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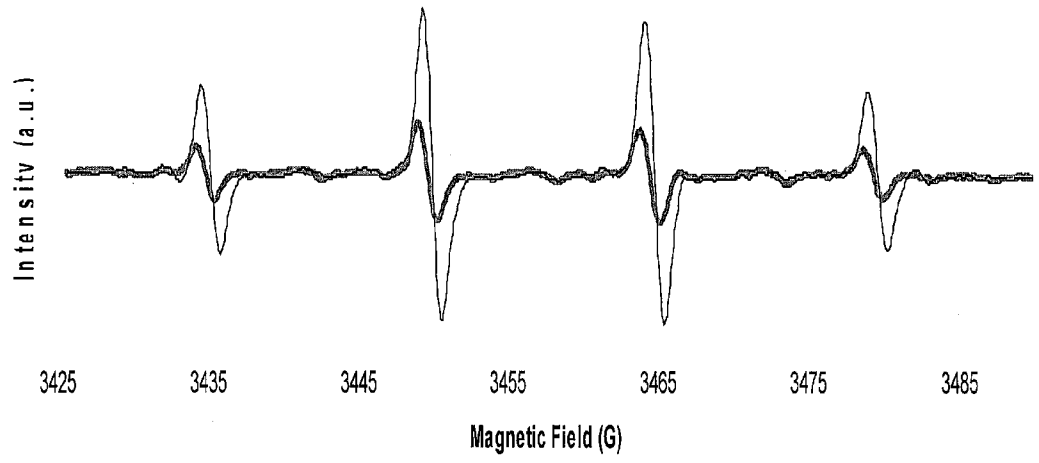
(54) **Title:** COMPOSITION AND METHOD FOR CELL ACTIVATION AND TISSUE REPAIR USING NANOPARTICLES AND LIGHT

**Figure 1**




(57) **Abstract:** A composition and methods of use thereof, as well as methods of treatment, featuring combining administration of nanoparticles or microparticles with or without light stimulation to produce ROS for cell stimulation and tissue regeneration.

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# COMPOSITIONS AND METHODS FOR CELL ACTIVATION AND TISSUE REPAIR USING NANOPARTICLES AND LIGHT

## FIELD OF THE INVENTION

The present invention is of compositions and methods for inducing cell stimulation and in particular, such compositions and methods which feature sub-cytotoxic nanoparticles or microparticles and light.

## BACKGROUND OF THE INVENTION

Reactive oxygen species (ROS) include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons.

It is known that high amounts of ROS are lethal to the cell, a phenomenon exploited in photodynamic therapy (PDT), which is typically employed for cancer therapy and antibacterial treatment (1, 2). PDT employs exogenous photosensitizers, such as hematoporphyrin derivatives, which are introduced to the cells and then irradiated with an appropriate wavelength of visible or near infra-red (NIR) light. The activated photosenstizer molecules pass on their energy to surrounding molecular oxygen, resulting in the formation of ROS. Typically in PDT, a large amount of the photosensitizer compounds accumulate within the target tissue and generate high ROS concentrations upon irradiation.

Cytotoxic amounts of photosensitizers or nanoparticles which destroy recipient cells via generation of cytotoxic ROS may be used for treating diseases such as cancer, viral and bacterial infections (3-6).

At concentrations below those required for cytotoxicity, ROS have a wide range of positive stimulatory effects on the cell.

For example, ROS promote cell growth (7-10) which in the case of fibroblasts can be exploited for wound healing and skin rejuvenation. ROS can induce differentiation of neurons (11) which could be used for nerve regeneration. In spermatozoa, ROS, such as the superoxide anion and $H_2O_2$, were found to induce sperm hypercapacitation and the acrosome reaction (12) which lead to enhanced fertilization rates. In cardiac cells and in cardiomyocytes, ROS function as an important regulator of cell survival (13), and small amounts of ROS were suggested to play a role in preconditioning against myocardial stunning (14). In addition, recent

experiments have shown that ROS stimulate signal transduction processes for transcription factor activation, gene expression, muscle contraction and cell growth (15, 16). The regulatory function of ROS was suggested as being connected to the increased activity of guanosine triphosphate (GTP)-binding proteins such as GTPase Rac-1 (17), which play a pivotal role in multiple signal-transduction pathways (18).

Furthermore ROS may increase nitric oxide (NO) synthesis (19-21). NO is a simple diatomic, semi-stable molecule that has been found to be an important biological messenger. NO is perhaps best known as a mediator of relaxation of vascular smooth muscle (VSM), causing dilation of blood vessels, and was known in this role as endothelium-derived relaxation factor and therefore used for relieving ischemic pain (19-21).

Lasers, light-emitting diodes (LEDs) and broadband light sources in the visible and near IR ranges have emerged in recent years as having therapeutic uses such as nerve regeneration (22, 23), pain relief (24) and more.

The stimulatory effects of light in the visible and near IR regions is termed "photobiomodulation" and has been reported to induce many cell processes, such as proliferation (25-28), spermatozoa fertilization (29) and motility (30), action potentials (31), cell differentiation (32), protection of cells from damage (33), and recovery of damaged cells (34). Other reported effects are the stimulation of collagen synthesis (35), release of cytokines (36) and growth factors (37, 38).

Moreover Oron et al (90) have recently demonstrated the ability of visible light to promote proliferation of mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) in vitro. These results may have an important impact on regenerative medicine.

The mechanism of photobiostimulation is based on the finding that ROS are produced by irradiated cellular endogenous photosensitizers such as cytochromes and flavins; and that ROS have biological effects as discussed above (39-43).

## SUMMARY OF THE INVENTION

The background art does not teach or suggest the use of sub-cytotoxic amounts of nanoparticles or microparticles, either alone or with light stimulation to produce cell-stimulatory amounts of ROS for cell stimulation and tissue regeneration.

The present invention overcomes these deficiencies of the background art by providing compositions and methods of use thereof, as well as methods of treatment, featuring administration of nanoparticles or microparticles, either alone or in combination with light stimulation to produce controlled, limited amounts of ROS for cell stimulation and tissue regeneration. The nanoparticles or microparticles are introduced to the subject exogeneously, as described in greater detail below.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

The term "comprising" means that other steps and ingredients that do not affect the final result can be added. This term encompasses the terms "consisting of" and "consisting essentially of".

The phrase "consisting essentially of" means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the claimed composition or method.

As used herein, the term "nanoparticles" refers to particles having a diameter of the order of about 200 nm or less. Such particles exhibit properties different from those of the bulk material. Because of their ultra-small size, nanoparticles can penetrate cell membranes and integrate themselves into larger molecules. They can resist cellular defense systems but are large enough to interfere with cell processes.

As used herein, the term "microparticles" refers to particles of diameter in the range of from about 1 to about 200 μm.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein, either compounds or physiologically acceptable salts thereof, with other chemical components such as traditional drugs, physiologically suitable carriers and excipients.

As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Herein, the phrases "physiologically suitable carrier" and "pharmaceutically acceptable carrier" are interchangeably used and refer to an approved carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered conjugate.

As used herein, the term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate processes and administration of the active ingredients.

Also as described in greater detail below, a delicate balance must be maintained with regard to the properties of the nanoparticles or microparticles, the amount administered, the type of light stimulation, the amount of light stimulation and localization of the nanoparticles or microparticles and/or of the light stimulation, in order to provide the most beneficial properties to the subject to be treated All of these aspects are described herein collectively as "ROS treatment parameters", as they impact upon the amount of ROS generated, as well as the location at which they are generated.

The present invention, in some embodiments, preferably provides a method for generating ROS for stimulatory purposes in cells and tissues, more preferably increasing their concentration, and hence their activity, in deep lying tissues by irradiating them with light in the visible and near IR region.

These embodiments of the present invention, as well as the present invention overall, may be contrasted with the background art, both as noted above and also as described in greater detail below.

For example, in U.S. Patent No. 6,379,376, co-owned and by the present inventors, it is demonstrated and claimed that broad band visible and near IR light induces in vitro and in vivo growth of cells and tissue, and also controls bacterial infections. This is based on the finding that endogenous photosensitizers can produce ROS. It is also claimed that the effect of light could be enhanced by introducing

small amounts of exogenous photosensitizers such as those used in PDT. While correct, there is limited penetration of the photosensitizers into the cells and bacteria.

