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(54) Title: MULTIFUNCTIONAL NANOPARTICLE COMPOSITIONS AND USES THEREOF

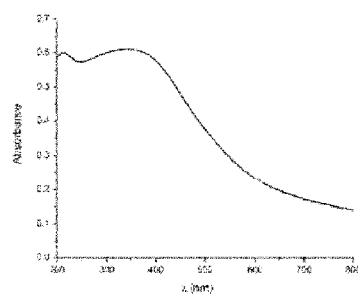


Fig. 1a

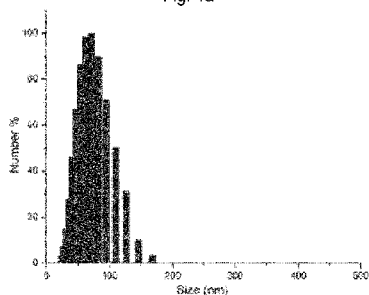


Fig. 1b

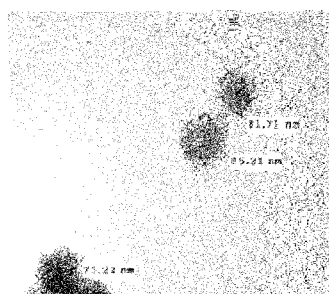


Fig. 1c

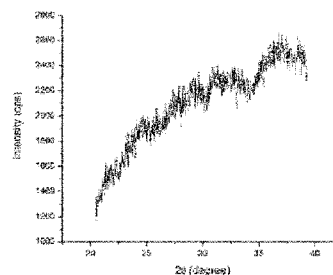


Fig. 1d

(57) Abstract: Disclosed herein are multifunctional nanoparticle compositions. The compositions can be useful for the treatment of cancer by enhancing the anti-tumor effectiveness of radiation directed to a tissue, cell or a tumor and the methods of use thereof. The multifunctional nanoparticle composition comprises a metal oxide nanoparticle core; a functional coating on the surface of the metal oxide nanoparticle core; and a matrix carrier in which the coated nanoparticle is embedded.



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GW, KM, ML, MR, NE, SN, TD, TG).

**MULTIFUNCTIONAL NANOPARTICLE COMPOSITIONS
AND USES THEREOF**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/974,878, filed on April 3, 2014, which is incorporated herein in its entirety by reference.

**STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT**

[0002] Not applicable.

BACKGROUND

[0003] Insufficient oxygenation (hypoxia), acidic pH (acidosis), and elevated levels of reactive oxygen species (ROS), such as H₂O₂, are characteristic abnormalities of the tumor microenvironment (TME). These abnormalities promote tumor aggressiveness, metastasis and resistance to therapies. To date, there is no treatment available for comprehensive modulation of the TME. Approaches so far have been limited to regulating hypoxia, acidosis or ROS individually, without accounting for their interdependent effects on tumor progression and response to treatments.

[0004] In solid tumors, hypoxia (low oxygenation) often occurs as a consequence of a disrupted balance between the supply and consumption of O₂, owing in part to tumor growth and vascular abnormalities, the latter also affecting O₂ transport insufficiencies.¹ Hypoxia, a characteristic of the TME, has been shown to contribute to the cancer resistance to radiation therapy (RT) and to promote clinically aggressive phenotypes in cancer.^{2,3} It has been shown that clinically relevant hypoxic levels are detected in 30-90% prostate cancers and nearly 40% of breast cancers with oxygen concentrations below that required for half-maximal radiosensitivity, which significantly reduces the effectiveness of ionizing radiation therapy^{4,5}.

[0005] Hypoxia also leads to chronic over activation of hypoxia-inducible-factor-1 (HIF-1) which plays a pivotal role in adaptive responses to hypoxia by modulating various cellular functions like proliferation, apoptosis, angiogenesis, pH balance and anaerobic glycolysis.^{6,7} Upon activation, HIF-1 binds to the hypoxic responsive element, thereby promoting transcription of various genes including VEGF (vascular endothelial growth

factor) and genes encoding for glucose transporters⁸. The expression of VEGF further induces angiogenesis and plays a key role in promoting malignant tumor growth.^{9, 10} HIF-1 also mediates the switch from aerobic to anaerobic metabolism in hypoxic tumors for energy preservation by activating glucose transporters and glycolytic enzymes leading to an increase in levels of lactic acid and acidosis (lower extracellular pH, $pHe < 6.9$).^{11, 12} In addition, hypoxia and high proliferation of cancer cells produce excess amounts of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2).¹³ Together, hypoxia, acidosis and ROS promote mutagenesis, metastasis of cancer cells, angiogenesis and resistance to therapies, contributing to treatment failures.

