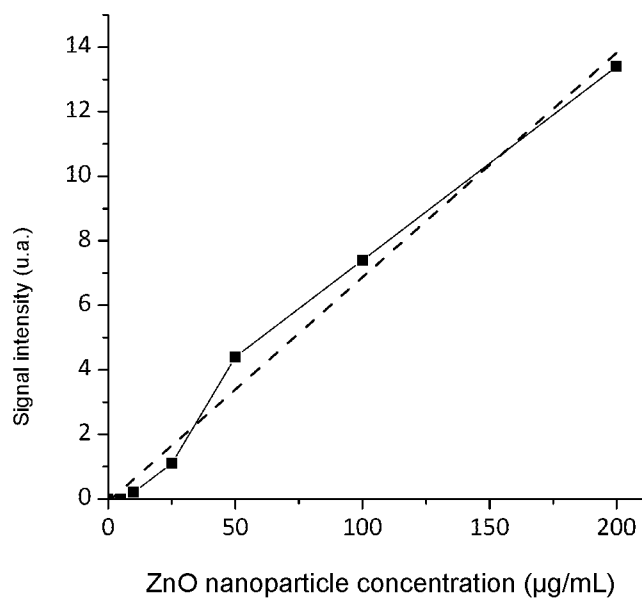


Fig. 8

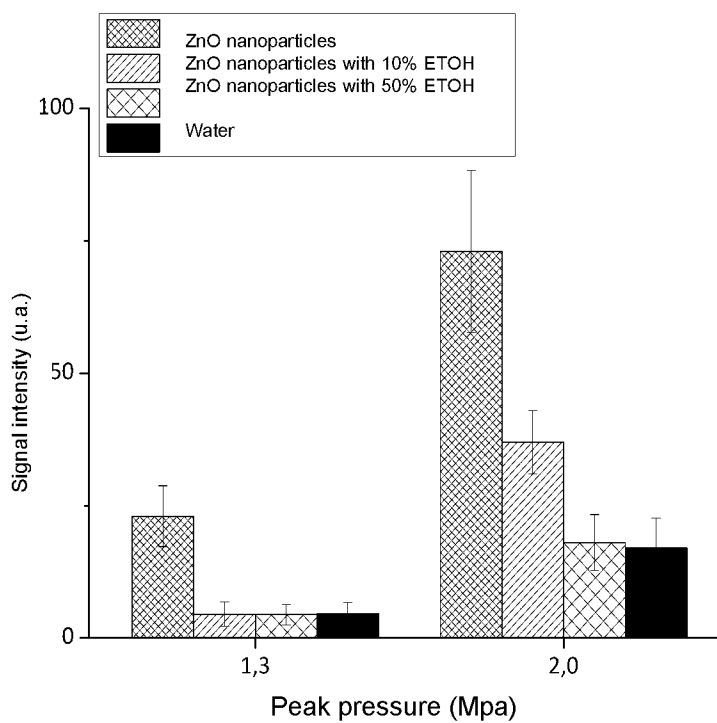


Fig. 9

Water

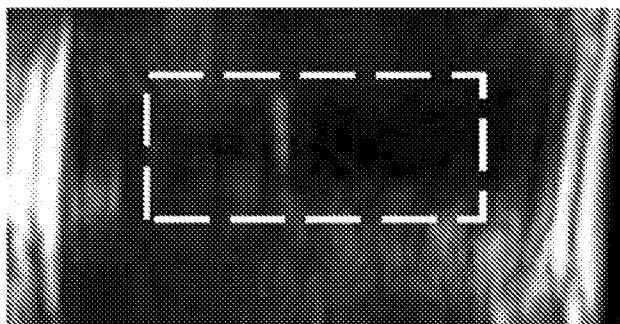


Fig. 10A

ZnO nanoparticles

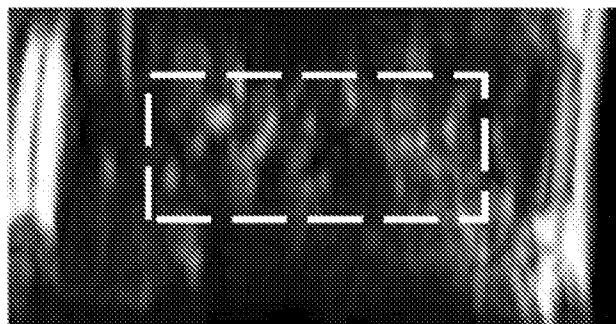


Fig. 10B

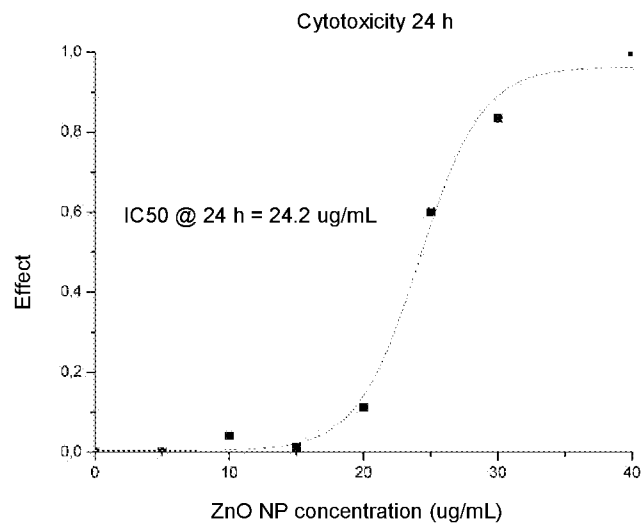


Fig. 11A

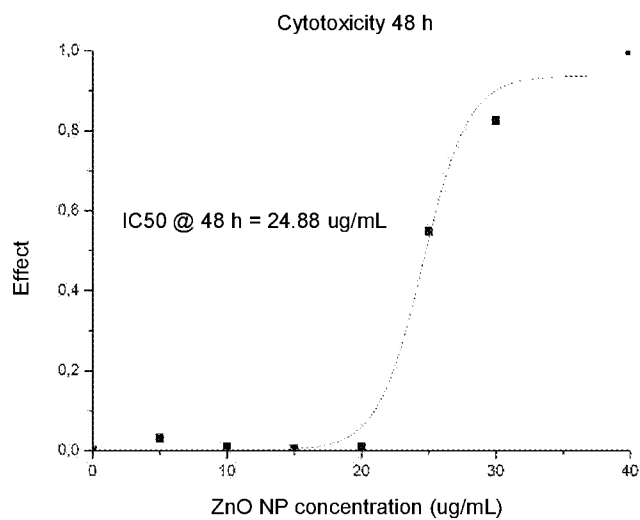


Fig. 11B

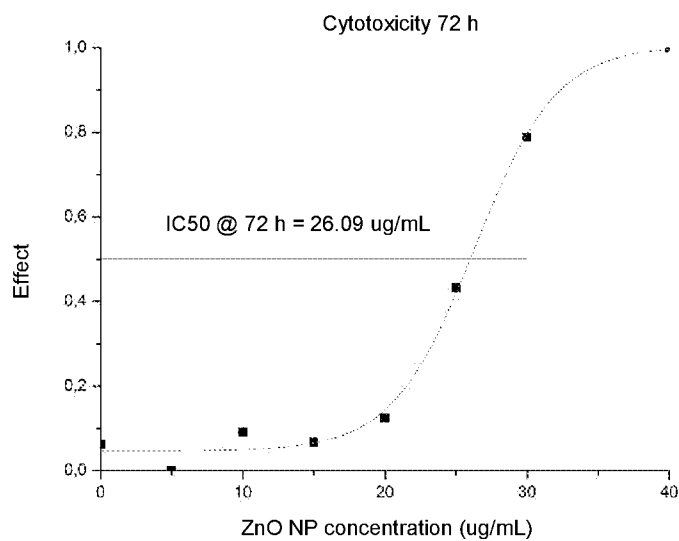


Fig. 11C

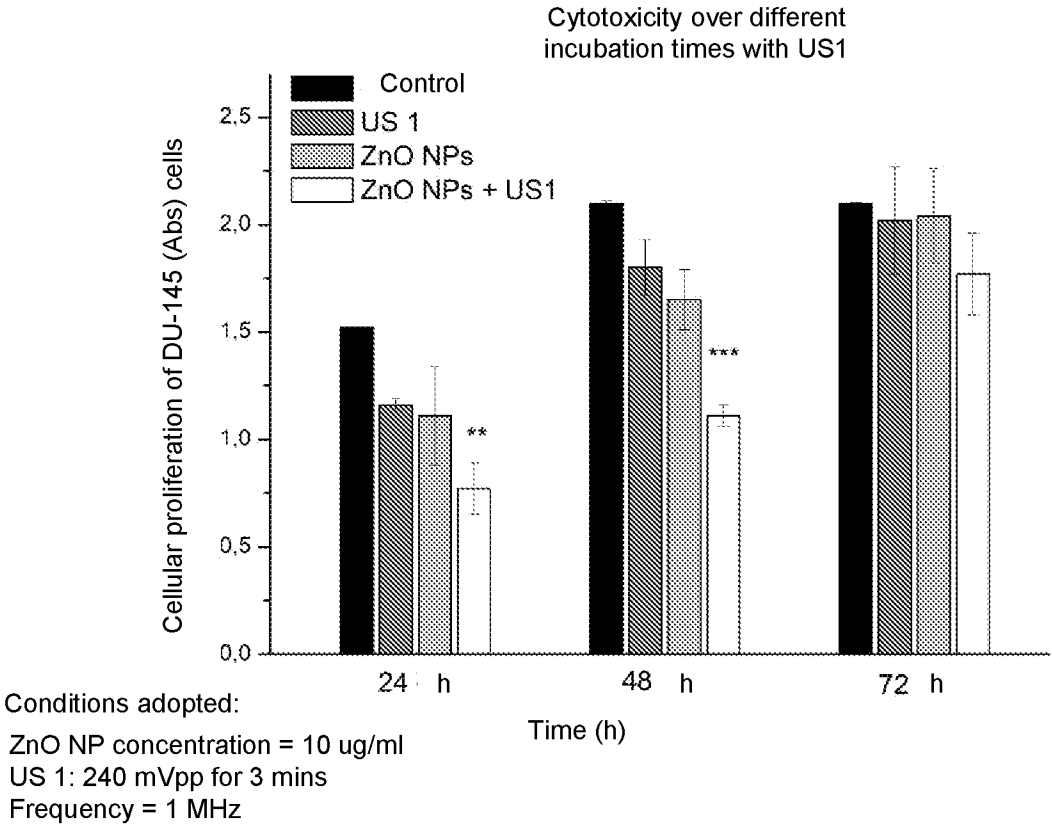


Fig. 12A

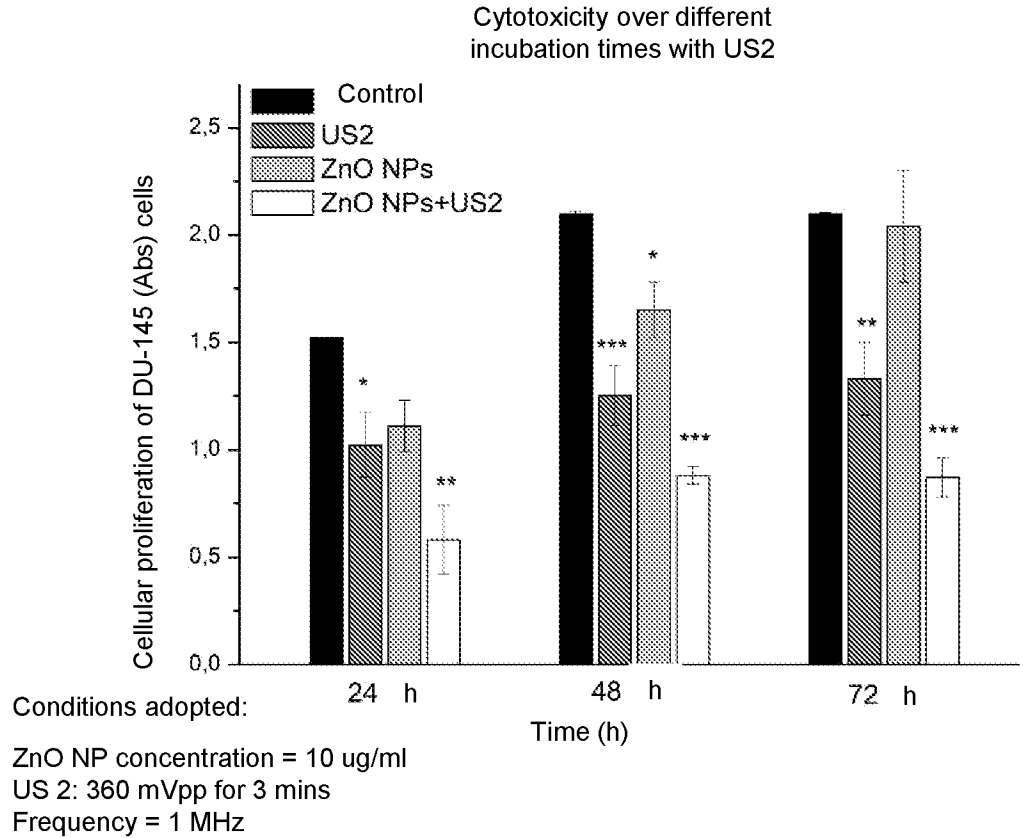


Fig. 12B

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2020/052014

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K41/13 A61K49/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANDREA ANCONA ET AL: "The 24th European symposium on