Moreover it has been found (44) that the main wavelengths responsible for ROS production by endogenous photosensitizers are in the blue region (400-500nm). The disadvantage of this is the low penetration depth of these wavelengths.

Thus, the present invention is able to overcome these known deficiencies of the background art, as well as to provide many other benefits, some of which are described in greater detail below.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in order to provide what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

Figure 1 shows an electon paramagnetic resonance (EPR) spectrum of zinc oxide in water, in which hydroxyl and super oxide anion radicals formation is clearly monitored. Application of white light increases the ROS amount;

Figure 2 shows that nanoparticles such as TiO2 can generate ROS , the amount of which can be increased by white light; and

Figure 3 shows that incubation of fibroblasts with $TiO_2$ at 50uM results in a stimulation of 40% in the cell number.

## DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides compositions and methods of use thereof, as well as methods of treatment, featuring nanoparticles or microparticles, either alone or

in combination with light stimulation to produce limited amounts of ROS for cell stimulation and tissue regeneration.

The use of nanoparticles to produce a cytotoxic effect via generation of high levels of ROS is known, and has been used for treatment of cancer, and viral and bacterial infections, as discussed in the Background section above. The present inventors have surprisingly found that lower amounts of nanoparticles and microparticles than those which result in cytotoxicity can cause stimulation of cellular activity, including cell activation and tissue repair.

The nanoparticles or microparticles themselves contain stable ROS, including but not limited to hydroxyl, singlet oxygen and super oxide anion, hydrogen peroxide, and nitric oxide which stimulate cell activity. Upon introduction to the tissue, the nanoparticles or microparticles cause cell stimulation and tissue regeneration; further light stimulation increases the amount of ROS produced.

The nanoparticles can penetrate easily into cells, while microparticles generate ROS at the cell membrane.

According to some embodiments, there is provided the use of a particle selected from the group consisting of a nanoparticle and a microparticle for inducing cell stimulation.

According to some embodiments, there is provided a method of inducing cellular activity, the method comprising administering to a subject a particle consisting of a nanoparticle and a microparticle.

According to some embodiments, there is provided a composition for inducing cell stimulation, the composition comprising a particle selected from the group consisting of a nanoparticle and a microparticle and a pharmaceutically acceptable carrier.

The nanoparticles or microparticles are preferably present in sub-cytotoxic concentrations.

The sub-cytotoxic concentration varies according to the particular material and size of the nanoparticle or microparticle.

The present inventors have found that nanoparticles or microparticles at concentrations in the range of 1-100 μg/l can stimulate skin cells.

ZnO at 1mM or 80 ug/ml was found to have antibacterial effect on a broad spectrum of microorganisms (88); while $TiO_2$ at 0.1-1 mg/ml was found to have a photocatalytic effect on E. coli (89).

According to some embodiments, the nanoparticles or microparticles may optionally comprise one or more metal oxides, more preferably metal dioxides of biologically compatible metals, semiconductors and metal oxides doped in silica. Examples of suitable metal oxides include ZnO, $TiO_2$, FeO, CuO $Ag_2O$, $Co_3O_4$, $Mn_3O_4$ or other metal oxides doped in silica, including but not limited to $Fe_2O_3$ /$SiO_2$.

Alternatively or additionally, a fullerene, carbon, a heterocrystal mineral, or a combination thereof may be used.

The heterocrystal mineral optionally includes but is not limited to rutile, sphere, loparite, perowskite, anatase, ilmenite, leukoxen, ferrite, argyrite, graphite, CaO, phosphoritemonooxides phosphoritedioxide, or CdSe/ZnS.

Without wishing to be limited by a single hypothesis, the capability of certain semiconductor nanoparticles or microparticles, such as, for example, those comprising ZnO to generate stable oxygen radicals is mainly attributed to their crystal defects. It is becoming increasingly established that the functional properties of nano- and micro scale materials depend not only on their bulk composition and morphology but also on their defect structure (46).

The bandgap size and consequently the electronic properties are very much dependent on the preparation of the nanoscale or microscale semiconductor.

The nanoparticle diameters are in the range of up to about 1000 nm. Preferably, the dimensions are in the range of from about 0.5 to about 200 nm, more preferably from about 0.5 to about 50 nm.

The microparticle diameters are preferably in the range of 1 to about 200 μm, more preferably in the range of from about 1 to about 10 μm.

Optionally, mixtures of different sized nanoparticles and microparticles at different ratios may be used.

According to some embodiments, the nanoparticles and microparticles of the present invention may have spherical and /or rod shapes.

The nanoparticles or microparticles are optionally coated with polyvinyl alcohol (PVA), poly-(N-vinyl-2-pyrrolidone) (PVP) Polyethylene Glycol (PEG), and/or other coatings to avoid aggregation.

To control the microenvironment of the nanoparticle or microparticle in the cell, the nanoparticles and microparticles may optionally be administered to the cells by means of binding them to units (such as proteins) targeted to a specific location.

For example, magnetic nanoparticles such as $Fe_2O_3$ can be targeted to a specific cell through an external static magnetic field.

According to some embodiments of the present invention, there are provided coated nanoparticles or microparticles which form "quantum dots". These nanoparticles comprise a metal or metals, and/or carbon, and are coated with photosensitizers such as porphyrins derivatives or flavins which are excited by visible and near IR light and/or which have poor solubility in water and tend to aggregate in aqueous solutions (47, 48). The nanoparticles facilitate penetration of the photosensitizers into the cells. Nanoparticles and microparticles can serve as ideal carriers for photosensitizer molecules for photobiostimulation and tissue repair.

According to some embodiments, the use or method of the present invention further comprises irradiating the particles with light.

For each nanoparticle and microparticle, there is an optimal wavelength or a band of wavelengths in the visible and near IR region with regard to stimulation of ROS. The optimal wavelength can be identified by methods known in the art, such as, for example, electron paramagnetic resonance (EPR) methods. This optimal wavelength is preferably balanced against one or more other treatment parameters, such as for example the need to penetrate deeply into tissue.

The irradiation can take place before, during or after introducing the nanoparticle or microrparticle to the cells and/or tissue.

Without wishing to be limited by a single hypothesis, the suggested mechanism for light induced ROS in semiconductors such as ZnO, $TiO_2$ is as follows. Upon ultraviolet or visible light excitation, the photon energy excites valance electrons and generates pairs of electrons and holes (electron vacancy in a valence band) that diffuse and become trapped on or near the nanoparticle or microparticle surface. These excited electrons and holes have strong reducing and oxidizing activities and react with water and oxygen to yield active oxygen species, such as hydroxyl radicals ( $\cdot OH$) and superoxide anions ($O_2^-$) (49).

Among the advantages of using nanoparticles or microparticles and light for cellular stimulation, in comparison to previous techniques using light alone for photobiostimulation, is the possibility of using less intense and yet more penetrative light to obtain the same ROS amounts. Use of such light provides the possibility of optionally treating deeper tissues, including but not limited to bone, muscle and nerves. Furthermore, the use of light provides the optional ability to regulate ROS

concentrations within the cell for various time intervals. This permits the introduction of very small concentrations of nonactive nanoparticles and microparticles (less than about 1 mM) to the tissue, which are then activated through controlled administration of light.

According to some embodiments, a light source in the visible and near infrared range (400-3000nm), continuous wave (CW) and/or pulsed, is preferably used. The light could optionally be coherent, polarized or 400-500nm.

In order to shift the sensitivity of certain nanoparticles from the ultraviolet or the blue region to the red and near infrared range (500-900nm) wherein light penetration depth is higher, it is possible to use nitrogen- and carbon-doped semiconductors like N-doped $TiO_2$. This leads to ROS generation in deep lying tissues, as red and near infrared light to penetrate the tissue to a high extent.

The light source is preferably selected from the group consisting of lasers, LEDs and broad band visible light devices. The limit of the light intensity is such that it does not lead to a significant thermal effect to skin cells, when applied on the skin. For example, it may cause a 5°C increase in the tissue temperature, which is not considered to have a significant thermal effect on the tissue. The term "skin" as used herein, refers to the outlining tissue of a human or mammalian body, as well as other epithelial tissue.

The nanoparticles or microparticles are introduced to the subject exogeneously.

By "subject" it is meant any living cells or tissue, whether in vivo or ex vivo.

The subject may optionally comprise any mammal or non-mammal. The term "mammal" preferably includes any commercially important animal as well as humans.