[0006] Known effects of the TME are the culmination of tumor resistance to RT and chemotherapy (CT). TME-related chemoresistance is attributed to several intrinsic cellular mechanisms, mostly to the increase in ABC transporters and gene transcription induced by HIFs.¹⁴ HIF-1 α and ROS are crucial mediators of hypoxia-mediated ABC transporter gene expression¹⁴. ROS may contribute to hypoxia-independent upregulation and activation of HIFs or nuclear factor kappa B (NF- κ B). ROS may also induce multidrug resistance protein-1 (MDR1) expression through functional HIF-1 α or NF- κ B transcriptional binding sites on the promoter of MDR1^{15, 16}. On top of hypoxia and ROS, tumor acidosis may render cancer cells chemoresistant as many cancer drugs are mildly basic ($pKa > 7.5$) and their protonation in the extracellular space of tumors may decrease the drug permeation through cell membranes (ion trapping effect). *In vivo* studies have shown that the uptake and efficacy of several clinically used cancer drugs are reduced by the acidic pHe of solid tumors¹⁷. The physiologic changes cancer cells undergo in response to low pHe also contribute to chemoresistance, including reduced apoptotic potential, genetic alteration (p53 mutations) and elevated activity of P-glycoprotein 1 (Pgp). The radioresistance, on the other hand, can be directly caused by hypoxia. Radiation and oxygen combine to “fix” DNA thus inhibiting intrinsic DNA repair mechanisms to make DNA damage permanent.

[0007] Besides its direct effect on chemoresistance, acidosis alone exhibits many effects on cancer malignancy, tumor development, and immune cell proliferation and activity against cancer cells. The acidic TME regulates apoptosis, cell proliferation, clonal evolution, cancer cell metastasis, and angiogenesis and also modulates tumor inflammatory response and anti-tumor immunity (immune cell function)¹⁷. It has been demonstrated that tumor acidosis reduces the inflammatory and immune response in the tumor. Acidosis decreases the

activity of natural killer (NK) and lymphokine-activated killer (LAK) cells. Acidosis also reduces the release of tumor necrosis factor (TNF- α), which primary role is the regulation of immune cells¹⁹. Significantly elevated serum lactate levels were detected in cancer pateints with high tumor burden and lactate was found to inhibit T-cell proliferation and cytotoxic activity. Counteracting tumor acidosis (i.e., by neutralization of the acidic tumor pH) can improve tumor prognosis by promoting tumor self-destructive mechanisms.

[0008] Another aspect of the TME is the effect of excessive ROS, such as H₂O₂. Increased H₂O₂ levels in the solid tumor induce the activation of HIF-1 α , which in turn triggers a cascade of expression of numerous genes and proteins associated with acquisition of a more malignant behavior, treatment resistance, and disease relapse. It has been demonstrated that the malignant phenotype of cancer cells can be reversed just by decreasing the cellular levels of H₂O₂¹³. Hydrogen peroxide has been identified as a “fertilizer” to promote tumor initiation, growth, progression, recurrence and metastasis. Abnormal production of hydrogen peroxide by cancer cells and cancer-associated fibroblasts has been shown to drive accelerated DNA damage, inflammation, and altered metabolism in the TME²¹. It is known that cancer cells release H₂O₂ which can trigger oxidative stress in neighboring fibroblasts and activation of nuclear factor kappa B (NF- κ B) and HIF, driving to cancer stromal inflammation and aerobic glycolysis. The H₂O₂-activated glycolytic fibroblasts will then produce high-energy nutrients such as lactate to promote tumor growth²¹.

[0009] Manganese (Mn) is the second most abundant transition metal on Earth occurring in multiple valence states in the environment, and is an essential micronutrient for most organisms. Its oxo-compounds (which include oxides, hydroxides, and oxyhydroxides) are some of the strongest oxidants naturally found in the environment showing a wide range of biological and technological applications²⁰. Besides its traditional applications in catalysis and electrodes in lithium batteries, manganese dioxide (MD) NPs have also been employed in the field of glucose biosensors⁹⁸. Such applications are based on the high reactivity of MD NPs towards H₂O₂. Thus, the high reactivity and specificity of MD NPs towards H₂O₂ can be used for the simultaneous and sustained production of O₂ and regulation of pH. Another advantage of MD NPs is their dual functions as both catalyst and reactant. In the latter case, they are decomposed to harmless, water-soluble Mn²⁺ ions²⁶, avoiding the *in vivo* accumulation of the metal oxide commonly observed for other metal-based nanoparticle (NP) systems²⁷. Compared with other metal NPs extensively explored for biological applications,

MDNPs have been limited to use in biochemical sensors^{26, 28} and bioassays²⁹ and their reactivity towards *in vivo*, endogenous H₂O₂ has not been studied for *in vivo* ROS reduction, O₂ production or for the regulation of pH in biological systems.

[0010] To date, various strategies have been proposed to modify the TME, aimed at the (systemic) improvement of tumor oxygenation to surmount hypoxia-associated radioresistance. These strategies include hypoxia by hyperbaric oxygen therapy,²² artificial blood substitutes,²³ and drugs which preferentially kill or sensitize hypoxic cells to radiation.²⁴ Unfortunately, the utility of such methods in clinical settings is limited due to safety concerns, reagent stability and/or inconsistent clinical response. Therefore, there is a continued and urgent need for new strategies to improve tumor oxygenation *in vivo* to enhance the radiation response in solid tumors.