Ultrasound Contrast Imaging 1 Focused ultrasound induced inertial cavitation by amino- propyl functionalized ZnO nanoparticles", THE 24TH EUROPEAN SYMPOSIUM ON ULTRASOUND CONTRAST IMAGING, 17 January 2019 (2019-01-17), XP055598873, page 1, paragraph 2 - page 2, paragraph 2 ----- -/--	1,2,7-9, 11-21



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

30 October 2020

Date of mailing of the international search report

09/11/2020

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Langer, Miren

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2020/052014

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 2016/302420 A1 (GEDANKEN AHARON [IL] ET AL) 20 October 2016 (2016-10-20) ----- -/--	1-21

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2020/052014

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUMAR R V ET AL: "Synthesis and characterization of a micro scale zinc oxide-PVA composite by ultrasound irradiation and the effect of composite on the crystal growth of zinc oxide", JOURNAL OF CRYSTAL GROWTH, ELSEVIER, AMSTERDAM, NL, vol. 250, no. 3-4, 1 April 2003 (2003-04-01), pages 409-417, XP004411735, ISSN: 0022-0248, DOI: 10.1016/S0022-0248(02)02411-9 -----	1-21
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Information on patent family members

International application No

PCT/IB2020/052014

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		US 2020330616 A1	22-10-2020
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