According to some embodiments, pharmaceutical carriers of the composition of the present invention can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

According to some embodiments of the present invention, the pharmaceutically acceptable carrier of compositions of the present invention

optionally comprises one or more of a physiological salt solution, dimethyl sulfoxide (DMSO), a solution of colloid liquid protein, a polymer solution, a suitable suspension etc. The nanoparticles or microparticles could also optionally be added to a powder which would then be administered to the subject.

The pharmaceutical composition may optionally further comprise one or more components selected from binding agents, stabilizers, diluents, excipients, surfactants, , and odorants.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, levigating, emulsifying, entrapping or lyophilizing processes.

Suitable pharmaceutical excipients include without limitation, calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, sodium stearate, glycerol monostearate, talc, sodium chloride, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, powders, sustained-release formulations and the like.

Further techniques for formulation and administration of active ingredients may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference as if fully set forth herein.

The pharmaceutical compositions herein described may also comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more pharmaceutically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically.

Proper formulation is dependent upon the route of administration chosen. Preferably, administration is by the transdermal route.

Formulations for oral delivery can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium

saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should be suitable for the mode of administration.

The composition for transdermal administration can be formulated in a form of a gel, a cream, an ointment, a paste, a lotion, a milk, a suspension, an aerosol, a spray, a foam, a serum, a swab, a pledget, a pad or a patch. Formulations for transdermal delivery can typically include carriers such as water, liquid alcohols, liquid glycols, liquid polyalkylene glycols, liquid esters, liquid amides, liquid protein hydrolysates, liquid alkylated protein hydrolysates, liquid lanolin, lanolin derivatives, glycerin, mineral oil, silicone, petroleum jelly, lanolin, fatty acids, vegetable oils, parabens, waxes, and like materials commonly employed in topical compositions.

Various additives, known to those skilled in the art, may be included in the transdermal formulations of the invention. For example, solvents may be used to solubilize certain active ingredients substances. Other optional additives include skin permeation enhancers, opacifiers, anti-oxidants, gelling agents, thickening agents, stabilizers, and the like.

The pharmaceutical compositions herein described may also comprise suitable solid of gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

Compositions comprising the nanoparticles or microparticles of the invention, and optionally other active ingredients, formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is detailed herein.

According to some embodiments, the cell stimulation induced by the present invention is one or more of cell growth, cell proliferation and differentiation, tissue regeneration (including regeneration of skin, stem cells, muscle, cartilage, connective tissue, epithelial tissue, heart and bone), skin rejuvenation, blood vessel dilation, and muscle relaxation.

Recent evidence has suggested that ROS might act as modulators of neural processes, including synaptic transmission (50); also the kinetics of ROS production may determine whether nerve cells will differentiate or proliferate (51). Moreover it has been suggested that preconditioning-induced neuroprotection is mediated by ROS through activation of the transcription factor nuclear factor kappaβ (52). The nanoparticles and microparticles of the present invention may thus be used to modulate synaptic transmission, differentiation and proliferation of nerve cells, and provide neuroprotection.

Treatment of the sciatic nerve of the rat with low energy He-Ne laser caused a significant increase in the amplitude of the action potential recorded in the corresponding gastrocnemius relative to the action potential of injured but not treated nerves and was accompanied by a diminution of the size of the scar tissue from these nerves (53, 54). As low energy visible light effects are attributed to ROS formation, the present invention may be used for nerve regeneration, the advantage of which is the generation of ROS in nerves lying not only in superficial sites.

Embodiments of the present invention may be used for treatment of any condition which is treatable by phototherapy.

For example, phototherapy in the visible range has been shown to be beneficial in the treatment of tissue regeneration which leads to the healing of chronic and acute wounds (55). Studies with fibroblasts and keratinocytes indicate that at specified relatively low energy doses of He-Ne laser or 780 nm diode laser, accelerated mitosis occurs (56).

Phototherapy also has beneficial effects on mouse, rat, dog and pig ischemic heart models. Phototherapy has a markedly beneficial effect on repair processes after injury or ischemia in skeletal and heart muscles (57).

Animal studies on the enhanced wound healing, bone, cartilage and muscle repair of laser light of low power density have been performed in toads, mice, rats, guinea pigs, and swine (58, 59). Human studies with laser light have demonstrated greater amounts of epithelialization for wound closure and stimulation of skin graft healing (60, 61).

Hence, according to some embodiments, the methods and compositions of the present invention may be used for wound healing, including healing of chronic and acute wounds.

The present inventors have previously shown, by using the electron paramagnetic resonance (EPR) coupled with the probe trapping technique, that the first step in photobiostimulation including stimulation of skin, sperm, and cardiac cells is ROS formation following irradiation (62, 63).

According to some embodiments of the present invention, there is provided a method and compositions for tissue regeneration, for example for regeneration of skin, epithelial cells, muscle, heart and bone, through the generation of small, controlled amounts of ROS using nanoparticles or microparticles, with or without light.

Considerable amounts of research have been performed in the field of non-ablative skin rejuvenation, in response to the continuous demand for a simple method of treating, for example, rhytids, UV exposure and acne scars. Numerous studies involve visible light systems operating at energy doses that do not burn the skin (64).

In a recent study by the present inventors, it was found that the mechanism for photorejuvenation is based on light induced Reactive Oxygen Species (ROS) formation (65). In this study, collagen was irradiated in-vitro with a broad band of visible light, 400-800 nm, 24-72J/cm$^2$, after which the spin trapping method coupled with electron paramagnetic resonance (EPR) spectroscopy was used to detect ROS. Irradiated collagen resulted in hydroxyl radical formation.

The results of the study suggested that visible light at the energy doses used for skin rejuvenation (20-30J/cm$^2$), produces ROS which destroy old collagen fibers, encouraging the formation of new ones. In places where low amounts of ROS are formed, they stimulate fibroblast proliferation, thereby regenerating the skin (66-69).

According to preferred embodiments of the present invention, there is provided a method and compositions for skin rejuvenation, using nanoparticles or microparticles, with or without light. The method and composition according to these embodiments may be used for treatment of rhytids, ultraviolet exposure, and acne scars.

Mammalian sperm undergo acrosome reaction before fertilization in order to penetrate the oocyte zona pellucida (ZP). This process occurs following binding to the ZP only if the spermatozoa have previously undergone a poorly-defined maturation process known as capacitation (70). The binding of the capacitated spermatozoa to the oocyte ZP activates a low-voltage $Ca^{2+}$ channel (71) and a store-operated $Ca^{2+}$ channel, which causes a sustained elevation of intracellular $Ca^{2+}$, leading to the acrosome reaction (72). The priming of spermatozoa to such calcium signals during capacitation involves many changes, including cholesterol efflux from the plasma membrane (73, 74), and increases in intracellular free $Ca^{2+}$ (75-77), cAMP (78-80) pH (81), protein tyrosine phosphorylation (82, 83), and actin polymerization (84). It has been found that reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion are involved in the regulation of human sperm capacitation and protein tyrosine phosphorylation (85-87).

The present inventors have previously found that He-Ne laser enhances intracellular calcium levels and the fertilizing potential of mouse spermatozoa. It was demonstrated that the effect of light is mediated through the generation of ROS by the spermatozoa and that this effect plays a significant role in the augmentation of the sperm cells' capability to fertilize metaphase II-arrested eggs in vitro (91).

In another study, the present inventors showed, using the hamster egg penetration essay (SPA), that He-Ne radiation may improve poor human sperm egg penetration ability. The present inventors also demonstrated, by using the Electron Paramagnetic Resonance (EPR) technique, that ROS are formed in illuminated sperm cells, which again proves the involvement of ROS in increasing the fertilization rates (92).

U.S. Patent No. 6,379,939 discloses that visible light enhances fertilization.

According to some embodiments of the present invention, there is provided a method and compositions comprising nanoparticles and microparticles for increasing fertilization rates by sperm of animals, including humans, with or without light.

Methods for relieving pain from pain-affected body areas of a patient, by radiating light, are known in the art.

The pain affected area is irradiated with a beam of laser light, or a LED, of a particular wavelength in the visible or near IR region. For example, He-Ne (632nm) and GaAlAs (830nm) lasers are used for pain relief. An updated summary concerning this modality has been written by Bijordal et al, review, Photoirradiation in acute pain.(2006), Photomedicine and Laser Surgery, 24, 2, 158-168.

The mechanism of light induced pain relief seems to be related to ROS formed by the irradiated nerve.