SUMMARY

[0011] In its first aspect, the present disclosure relates to multifunctional nanoparticle compositions. In certain applications, the compositions are useful for the treatment of cancer by enhancing the anti-tumor effectiveness of radiation directed to a tissue or a tumor. The multifunctional nanoparticle composition can include a coated nanoparticle embedded in a matrix. The coated nanoparticle can include a metal oxide nanoparticle and a functional coating on the surface of the metal oxide nanoparticle. The coated nanoparticle embedded in the matrix can be in nanoparticulate form.

[0012] In some embodiments, the matrix can include organic groups, inorganic compounds, proteins, nucleic acids, polymers, lipids and the mixtures thereof.

[0013] In some embodiments, the coated nanoparticle embedded in the matrix further comprises a functional moiety, such as a targeting moiety, a detectable moiety, a radioisotope, a radionuclide, a labeling agent, an imaging agent and the mixtures thereof.

[0014] In some embodiments, the metal oxide nanoparticle is a manganese dioxide nanoparticle (MD NP).

[0015] In some embodiments, the functional coating is selected from the group consisting of a biocompatibility coating, a colloidal coating, an organic coating, an inorganic coating, a hydrophilic coating, and the mixtures thereof.

- 1 **[0016]** In some embodiments, the functional coating comprises a first layer. For
2 example, the first layer can be poly(allylamine hydrochloride) (PAH). The first layer may
3 also be linked to an oleic acid (o) introduced by covalent bond or physical attachment.
- 4 **[0017]** In some embodiments, the multifunctional nanoparticle composition is A-MD
5 NP (Formulation #1) in the size of about 20 nm to about 1000 nm in diameter.
- 6 **[0018]** In some embodiments, the multifunctional nanoparticle composition is TER-
7 MD NP (Formulation #2) in the size of about 50 nm to about 1000 nm in diameter.
- 8 **[0019]** In some embodiments, the multifunctional nanoparticle composition is a PEG-
9 TER-MD NP (Formulation #3) in the size of about 50 nm to about 1000 nm in diameter.
- 10 **[0020]** In some embodiments, the multifunctional nanoparticle composition is L-MD
11 NP (Formulation #4) in the size of about 50 nm to about 1000 nm in diameter.
- 12 **[0021]** In some embodiments, the multifunctional nanoparticle composition is PMA-
13 MD NP (Formulation #5) in the size of about 50 nm to about 1000 nm in diameter.
- 14 **[0022]** In some embodiments, the nanoparticles are mixed with a pharmaceutically
15 acceptable vehicle is in the form suitable for intratumoral injection, peritumoral injection or
16 intravenous injection.
- 17 **[0023]** In its second aspect, the present disclosure relates to methods of preparing a
18 multifunctional nanoparticle composition for the treatment of cancer by enhancing the anti-
19 tumor effectiveness of radiation directed to a tissue or a tumor, the method comprising the
20 steps of (1) preparing a metal oxide nanoparticle, (2) treating the metal oxide nanoparticle of
21 step (1) with a first functional layer to form a single-coated functional metal oxide
22 nanoparticle; and (3) loading single-coated functional metal oxide nanoparticles of step (2) in
23 a matrix to form a particle loaded with one or more functional metal oxide nanoparticles.
- 24 **[0024]** In some embodiments, the method may further comprise a step of treating the
25 particle loaded with one or more coated functional metal oxide nanoparticles of step (3) with
26 a functional moiety to form a functional loaded particle.
- 27 **[0025]** In its third aspect, the present disclosure relates to methods of treating cancer
28 disease in a subject comprising the steps of administering an effective amount of a
29 multifunctional nanoparticle composition disclosed herein to the subject, and applying an
30 ionizing radiation treatment to the subject, wherein the cancer disease is treated.
- 31 **[0026]** In some embodiments, the administration of the composition is prior to the
32 application of the radiation treatment. More preferably, the composition is intravenously

1 injected to the subject about 1 to about 24 hours prior to the radiation, or the composition is
2 intratumorally injected to the subject about 5 minutes to about 3 hours prior to the radiation.

3 **[0027]** In its fourth aspect, the present disclosure relates to methods of enhancing a
4 radiation directed to a tissue or a tumor in a subject comprising the steps of (1) administering
5 an effective amount of a multifunctional nanoparticle composition disclosed herein to the
6 subject, and (2) applying radiation to the subject, wherein the radiation is enhanced in the
7 subject.

8 **[0028]** In its fifth aspect, the present disclosure relates to the use of a multifunctional
9 nanoparticle composition as described herein for the manufacture of a medicament for the
10 treatment of cancer in combination with radiation in a subject.

11 **[0029]** In its sixth aspect, the present disclosure relates to the use of a multifunctional
12 nanoparticle composition as described herein for the manufacture of a medicament for the
13 enhancement of a radiation directed to a tissue or a tumor in a subject.