For example it has been shown (93) that a high power 300mW, 700mW/cm2 830nm laser, which had an analgesic effect, caused a decrease in the mitochondrial membrane potential and the ATP of rat neurons. Such an effect could be explained by high ROS concentrations generated in the cell, inhibiting the cellular respiratory chain (RC), and hence inhibit Adenosine Tri-Phosphate (ATP) production (94).

It is also known that ROS increase Nitric oxide (NO) synthesis which has an important role in ischemic pain relief.

According to some embodiments of the present invention, there is provided a use, method and composition for providing pain relief in a subject in need thereof, through the generation of small, controlled amounts of ROS using microparticles or nanoparticles with or without light. Preferably, if light is used, the light is in the visible range. More preferably, sufficiently penetrative light is used to treat deep lying tissues for pain relief.

The present invention, in at least some embodiments, provides ex-vivo applications for cellular stimulation, for example for increasing the rate of cultivation of skin cells in order to rapidly obtain skin-like tissue for grafting onto burn wounds.

According to optional embodiments of the present invention, the subject is placed in a unit arranged to irradiate the subject with light, thereby activating the nanoparticles or microparticles

## EXAMPLES

Reference is now made to the following examples, which together with the above description, illustrate the invention in a non limiting fashion.

***Example 1: Formation of ROS (hydroxyl and super oxide anion radicals) in water suspensions of ZnO nanoparticles with and without white light irradiation.***

In order to detect $^{\bullet}OH$ and $O_2^{\bullet -}$, the EPR-spin trapping technique coupled with the spin trap DMPO was used. DMPO is a common spin probe that detects $^{\bullet}OH$ and $O_2^{\bullet -}$ to give the spin adduct DMPO-OH which yields a quartet EPR spectrum.

Samples containing ZnO nanoparticles (10nm) solution and DMPO were drawn by a syringe into a gas-permeable Teflon capillary (Zeus Industries, Raritan, NJ) and inserted into a narrow quartz tube that was open at both ends. Then the tube was placed into the EPR cavity and the spectra were recorded while or after illumination with white light (40mW/cm$^2$ for 1min) through the EPR cavity on a Bruker EPR 100d X-band spectrometer. The EPR measurement conditions were as follows: frequency: 9.75 GHz; microwave power: 20 mW; scan width: 60 G; resolution: 4096; receiver gain: $5\times10^5$; conversion time: 82 ms; time constant: 1310 sweep time: 335 s; scans: 2; modulation frequency 100 KHz.

As can be seen from Figure 1, a characteristic DMPO-OH spin adduct with hyperfine splitting constant (HFSC), giving rise to 4 resolved peaks ($A_N = A_H^{\beta} = 1.49$ mT) was obtained. These HFSC values suggest that $HO^{\bullet}$ and $O_2^{\bullet -}$ were generated from ZnO (Buettner, 1987) before (grey) and after (black) irradiation.

***Example 2: Formation of ROS (hydroxyl and super oxide anion radicals) in water suspensions of $TiO_2$ nanoparticles with and without white light irradiation.***

Samples containing $TiO_2$ nanoparticles (25nm) in water solution and DMPO were inserted into the EPR machine and the EPR spectra before and after illumination with white light (340mW/cm$^2$ for 1min) were recorded. As can be seen from Figure 2, a characteristic DMPO-OH spin adduct with hyperfine splitting constant (HFSC), giving rise to 4 resolved peaks ($A_N = A_H^{\beta} = 1.49$ mT) was obtained. These HFSC values suggest that $HO^{\bullet}$ and $O_2^{\bullet -}$ were generated from TiO2 before (grey) and after (black) irradiation.

***Example 3: Stimulation of fibroblast proliferation following incubation with $TiO_2$ nanoparticles***

Fibroblasts were incubated with $TiO_2$ nanoparticles (25nm) at concentration 2-150uM for 24h, and cell number was evaluated using the MTT method (95).

As can be seen from Fig. 3, TiO2 at concentrations up to 70 μM stimulates fibroblasts proliferation.

***Example 4: Nerve regeneration***

The sciatic nerve of injured rats is treated for 20 consecutive days, with the compositions of the present invention, with and without light, and the amplitude of the action potential is recorded in the corresponding gastrocnemius. For example ZnO (10nm) in an appropriate gel or ointment is smeared on the injured rat leg followed by blue light irradiation. An increase in the amplitude of the action potential recorded in the corresponding gastrocnemius relative to the action potential of injured but not treated nerves monitors nerve fibers recovery.

***Example 5: Effect on myocardial infarction (MI)***

In order to investigate the possibility of applying nanoparticles to the ischemic heart with or without light-laser application for improving heart function, the model of induction of MI to the rat heart is used (96).

Thirty minutes post MI (rat chest open) nanoparticles are injected into the infarcted area and its vicinity in a well controlled manner in a first group of rats. In a second group of rats, low level blue light irradiation is applied in addition to the nanoparticle injection. A third group of infarcted rats serve as sham (control) group and are injected only with the vehicle of the nanoparticles. Histological observations follow the changes in scar tissue of the treated hearts relative to non- treated ones.

***Example 6: Effect on muscle repair***

Ischemia/reperfusion type of injury is induced in the skeletal leg muscles (3 h of ischemia) of rats. $TiO_2$ nanoparticles in a cream or lotion are smeared or injected at the site of injury, followed by white light illumination. Muscle regeneration following nanoparticle administration alone or with light irradiation is examined by heat shock proteins (HSP-70i) content, and an increase in total antioxidants.

***Example 7: Skin rejuvenation***

Creams containing nanoparticles in the (ug/l range are smeared twice a day for a period of one month, on the skin of patients suffering from acne scars and the improvement of the scars is evaluated by the physician.

***Example 8: Effect on fertilization***

A suspension of $TiO_2$ nanoparticles in water in the ug/l range is added to human spermatozoa in the presence or absence of blue light at very low intensity (in the range of $mW/cm^2$) . The zona-free hamster egg (SPA) assay is used for evaluating the percentage of penetrated eggs (SPA percent) in treated sperms compared to that of the control.

***Example 9: Pain relief***

ZnO nanoparticles in a cream or lotion are smeared onto the wrist of patients suffering from Carpal Tunnel Syndrome (CTS) accompanied by an edema. White light irradiation follows the nanoparticle administration in some of the patients. The edema and the local tingling sensation are evaluated after 10 local treatments compared to non treated patients.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

References:

1. Malik Z, Hanania J, Nitzan Y. (1990) Bactericidal effects of photoactivated 1 porphyrins-an alternative approach to antimicrobial drugs. J Photochem. Photobiol. B. 5:281-93.

2. O'Neill JF, Hope CK, Wilson M. (2002) Oral bacteria in multi-species biofilms can be killed by red light in the presence of toluidine blue. Lasers Surg. Med. 31:86-90.

3. Orenstein A, Haik J, Tamir J, Winkler E, Trau H, Malik Z, Kostenich G. (2000) Photodynamic therapy of cutaneous lymphoma using 5-aminolevulinic acid topical application. Dermatol. Surg. Aug 26(8):765-9;

4. Ashkenazi H, Malik Z, Harth Y, Nitzan Y. (2003) Eradication of Propionibacterium acnes by its endogenic porphyrins after illumination with high intensity blue light. FEMS Immunol. Med. Microbiol. Jan 21;35(1):17-24.

5. Mitoraj D, Janczyk A, Strus M, Kisch H, Stochel G, Heczko PB, Macyk W. (2007) Visible light inactivation of bacteria and fungi by modified titanium dioxide. Photochem. Photobiol. Sci. Jun;6(6):642-8. Epub 2007 Apr 2

6. PCT Application PCT/EP2006/010384

7. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. (1995) Free Radic. Biol. Med. 18: 775-794.

8. Burdon RH, Alliangana D, Gill V. (1995) Hydrogen peroxide and the proliferation of BHK-21 cells. Free Radic. Biol. Med. 23: 471-486.

9. Murrell GAC, Francis MJO, Bromley L. (1990) Modulation of fibroblast proliferation by oxygen free radicals. Biochem. J. 265: 659-665.

10. Callaghan GA, Riordan C, Gilmore WS, McIntyre IA, Allen JM, Hannigan BM. (1996) Reactive oxygen species inducible by low-intensity laser irradiation alter DNA synthesis in the haemopoietic cell line U937. Lasers Surg. Med. 19: 201-206.

11. Suzukawa K, Miura K, Mitsushita J, Resau J, Hirose K, Crystal R, Kamata T. (2000) Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. J. Biol. Chem. 275: 13175-13178.

12. Aitken RJ, Fisher H. (1994) Reactive oxygen species generation and human spermatozoa: The balance of benefit and risk. Bioessays 1994; 1□: 259-267.

13. Das DK. (2001) Redox regulation of cardiomyocyte survival and death. Antioxid. Redox. Signal 3: 23-37.

14. Vanden Hoek TL, Becker LB, Shao ZH, Li CG, Schumacker PT. (1998) Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J. Biol. Chem. 273: 18092-18098.

15. Suzuki YJ, Ford GD. (1999) Redox regulation of signal transduction in cardiac and smooth muscle. J. Mol. Cell. Cardiol. 31: 345-353.

16. Rhee SG. (1999) Redox signaling: hydrogen peroxide as intracellular messenger. Exp. Mol. Med. 31: 53-59.

17. Pani G, Colavitti R, Bedogni B, Anzevino R, Borrello S, Galeotti T. (2000) A redox signaling mechanism for density-dependent inhibition of cell growth. J. Biol. Chem. 275: 38891-38899.

18. Sundaresan M, Yu Z-X, Ferrans VJ, Sulciner DJ, Gutkind SJ, Irani KG-CJP, Finkel T. (1996) Regulation of reactive-oxygen species generation in fibroblasts by Rac1. Biochem. J. 318: 379-382.

19. Thomas SR, Chen K, Keaney JFJ. (2002) Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and

dephosphorylation via phosphoinositide 3-kinase-dependent signaling pathway. J. Biol. Chem. 227:6017-6024.

20. Lopez-Ongil S, Hernandez-Perera O, Navarro-Antolin J, Perez de Lema G, Rodriguez-Puyol M, Lamas S, Rodriguez-Puyol D. (1998) Role of reactive oxygen species in the signaling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. Br. J. Pharmacol. 124:447-454.

21. Banan A, Fields JZ, Zhang Y, Keshavarzian A. (2001) iNOS upregulation mediates oxidant-induced disruption of F-actin and barrier of intestinal monolayers. Am. J. Physiol. Gastrointest. Liver Physiol. 280:G1234-G1246.

22. Anders JJ, Borke RC, Woolery SK, Van de Merwe WP. (1993) Low power laser irradiation alters the rate of regeneration of rat facial nerve. Lasers Surg. Med. 1993; 1□: 72-82.

23. Rochkind S, Nissan M, Alon M, Shamir M, Salame K. (2001) Effects of laser irradiation on the spinal cord for the regeneration of crushed peripheral nerve in rats. Lasers Surg. Med. □□: 216-219.

24. Naeser MA. (2006) Photobiomodulation of pain in carpal tunnel syndrome: review of seven laser therapy studies. Photomed. Laser Surg. 24:101-110

25. Ben-Dov N, Shefer G, Irintchev A, Wernig A, Oron U, Halevy O. (1999) Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. Biochim. Biophys. Acta 1448:372-380.

26. Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD. (2005) Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation in vitro. Lasers Surg. Med. 36:8-12.

27. Gulsoy M, Ozer GH, Bozkulak O, Tabakoglu HO, Aktas E, Deniz G, Ertan C. (2006) The biological effects of 632.8-nm low energy He-Ne laser on peripheral blood mononuclear cells in vitro. J. Photochem. Photobiol. B 82:199-202

28. Grbavac RA, Veeck EB, Bernard JP, Ramalho LM, Pinheiro AL. (2006) Effect of laser therapy in CO2 laser wounds in rats. Photomed. Laser Surg. 24:389-396.

29. Cohen N, Lubart R, Rubinstein S, Breitbart H. (1998) Light irradiation of mouse spermatozoa: stimulation of in vitro fertilization and calcium signals. Photochem. Photobiol. 68:407413.

30. Corral-Baques MI, Rigau T, Rivera M, Rodriguez JE, Rigau J. (2005) Effect of 655-nm diode laser on dog sperm motility. Lasers Med. Sci. 20:28-34.

31. Walker JB, Akhanjee LK. (1985) Laser-induced somatosensory evoked potentials: evidence of photosensitivity in peripheral nerves. Brain Res. 344:281-285.

32. Stein A, Benayahu D, Maltz L, Oron U. (2005) Low-level irradiation promotes proliferation and differentiation of human osteoblasts in vitro. Photomed. Laser Surg. 23:161-166.

33. Oron U. (2006) Photoengineering of tissue repair in skeletal and cardiac muscles Photomed. Laser Surg. 24:111-120.

34. Rochkind S, Ouaknine GE. (1992) New trend in neuroscience: low-power laser effect on peripheral and central nervous system (basic science, preclinical and clinical studies). Neurol. Res. 14:2-11.

35. Saperia D, Glassberg E, Lyons RF, Abergel RP, Baneux P, Castel JC, Dwyer RM, Uitto J. (1986) Demonstration of elevated type I and type III procollagen mRNA levels in cutaneous wounds treated with helium-neon laser. Proposed mechanism for enhanced wound healing. Biochem. Biophys. Res. Commun. 138:1123-1128.

36. Funk JO, Kruse A, Kirchner H. (1992) Cytokine production after helium-neon laser irradiation in cultures of human peripheral blood mononuclear cells. J. Photochem. Photobiol. B 16:347-355.

37. Schwartz F, Brodie C, Appel E, Kazimirsky G, Shainberg A. (2002) Effect of helium/neon laser irradiation on nerve growth factor synthesis and secretion in skeletal muscle cultures. J. Photochem. Photobiol. B 66:195-2000.

38. Yu HS, Wu CS, Yu CL, Kao YH, Chiou MH. (2003) Helium-neon laser irradiation stimulates migration and proliferation in melanocytes and induces repigmentation in segmental-type vitiligo. J. Invest. Dermatol. 120:56-64.

39. Lubart R, Malik Z, Rochkind S, Fisher T. (1990) A possible mechanism of low level laser-living cell interaction. Laser Therapy 2:65-70.

40. Grossman N, Schneid N, Reuveni H, Halevy S, Lubart R. (1998) 780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: involvement of reactive oxygen species. *Lasers Surg. Med.* 22:212-218.

41. Lavi R, Shainberg A, Friedmann H, Shneyvas V, Rickover O, Eichler M, Kaplan D, Lubart R. (2003) Low energy visible light induces reactive oxygen species generation and stimulates an increase of intracellular calcium concentration in cardiac cells. J. Biol. Chem. 278:40917-40922.

42. Lavi R, Sinyakov M, Samuni A, Shatz S, Friedmann H, Shainberg A, Breitbart H, Lubart R. (2004) ESR detection of 1O2 reveals enhanced redox activity in illuminated cell cultures. Free Radic. Res. 38:893-902.

43. Lubart R., Eichler M., Lavi R., Friedmann H., and Shainberg A. (2005) Low-Energy laser irradiation promotes cellular redox activity. Photomedicine and Laser Surgery, 23, 3-9.

44. Eichler M, Shainberg A, Lavie R and Lubart R. (2005) Flavins are source of visible-light-induced free radical formation in cells. Lasers Surg. Med. 37, 14-9.

45. Torimoto T, Murakami SY, Sakuraoka M, Iwasaki K, Okazaki K, Shibayama T, Ohtani B. (2006) J. Phys. Chem. B Condens. Matter Mater Surf Interfaces Biophys. 13: 110(27):13314-8.

46. Vladislav I, et al. (2005) Advanced functional materials. 15,1945-1954.

47. Samia, A, Hone CS, Walker DCPI, Evans-Growing R, FitzGerald S, Beeby A, Chambrier I, Cook MJ, and Russell DA. (2002) Generation of cytotoxic singlet oxygen via phthalocyanine-stabilized gold nanoparticles: A potential delivery vehicle for photodynamic therapy. Langmuir 18, 2985-2987.

48. Roy I, Ohulchanskyy TY, Pudavar HE, Bergey EJ, Oseroff AR, Morgan J, Dougherty TJ, and Prasad PN. (2003) Ceramic-based nanoparticles entrapping water-insoluble photosensitizing anticancer drugs: A novel drug-carrier system for photodynamic therapy. J. Am. Chem. Soc. 125, 7860-7865.

49. Fujishima A, and Honda K. (1972). Electrochemical photolysis of water at a semiconductor electrode. Nature 238:37-38.