14 15 BRIEF DESCRIPTION OF THE DRAWINGS

16 **[0030]** Figure 1 shows characterization of bare MnO₂ (bMD) NPs. **(a)** UV-Vis
17 spectrum; **(b)** size distribution; **(c)** TEM image; and **(d)** X-ray powder diffraction (XRD) of
18 obtained bMD NPs.

19 **[0031]** Figure 2 shows characterization of polyelectrolyte-coated MnO₂ (pMD p =
20 PAH or PARG) NPs and BSA-complexed A-MD NPs **(a)** UV-Vis absorption spectra of
21 KMnO₄ solution and MD NPs prepared at various molar ratios between PAH and MD. **(b)**
22 Effect of coating of pMD NPs with BSA on zeta potential for various BSA/NPs ratios. By
23 adding BSA to a pMD NP aqueous suspension, the zeta potential of the NPs decreased from
24 +30 mV to -25 mV. **(c)** and **(d)** Size distribution of pMD NPs before and after complexation
25 with BSA.

26 **[0032]** Figure 3 shows **(a)** diagram and **(b)** TEM images of polyelectrolyte-coated
27 MnO₂ (pMD) NPs and BSA-complexed A-MD NPs. MD NPs (~15 nm) are stabilized by
28 positively charged polyelectrolyte (PAH or PARG). In A-MD (~50 nm), several MD particles
29 are entrapped in a PAH/BSA or PARG/BSA complex due to strong electrostatic interaction
30 between the protein and the polymer. The scale bars in the TEM pictures correspond to 100
31 nm.

1 [0033] Figures 4a-d show the characterization of A-MD NPs: (a) Diagram & TEM
 2 images of MD and A-MD NPs. Precursor pMD NPs (~15 nm) are stabilized by positively
 3 charged PAH. In A-MD (~50 nm) several MD particles are entrapped in a PHA/BSA
 4 complex due to strong electrostatic interaction between the protein and the polymer. (b) Size
 5 distribution of NPs. (c) Effect of coating of pMD NPs with BSA on zeta potential for various
 6 BSA/NPs ratios. By adding BSA to a pMD NP aqueous suspension, the zeta potential of the
 7 NPs decreased from +30 mV to -25 mV. (d) Picture of pMD NPs (left) and A-MD NPs
 8 (right) (1mM) in various aqueous media: DDI water, normal saline (0.9% NaCl) and α MEM
 9 cell medium containing 10% fetal bovine serum (FBS). pMD NPs undergo aggregation in
 10 saline or cell culture medium, while A-MD NPs are stable in these media. The red color
 11 observed in the vials comes from the pH indicator in the α MEM medium.

12 [0034] Figures 5a-e show the *in vitro* reactivity of A-MD NPs towards hydrogen
 13 peroxide. (a) Reaction scheme showing the reactivity of MD towards H_2O_2 for the production
 14 of O_2 and removal of protons. (b) Quenching of endogenous level H_2O_2 (1 mM) by A-MD
 15 NPs (45 μ M). (c) Oxygen generation at various A-MD NP contents (numbers indicate MD in
 16 μ M). (d) Simultaneous O_2 generation and pH increase vs. time by the A-MD NPs. (e) O_2
 17 generation by addition of H_2O_2 to an A-MD NP suspension. All experiments were performed
 18 (n = 3) in cell culture medium containing 10% FBS at 37°C. Error bars are standard error of
 19 the mean.

20 [0035] Figure 6 shows cytotoxicity and cellular uptake of various MD NP
 21 formulations. (a) Viability of murine EMT6 cancer cells (10^5 cells/ mL) exposed to various
 22 concentrations of A-MD NPs for 48 h. Viability of (b) human breast cancer MDA-MB 231
 23 and (c) murine breast cancer EMT6 incubated with L-MD and TER-MD NPs for 48 h.
 24 (d) Uptake of A-MD NPs at 37°C by EMT6 murine breast cancer cells after 1h incubation
 25 determined by fluorescence microscopy. (e) TEM images of cellular uptake of A-MD NPs.
 26 (f) and (g) show cellular uptake of different MD NPs formulations at various time points.
 27 TER-MD: MD NPs loaded in a terpolymer crosslinked denatured protein matrix; L-MD and
 28 PMA-MD: oleic acid-conjugated MD (oMD) loaded in PEG-lipid and amphiphilic polymer,
 29 respectively. Percent of cell viability was determined with MTT assay. (n=3) Error bars
 30 represent standard errors of the mean.

31 [0036] Figures 7a-c show the effect of A-MD NPs on tumor oxygenation: (a)
 32 Representative 2D photoacoustic images of EMT6 solid tumors showing parametric map of

1 estimated oxygen saturation (sO_2) pre- and post-i.t. injection of saline (control) or A-MD
 2 NPs. (b) Average total sO_2 in the tumor over time. (c) Comparison of average tumor sO_2
 3 levels before and after treatments ($n = 3$). Error bars represent standard errors of the mean.
 4 * = statistically significant increase (* $p = 1.8E-5$) in sO_2 as compared to saline (control)
 5 treated group.

6 **[0037]** Figure 8 graphically shows the calculation of pKa value for SNARF in
 7 biological phantoms. (a) Fluorescence images of SNARF-containing phantoms at different
 8 pH values, recorded using Xenogen microscope with a excitation wavelength of 535 nm (row
 9 (i) green channel, row (ii) red channel and row (iii) is overlay of the two channels). (b)
 10 Graphical representation of the ratios ($R = I_g/I_r$) versus pH where the ratio R was calculated
 11 from the values I_g and I_r which were obtained from fluorescent images in (a) using image-J
 12 program. (c) The Boltzmann fit of data points R using Eq.1, where the values for fit
 13 parameter were $R_a = 1.47$, $R_b = 0.60$, $pH_{infl} = 6.25$, and $\Delta pH = 0.3$. (d) Graphical representation
 14 of $-\log(\dots)$ term versus pH, where the ratio R was obtained from the Boltzmann fit in (c) and
 15 $I_{a(42)}$ and $I_{b(42)}$ are fluorescence intensities at 640nm (red channel) obtained from images
 16 provided in (a) at pH 4.5 and pH 8.5 using image-J program, $I_{a(42)} = 41.92$ and $I_{b(42)} = 63.11$.
 17 (d) Graphical representation of the intercept of linear fit of data points where pKawas
 18 calculated according to Eq. 2. The obtained pKa of SNARF was 6.39.

19 **[0038]** Figures 9a-b show the effect of A-MD NPs on tumor pH: (a) *Ex vivo* pH
 20 imaging of solid tumors treated with i.t. injection of A-MD NPs or saline (control). The
 21 tumors were cut in the central line and intratumoral pH was determined with multispectral
 22 fluorescence imaging (MSFI) using a pH-sensitive fluorescent dye (SNARF-4F) and the
 23 calibration curve. The bottom insert shows the pH scale obtained using the dye in biological
 24 phantoms of various pH values. (b) Tumor pH after treatment with A-MD NPs or saline
 25 (control) ($n = 3$) determined *ex vivo* by MSFI (black bars) or with a microneedle pH probe
 26 (gray bars). Error bars represent standard errors of the mean. *statistically significant increase
 27 (* $p = 0.004$, ** $p = 0.007$) in pH as compared to saline (control) treated group.

28 **[0039]** Figure 10 shows consumption of A-MnO₂ nanoparticles by H₂O₂. Upon
 29 reaction with hydrogen peroxide for the production of molecular oxygen, MnO₂ nanoparticles
 30 are consumed. Consumption of the MnO₂ NPs (90 μ M) by various endogenous
 31 concentrations of H₂O₂ (up to 1 mM) is shown. For the experiment, H₂O₂ was added to A-

MnO₂ (A-MD) in saline, incubated for 5 min at room temperature and the absorbance of the MnO₂ was measured at 360 nm.

[0040] Figures 11a-e show the tumor retention of A-MD NPs and effect on tumor hypoxia, HIF-1 α and VEGF. (a) Representative optical images of EMT6 tumor-bearing mouse with i.t. injected near infrared-labeled A-MD NPs at various times. (b) Representative immunohistochemistry in continuous sections from EMT6 tumors treated i.t. with saline (control) or A-MD NPs for 30 min, 60 min and 24 h. Tumor hypoxia was determined by pimonidazole binding HIF-1 α and VEGF antibody. Scale bars correspond to 85 μ m. (c-e) Quantification of tumor hypoxia, HIF-1 and VEGF after treatments, determined from classified images (not shown). (n=3). Error bars represent standard errors of the mean. *=statistically significant difference (*p = 6.9E-5, **p = 0.003, ***p = 4.5E-4) as compared to saline (control) treated group. Figures 11f-g show *in vivo* biodistribution, tumor accumulation and tumor retention of NIR fluorophore-labelled formulations (f) L-MD NPs and (g) PMA-MD NPs injected i.v. in Balb/c mice bearing orthotopically implanted EMT6 murine breast tumor cells. Fluorescence was determined prior to intravenous injection (base line), and at different time points post-injection up to 24 hours. Tumor is indicated with the arrow. Bottom rows showing *ex vivo* fluorescence images of different organs: liver, tumor, lungs, heart, kidneys, spleen, intestine and blood. Tissues were excised from the tumor-bearing mice at 4 and 24 hours post-NP injection.

[0041] Figures 12a-f show the effect on tumor growth after treatment with radiation and A-MD NPs: Tumors (n=3/ group) were treated with intratumoral injection of 1) Saline, 2) Saline+RT, 3) A-MD NP, or 4) A-MD NP + RT. A radiation dose of 10 Gy was given 30 minutes after saline or A-MD NP treatment. (a) Tumor volume measured over time following treatment. (b) *Ex vivo* measurement of tumor weight at the end of Day 5. (c) Quantification of % necrotic + apoptotic area in tumors after treatment. (d) Quantification of DNA DSBs as measured by γ -H2AX staining in tumors after treatment. (e) Quantification of DNA DSBs determined by measuring % of positive γ -H2AX cells in tumor tissue implanted in dorsal window chamber and treated with Saline and A-MD NPs. (f) Representative image of tumor implanted in dorsal window chamber and treated with Saline and A-MD NPs, and immunohistochemical image of tumor tissue stained with γ -H2AX. *=statistically significant difference (*p < 0.05) as compared to all other groups.