50. Chen BT, Avshalumov MV, and Rice ME. (2001) J. Neurophysiol. 85(6): 2468-76.

51. Goldsmit Y, Erlich S, and Pinkas-Kramarski R. (20010 Cell Mol Neurobiol. 21(6): 753-69.

52. Ravati A, Ahlemeyer B, and Becker A (2001) J. Neurochem. 78(4): 909-19.

53. Rochkind S, Barrnea L, Razon N, Bartal A, and Schwartz M. (1987) Stimulatory effect of He-Ne low dose laser on injured sciatic nerves of rats. Neurosurgery. 20(6):843-7.

54. Rochkind S, Nissan M, and Lubart R. (1989) A single trans-cutaneous light irradiation to injured peripheral nerves. Lasers in Medical Sciences,. 4. 259-263.

55. Conlan MJ, Rapley JW, and Cobb CM. (1996) Biostimulation of wound healing by low-energy laser irradiation. A review. J. Clin. Periodontol. 23: 492-496.

56. Grossman N, Schneid N, Reuveni H, Halevy S, and Lubart R. (1998) Enhanced proliferation of keratinocyte cultures following 780 nm low power diode laser irradiation. Involvment of reactive oxygen species. Lasers Surg. Med. 22: 212-218.

57. Oron U. (2006) Photoengineering of tissue repair in skeletal and cardiac muscles . Photomed. Laser Surg. 24(2):111-20

58. Bibikova A, and Oron, U. (1995) Regeneration in Denervated Toad (*Bufo viridis)* Gastrocnemius Muscle and the Promotion of the Process by Low Energy Laser Irradiation. The Anatomical Record. 241, 123-128.

59. Al-Watban F.A. (1997) Laser Acceleration of Open Skin Wound Closure in Rats and its Dosimetric Dependence. Lasers in the Life Sciences. 7(4), 237-47.

60. Miller M, and Truhe T. (1993) Lasers in dentistry: An overview. Journal of the ADA. 124, 32-35.

61. Conlan M.J., and Rapley J.W., Cobb C.M. (1996) Biostimulation of Wound Healing by Low-Energy Laser Irradiation. J. Clin. Periodont. 23, 492-496.

62. Lubart R, Malik Z, Rochkind S, and Fisher T. (1990) A possible mechanism of Low Level Laser-Living Cell Interaction. Laser Therapy, 2, 65-70.

63. Lavi R, Shainberg A, Friedmann H, Shnevays V, Rikover O, Eichler M, Kaplan D and Lubart R.(2003). Low Energy Visible Light Induces Reactive Oxygen

Species Generation and Stimulates an Increase of Intracellular Calcium Concentration in Cardiac Cells. J Biol. Chem. 17;278(42):40917-22.

64. Goldberg DJ, Cutler KB. (2000 )Non-ablative treatment of rhytides with intense pulsed light. Lasers Surg. Med. 26:196-200.

65. Lubart R, Friedmann H, Lavie R, Longo L, Jacobi J , Baruchin O, and Baruchin AM. (2007), Lasers in Medical sciences, to be published. A reasonable mechanism for visible light induced skin rejuvenation.

66. Grossman N, Schneid N, Reuveni H, Halevy S, and Lubart R.(1998) 780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: involvement of reactive oxygen species. Lasers Surg. Med. 22:212-218.

67. Lubart R, Sinyakov M, Friedmann H, Grossman N, Solodiev I, Zurgil N, and Belotsky S. (1999) Photobiostimulation by visible light: Involvement of hydroperoxide. Trends in Photochemistry and Photobiology, 6, 169-174

68. Yamamoto Y, Kono T, Kotani H, Kasai S, and Mito M (1996) Effect of low power laser irradiation on procollagen synthesis in human fibroblasts. J. Clin. Laser Med. Surg. 14(3):129-132.

69. Reddy GK, Stehno-Bittel L, and Enwemeka CS (1998). Laser photo-stimulation of collagen production in healing rabbit Achilles tendon. Lasers Surg. Med. 22:187-281

70. Yanagimachi R. (1994). Mammalian fertilization. In: Knobil E, Neil JD (eds.), The Physiology of Reproduction, vol. 1. New York: Raven Press; 189–317

71. Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, and Florman HM (1999). Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc Natl Acad Sci U S A 1999 96:6757-6762.

72. O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, and Florman HM. (2000). $Ca^{2+}$ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol. Biol. Cell 11:1571-1584.

73. Langlais J, Roberts KD. (1985) A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Res. 1985 12:183-224.

74. Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, and Kopf GS. (1999) Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. J. Biol. Chem. 274:3235-3242.

75. Handrow RR, First NL, and Parrish JJ. (1989) Calcium requirement and increased association with bovine sperm during capacitation by heparin. J. Exp. Zool. 252:174-182.

76. Ruknudin A, and Silver IA. (1990) $Ca^{2+}$ uptake during capacitation of mouse spermatozoa and the effect of an anion transport inhibitor on $Ca^{2+}$ uptake. Mol. Reprod. Dev. 26:63-68.

77. Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, and Forti G. (1991) Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J. Androl. 12:323-330.

78. Parrish JJ, Susko-Parrish JL, Uguz C, and First NL. (1994) Differences in the role of cyclic adenosine 3',5'-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. Biol. Reprod. 51:1099-1108.

79. Parinaud J, and Milhet P. (1996) Progesterone induced $Ca^{2+}$ dependent 3',5'-cyclic adenosine monophosphate increase in human sperm. J. Clin. Endocrinol. Metab. 81:1357-1360.

80. Zhang Y, Ross EM, and Snell WJ. (1991) ATP-dependent regulation of flagellar adenylylcyclase in gametes of *Chlamydomonas reinhardtil*. J. Biol. Chem. 266:22954-22959.

81. Vredenburgh-Wilberg WL, and Parrish JJ. (1995) Intracellular pH of bovine sperm increases during capacitation. Mol. Reprod. Dev. 40:490-502.

82. Visconti PE, Bailey JL, Moore GD, Pan D, Old-Clarke P, and Kopf GS. (1995) Capacitation in mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995 121:1129-1137.

83. Galantino-Homer HL, Visconti PE, and Kopf GS. (1997) Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by cyclic adenosine 3',5'-monophosphate-dependent pathway. Biol. Reprod. 56:707-719.

84. Brener E, Rubinstein S, Cohen G, Shternall K, Rivlin J, and Breitbart H. (2003) Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol. Reprod. 68:837-845.

85. Aitken RJ, Buckingham DW, Harkiss D, Paterson M, Fisher H, and Irvine DS. (1996) The extragenomic action of progesterone on human spermatozoa is influenced by redox regulated changes in tyrosine phosphorylation during capacitation. Mol. Cell. Endocrinol. 117:83-93.

86. de Lamirande E, Leclerc P, and Gagnon C. (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol. Hum. Reprod. 3:175-194.

87. Leclerc P, de Lamirande E, and Gagnon C. (1997) Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radical Biol. Med. 22:643-656.

88. Jones L, Ray B, and Manna AC. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiology Letters , Volume 279, Issue 1, Pages 71-76.

89. Maness PC, Smolinski S, Blake DM, Huang Z, Wolfrum EJ, and Jacoby WA. (1999) Bactericidal Activity of Photocatalytic $TiO_2$ Reaction: toward an Understanding of Its Killing Mechanism . Applied and Environmental Microbiology, Vol. 65, No. 9, 4094-4098.

90. Tuby H, Maltz L, and Oron U. (2007) Low-level laser irradiation (LLLI) promotes proliferation of mesenchymal and cardiac stem cells in culture. Lasers Surg Med. 39(4):373-8.

91. Cohen N, Lubart R, Rubinstein S, and Breitbart H (1998). Light irradiation of mouse spermatozoa: stimulation of *in vitro* fertilization and intracellular calcium. Photochem. Photobiol., 68(3), 407-413.

92. Lubart R, Breitbart H, Soffer Y, and Lavie R. (1999) He-Ne irradiation of human spermatozoa: Enhancement in Hamster Egg Penetration. Laser Therapy, 11, 171-176.

93. Chow R. (2004). Effect of 830nm laser on cultured rat dorsal root ganglia: implication for the analgestic effect of laser. Lasers in Surgery and Medicine supplement 16, p5.

94. Friedman H, and Lubart R. (1996) Competition Between Activating and Inhibitory Processes in Photobiology", SPIE 2630, 60-64.