1 [0042] Figure 13 is a schematic showing the steps for preparation of A-MD NPs,
2 denatured A-MD NPs and terpolymercrosslinked denatured A-MD (TER-MD) NPs.

3 [0043] Figure 14 shows (a) size distribution and (b) TEM image of pMD NPs loaded
4 in a terpolymer crosslinked denatured BSA matrix (TER-MD).

5 [0044] Figure 15 shows phase transfer of pMD NPs from hydrophilic to hydrophobic
6 phase through covalent conjugation of oleic acid on the surface of the NPs (oMD NPs) (left).
7 (a) pMD NPs before phase transfer (in DDI water phase) and after phase transfer (in
8 chloroform phase). (b) oMD NPs dispersion in DDI water (left) and in chloroform (right).
9 Very stable particles were obtained after conjugation of oleic acid and no aggregation was
10 observed. (c) UV-Vis spectrum of NPs before and after conjugation with oleic acid. The new
11 sharp peak at around 280 nm corresponds to oleic acid conjugated to the NPs surface.

12 [0045] Figure 16 is a schematic diagram illustrating the different steps during the
13 preparation of oMD NPs L-MD NPs or PMA-MD NPs.

14 [0046] Figure 17 shows (a) TEM image and (b) size distribution oMD NPs loaded in
15 a PEG-lipid matrix (L-MD NPs). (c) Stability of L-MD NPs in α -medium with 10%FBS,
16 medium with 50%FBS and in pH 7.4 weak buffer (PBS) in the presence of 250 μ M H₂O₂.

17 [0047] Figure 18 shows (a) TEM image and (b) size distribution of oMD NPs loaded
18 in an amphiphilic polymer matrix (PMA-MD NPs). (c) Stability of PMA-MD NPs in α -
19 medium with 10% FBS, or 50% FBS and in pH 7.4 weak buffer (PBS) in the presence of 250
20 μ M H₂O₂.

21 [0048] Figure 19 shows a schematic of the apparatus for measurement of generated
22 Oxygen.

23 [0049] Figure 20 shows O₂ generation by addition of H₂O₂ (250 μ M) to various MD
24 NP formulations. A-MD, TER-MD, L-MD and PMA-MD NPs. Measurements were
25 performed (n = 3) in cell culture medium containing 10% FBS at 37°C.

26 [0050] Figure 21 shows a schematic of the apparatus for measurement of generated
27 Oxygen with suspended hypoxic cells.

28 [0051] Figure 22 shows O₂ generation by A-MD NPs incubated with suspended
29 hypoxic cancer cells (n = 3). Suspended cells are made hypoxic and upon addition of NPs to
30 the culture oxygen is generated by the reactivity of the MD content of the NPs towards H₂O₂
31 released by hypoxic cancer cells. Measurements were performed (n = 3) in cell culture
32 medium containing 10% FBS at 37°C.

[0052] Figure 23 shows effect on tumor growth of treatment with various MD-containing NP formulations and NPs + RT up to 5 days. Tumors (n=3/ group) were treated with a single intratumoral injection of 1) saline, 2) saline+RT, 3) TER-MD, 4) TER-MD + RT, 5) L-MD, and 6) L-MD + RT. A radiation dose of 10 Gy was applied to the tumors 30 minutes post injections. (a) Tumor volume measured using a caliper over time at various times. (b) Percent of tumor weight change at Day 5 post treatment after the mice were sacrificed. i.t. injection of saline with or without RT was used as a control.

[0053] Figure 24 shows effect on tumor weight of a single treatment with various MD-containing NP formulations and NPs + RT at Day 5 post the treatment. (a) *Ex vivo* measurement of tumor weight at the end of Day 5. (b) Pictures of tumors taken 5 days post treatment. (c) Images of three tumor samples taken from the same treatment group (not to scale).

[0054] Figure 25 shows effect of the combination of irradiation with L-MD and TER-MD NPs on tumor necrosis. **Panel:** Representative Hematoxylin and Eosin (H&E)-stained tissue sections of EMT6 tumors after treatment with NPs + RT - 5 days post-treatment (saline was used as a control). Tumors treated with NPs + RT showed massive necrosis. Overall, L-MD NPs + RT group are the smallest in the group and by far the most necrotic. Scale bar = 5 mm.

[0055] Figure 26 shows evaluation of the acute effect of MD-containing NP formulations and NPs + RT – 24 h treatment. (a) Quantification of DNA DSBs determined by measuring % of positive gamma-H2AX cells in EMT6 tumors treated with Saline, L-MD NPs and TER-MD NPs. (b) Quantification of cell proliferation marker Ki-67.