95. Mosmann,T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival:Application to Proliferation and Cytotoxity Assay.J. Immunol. Meth.,65,55-63).

96. Oron U, Yaakobi T, Oron A, Mordechovitz D, Shofti R, Hayam G, Dror U, Gepstein L, Wolf T, Haudenschild C, and Ben Haim SA (2001). Low energy laser

irradiation reduces formation of scar tissue following myocardial infarction in rats and dogs. Circulation 103: 296-301.

## CLAIMS

1. Use of a particle selected from the group consisting of a nanoparticle and a microparticle for inducing cell stimulation.

2. A method of inducing cell stimulation, the method comprising administering to a subject a particle consisting of a nanoparticle and a microparticle.

3. A composition for inducing cell stimulation, the composition comprising a particle selected from the group consisting of a nanoparticle and a microparticle and a pharmaceutically acceptable carrier.

4. The use, method or composition of any of claims 1 to 3, wherein said nanoparticle or microparticle is present in a sub-cytotoxic amount.

5. The use, method or composition of claim 4, wherein said sub-cytotoxic amount is in the range of from about 0.1 μg/l to about 100 mg/l.

6. The use, method or composition of claim 4 wherein a concentration of said sub-cytotoxic amount is less than about 1 mM.

7. The use or method of any of claims 1 to 6, further comprising irradiating said particle with light.

8. The use, method or composition of any of claims 1 to 7, wherein said particles comprise a material selected from the group consisting of a metal oxide, a fullerene, carbon, and a heterocrystal mineral.

9. The use, method or composition of claim 8, wherein said metal oxide is selected from the group consisting of ZnO, $TiO_2$, FeO, CuO $Ag_2O$, $Co_3O_4$, $Mn_3O_4$, and $Fe_2O_3$ /$SiO_2$

10. The use, method or composition of claim 8, wherein said heterocrystal mineral is selected from the group consisting of rutile, sphere, loparite, perowskite,

anatase, ilmenite, leukoxen, ferrite, argyrite, graphite, CaO, phosphoritemonooxide, phosphoritedioxide, and CdSe/ZnS.

11. The use, method or composition of any of claims 1 to 10, wherein a diameter of said nanoparticles is less than about 1000 nm.

12. The use, method or composition of claim 11, wherein said diameter is in the range of from about 0.5 to about 200 nm.

13. The use, method or composition of claim 12, wherein said diameter is in the range of from about 0.5 to about 50 nm.

14. The use, method or composition of any of claims 1 to 10, wherein a diameter of said microparticles is in the range of from about 1 to about 200 μm.

15. The use, method or composition of claim 14, wherein a diameter of said microparticles is in the range of from about 1 to about 10 μm.

16. The use, method or composition of any of claims 1 to 15, wherein said particles comprise a coating comprising a photosensitizer.

17. The use, method or composition of any of claims 1 to 16, wherein said particles further comprise an anti-aggregation coating.

18. The use, method or composition of claim 17, wherein said anti-aggregation coating is selected from the group consisting of polyvinyl alcohol (PVA), poly-(N-vinyl-2-pyrrolidone) (PVP) Polyethylene Glycol (PEG).

19. The use, method or composition of any of claims 1 to 18, wherein a shape of said particles is selected from the group consisting of a sphere and a rod.

20. The use, method or composition of claim 7, wherein said light has a wavelength in the range of from about 400 to about 3000 nm.

21. The use, method or composition of claim 20, wherein said wavelength is in the range of from about 400 to about 500 nm.

22. The use, method or composition of claim 20, wherein said light is selected from the group consisting of continuous wave and pulsed light.

23. The use, method or composition of claim 20, wherein said light is selected from the group consisting of coherent, polarized or monochromatic light.

24. The use, method or composition of claim 7, wherein said light is provided by a source selected from the group consisting of a laser, a light-emitting diode, and a broad band visible light source.

25. The use, method or composition of any of claims 1 to 24, wherein said cell stimulation is selected from the group consisting of cell growth, cell differentiation and proliferation, tissue regeneration, blood vessel dilation, muscle relaxation, synaptic transmission, neuroprotection, or combinations thereof.

26. The use, method or composition of claim 25, wherein said tissue regeneration is selected from the group consisting of regeneration of skin, stem cells, muscle, cartilage, connective tissue, epithelial tissue, heart and bone, or combinations thereof.

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27. The use, method or composition of any of claims 1 to 24, for use in an ex vivo application.

28. The use, method or composition of claim 27, wherein said ex vivo application comprises cultivation of skin cells for graft preparation.

29. The use, method or composition of any of claims 1 to 24, wherein said cell stimulation comprises skin rejuvenation.

30. The use, method or composition of claim 29, for treatment of a condition selected from the group consisting of rhytids, damage caused by exposure to ultraviolet light, and acne scars.

31. The use, method or composition of any of claims 1 to 24, wherein said cell stimulation comprises fertilization by sperm.

32. Use of a particle selected from the group consisting of a nanoparticle and a microparticle for providing pain relief in a subject in need thereof.

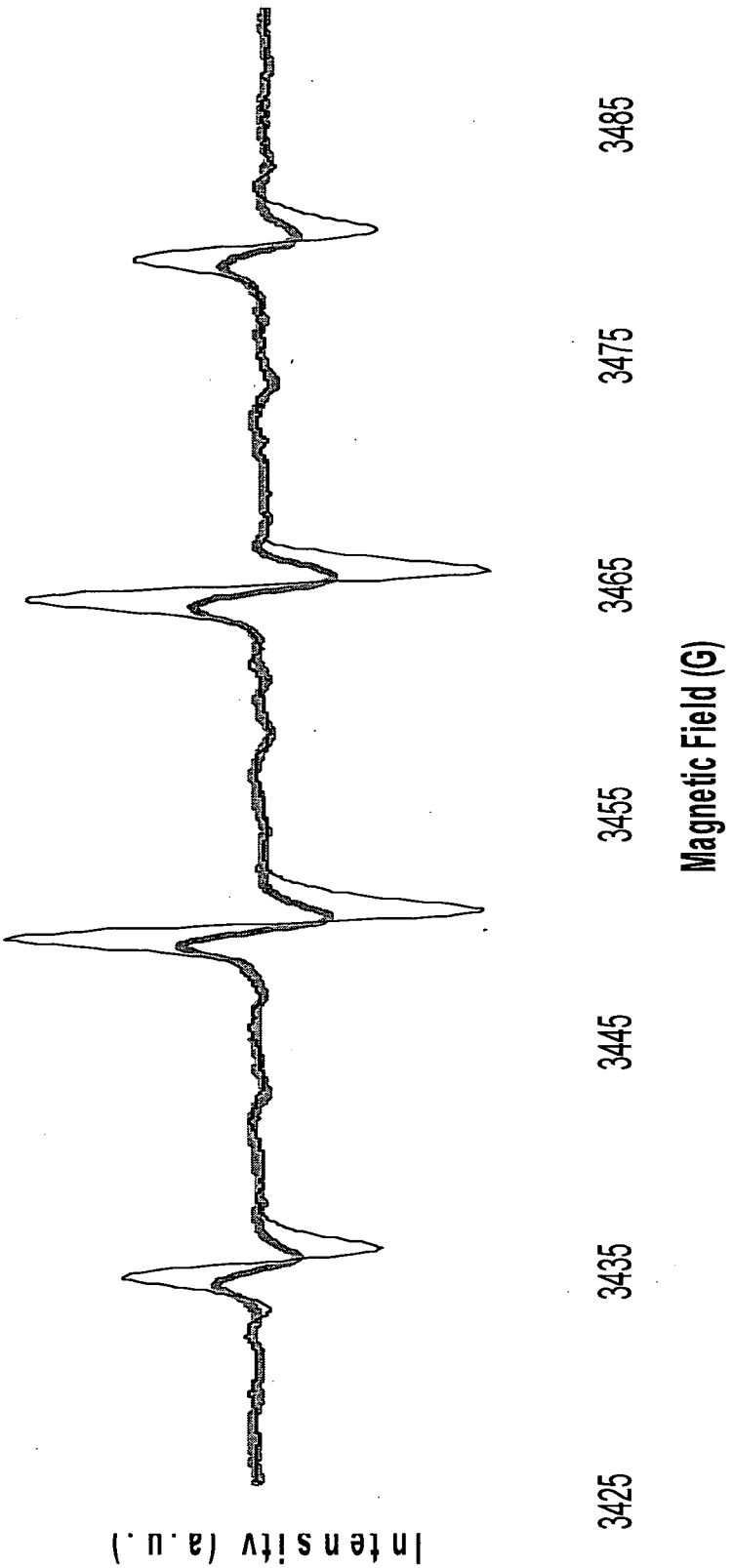
33. A method of inducing pain relief in a subject in need thereof, the method comprising administering to a subject a particle consisting of a nanoparticle and a microparticle.