[0056] Figure 27 shows the effects of L-MD NPs on human prostate cancer cells *in vitro* (a) and *in vivo* (b). (a) Graphical representation of cell viability data obtained following treatment of PC3 human prostate cells with saline control, TER-MD NPs, or L-MD NPs under normoxic (left) and hypoxic (right) conditions and following radiation. Figure 26b shows increased radiation-induced necrosis in human PC3 prostate cancer cells treated with L-MD NPs *in vitro*. Representative Hematoxylin and Eosin (H&E)-stained tissue sections of PC3 tumors after treatment with saline + RT (**Left Panels**) or L-MD NPs + RT (**Right Panels**) 5 days post-treatment. Tumors treated with NPs + RT showed significant necrosis while there was no histological evidence of necrosis in the saline control group. Scale bar = 4 mm (1.25×) and 1 mm (3×). (c) The *in vivo* biodistribution and tumor accumulation of ICG-

1 loaded L-MD nanoparticles in a male SCID mouse bearing PC3 prostate tumor implanted
2 into abdominal side. Images were recorded both at the (i) prone and (ii) supine positions.
3 Fluorescence images of tumor-bearing micewere obtained prior to i.v. (base line), and at
4 different time points up to 24 hours post-injection. Tumor is shown with arrow. d) *Ex vivo*
5 fluorescence images of different organs collected after 24 hours of nanoparticle
6 administration.

8 DETAILED DESCRIPTION

9 [0057] The present disclosure relates to multifunctional nanoparticle compositions
10 and methods of using the compositions for the treatment of cancer by enhancing the anti-
11 tumor effectiveness of ionizing radiation directed to a tissue or a cell of a subject.

12 [0058] The present disclosure provides engineered new multifunctional and
13 colloidally stable bioinorganic nanoparticles composed of bioreactive MnO₂ nanoparticles
14 (MD NPs) loaded into a biocompatible polymeric and/or lipidic matrix such as
15 polyelectrolyte-albumin complex, graft terpolymer or poly(methacrylic acid)-polysorbate 80-
16 starch (TER) crosslinked albumin, and amphiphilic polymer, and fatty acids. The reactivity of
17 MD towards peroxides for regulation of the TME with simultaneous H₂O₂ quenching, oxygen
18 generation and pH increase was utilized. *In vitro* studies showed that these NPs can generate
19 oxygen by reacting with H₂O₂ produced by cancer cells under hypoxic conditions. In a
20 murine orthotopic breast tumor model, albumin-based MD (A-MD) NPs increased tumor
21 oxygenation by 45% by reacting with endogenous H₂O₂ produced within the tumor while
22 increasing tumor pH from pH 6.7 to pH 7.2. Intratumoral treatment with A-MD NPs led to
23 the downregulation of two major regulators in tumor progression and aggressiveness, i.e.,
24 hypoxia-inducible factor-1 alpha (HIF-1 α) and vascular endothelial growth factor (VEGF) in
25 the tumor. Combination treatment of the tumors with MD NPs and ionizing radiation
26 significantly inhibited tumor growth, increased DNA double-strand breaks and cancer cell
27 death as compared to radiation therapy (RT) alone. Combination of RT with terpolymer
28 cross-linked denatured albumin-based MD (TER-MD) NPs or lipid-based (L-MD) NPs also
29 caused more death of hypoxic prostate cancer cells and more tumor necrosis than radiation
30 alone. These results suggest the usefulness of MD NPs for modulation of the TME and
31 enhancement of radiation therapeutic response in the treatment of cancer.

1 [0059] The inventors unexpectedly discovered the high reactivity and specificity of
2 metal oxide nanoparticles, particularly, manganese dioxide nanoparticles (MD NPs), towards
3 tumor H_2O_2 to modulate the tumor microenvironment (TME) by the simultaneous and
4 sustained production of O_2 and regulation of pH^{25, 26}. Unlike other experimental and clinical
5 strategies to increase tumor oxygenation, which are mostly achieved by the delivery of
6 molecular oxygen by nanoparticles with limited O_2 loading capacity or hyperbaric treatment
7 strategies,² MD NPs are able to generate O_2 *in situ* for a prolonged time by reacting with
8 undesirable and abundantly available tumor metabolites (H_2O_2 and H^+).

9 [0060] The inventors also found that MD NPs have dual functions as both catalyst
10 and reactant *in vivo*. In the latter case, MD NPs are decomposed to harmless, water-soluble
11 Mn^{2+} ions,²⁶ avoiding the *in vivo* accumulation of the metal oxide commonly observed for
12 other metal-based nanoparticle (NP) systems.²⁷ So far, the reactivity of MD NPs towards
13 tumor H_2O_2 has not been studied for *in vivo* ROS reduction, O_2 production or for the
14 regulation of pH in biological systems.