34. A composition for inducing pain relief in a subject in need thereof, the composition comprising a particle selected from the group consisting of a nanoparticle and a microparticle and a pharmaceutically acceptable carrier.

35. The composition of claim 3, wherein said pharmaceutically acceptable carrier is selected from the group consisting of a physiological salt solution, dimethyl sulfoxide, a solution of colloid liquid protein and a polymer solution.

36. The use, method or composition of any of claims 1 to 35, wherein said particle is comprised in a form selected from the group consisting of gel, a cream, an ointment, a paste, a lotion, a milk, a suspension, an aerosol, a spray, a foam, a serum, a swab, a pledget, a pad and a patch.

Figure 1


Intensity (a.u.)
3425
3435
3445
3455
3465
3475
3485
Magnetic Field (G)

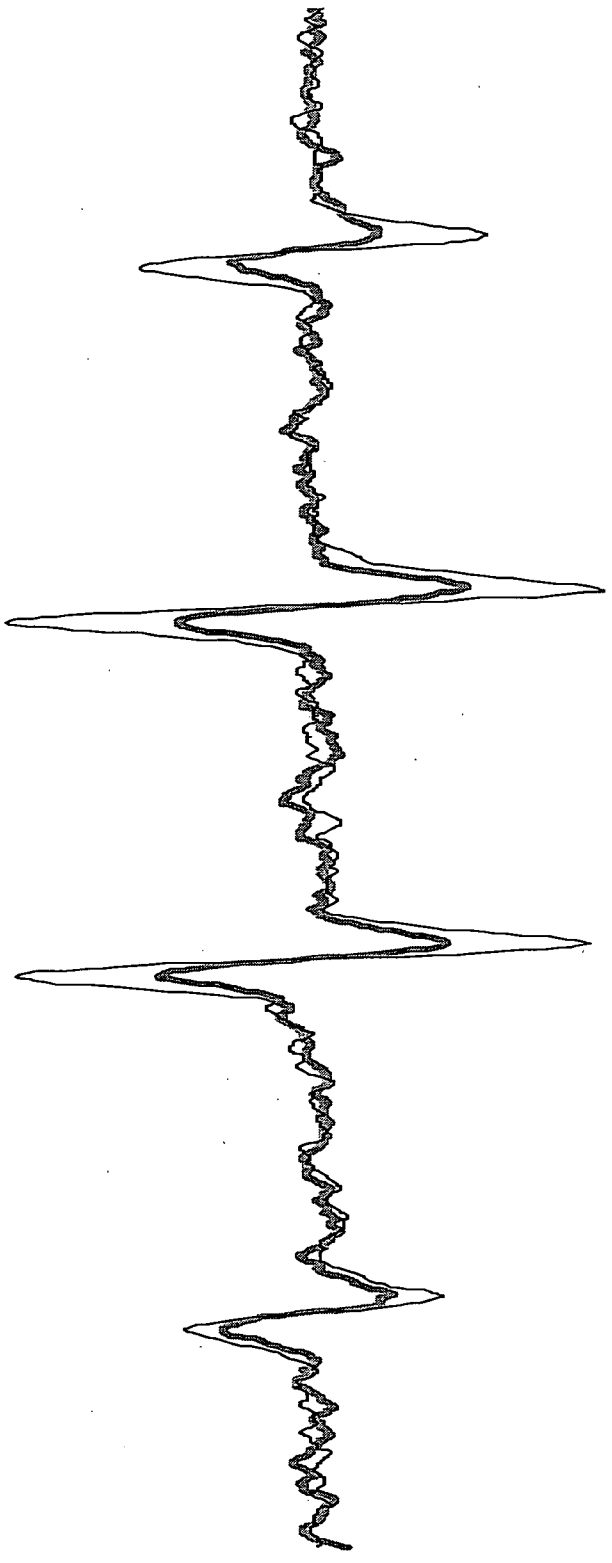
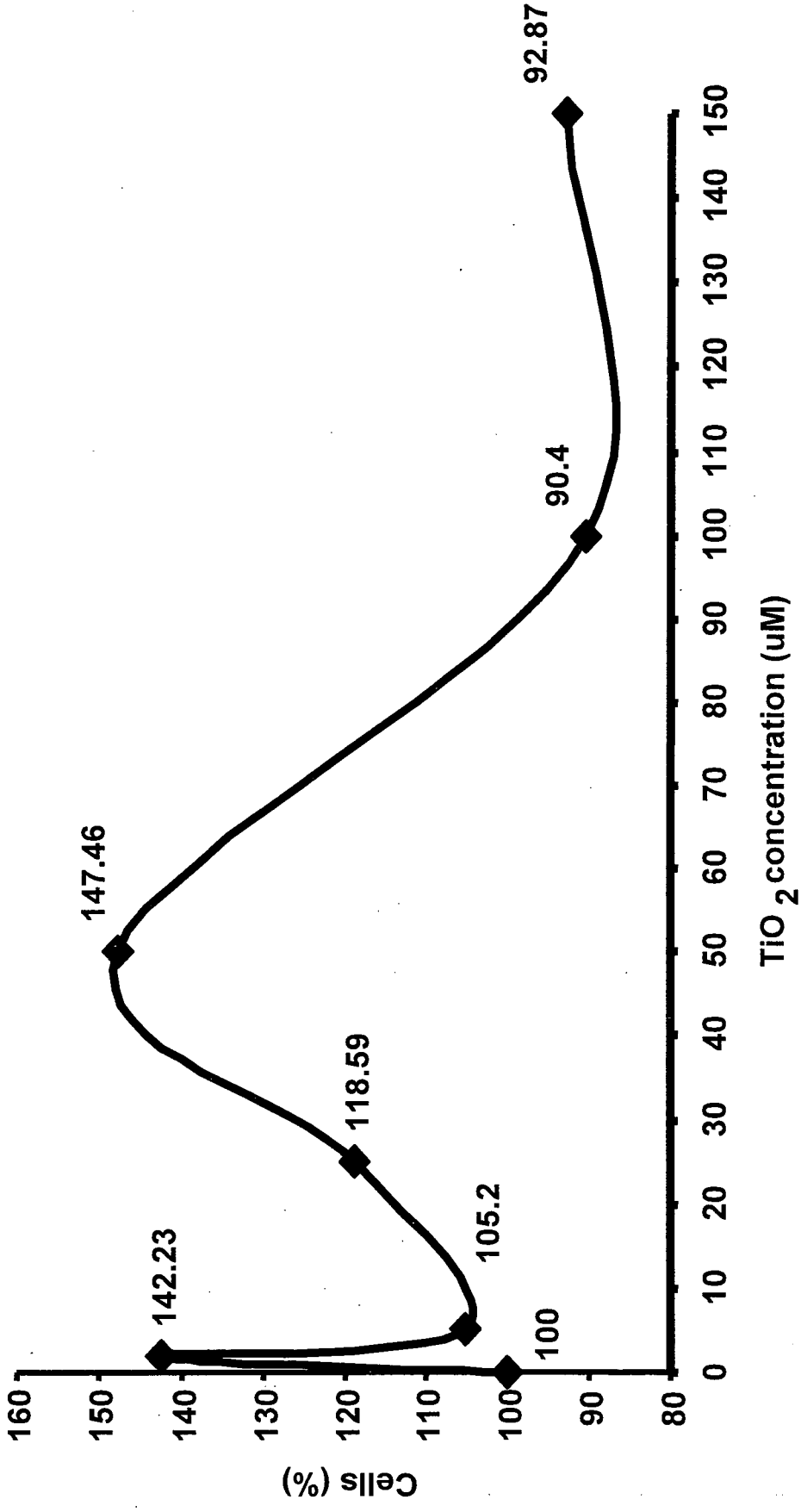


Figure 2

Figure 3


Cells (%)
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150
140
130
120
110
100
90
80
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
TiO 2 concentration (uM)
142.23
147.46
118.59
105.2
100
90.4
92.87