15 [0061] Thus, for the first time, the inventors developed compositions based on
16 multifunctional metal oxide nanoparticles, particularly manganese dioxide nanoparticles (MD
17 NPs) and use them for the modulation of hypoxia of the TME, and for enhancement of
18 radiation-induced tumor cell cytotoxicity in a murine breast tumor animal model. As the
19 majority of cancer treatment and therapy focus on developing new drugs and modalities
20 against tumor cells, the inventors envision that this technology can solve a bottleneck
21 challenge in tumor response to radiation therapy and can be used as a platform technology to
22 enhance the effectiveness of radiation treatment and associated therapies.

23 [0062] In its first aspect, the present disclosure provides multifunctional nanoparticle
24 compositions for the treatment of cancer by enhancing the anti-tumor effectiveness of
25 radiation directed to a tissue, cell or a tumor. Specifically, the multifunctional nanoparticle
26 composition can include a coated nanoparticle embedded in a matrix. The coated
27 nanoparticle can include a metal oxide nanoparticle and a functional coating on the surface of
28 the metal oxide nanoparticle.

29 [0063] The term “metal oxide nanoparticle” as used herein refers to a particle
30 comprising a solid core of an inorganic metal oxide material comprising at least partially
31 ordered three dimensional array of metallic cations and oxide anions. The nanoparticle size
32 desired by this disclosure can vary widely, and essentially any particle size in the

nanoparticle size range (e.g., below 1,000 nm) can be used. The shape of the nanoparticles may be regular (column, cube, cylinder, pillar, pyramid, rod, sphere, tube, wire, flake, disk, etc.) or irregular/random, which for example can be controlled by adjusting the reaction dynamics and aging/ripening time.

[0064] There are no limitations on the base metal of the metal oxide nanoparticles for this disclosure. For example, the base metal may be one or more selected from Cu, Sn, Ti, V, Cr, Mn, Co, Fe, Ni, Zn, Al, Y, Zr, Mo, In, Mg, La, Ce, Nd, Sm, Eu, Gd, Si, Ge, Pb, Ag, Tl, Cs, Hf, and Bi. In certain applications, the base metal may be one or more selected from V, Cr, Mn, Co, Y, Zr, Mo, In, Mg, La, Ce, Nd, Sm, Eu, Gd, Ge, and Bi. In certain applications, the base metal may be one or more selected from V, Cr, Mn, Mo, and Co. In certain applications, the base metal may be one or more selected from V, Cr, Mn, Fe, and Co. In certain applications, the base metal may be one or more selected from Ti, Ag, and Mn. In a specific embodiment of the present disclosure, the base metal is Mn.

[0065] There are no limitations on the chemical composition of the metal oxide nanoparticles for this disclosure as well. For example, some suitable metal oxide nanoparticles have a well defined chemical composition (e.g., stoichiometric metal oxides having a well defined composition, such as MnO_2), while some “non-stoichiometric” metallic oxides have variable proportions of mixtures of the metal cations, such as for example $\text{BaTi}_{0.8}\text{Zr}_{0.2}\text{O}_3$ or those with metal and/or oxygen ion vacancies. In certain applications, the metal oxide nanoparticle may be one or more selected from Al_2O_3 , TiO_2 , ZrO_2 , MgO , NiO_2 , Co_3O_4 , AgO , CuO , BiO , Rb_2O , In_2O_3 , Tl_2O_3 , Cs_2O , and HfO_2 . In certain applications, the metal oxide nanoparticle may be one or more selected from MnO_2 , Mn_2O_3 , Mn_2O_7 , MnOOH , Mn_3O_4 , and $\text{Mn}(\text{C}_2\text{O}_4)$. In a specific embodiment of the present disclosure, the metal oxide nanoparticle is MnO_2 (MD NP).

[0066] To make metal oxide nanoparticles suitable for clinic use, the inventors designed and formulated the nanoparticles under the following consideration: (1) reducing toxicity; (2) improving colloidal stability under physiological conditions; (3) improving blood circulation and half-life of the nanoparticles; (4) increasing tumor uptake and retention; (5) modulating the kinetics of the nanoparticle reaction towards H_2O_2 and the rates of oxygen generation and pH changes. The inventors have found that these criteria can be achieved by modifying the coating material, the size, the morphology, the structure, the surface, the charge, the hydrophobicity and the chemistry of the nanoparticles.

1 [0067] In some embodiments, the surface of the metal oxide nanoparticle disclosed in
2 the present application is modified with a functional coating, including, but is not limited to, a
3 biocompatibility coating, a colloidal coating, an organic coating, an inorganic coating, a
4 hydrophilic coating, or the mixture thereof. These functional coatings are used to increase the
5 stability, biocompatibility and other functionality of the metal oxide nanoparticles. The
6 coating strategies and processes are well known in the art.

7 [0068] For example, biocompatible coating materials include, but are not limited to,
8 synthetic and biological polymers, copolymers and polymer blends, and inorganic materials.
9 Polymer materials may include various combinations of polymers of acrylates, siloxanes,
10 styrenes, acetates, esters, alkylene glycols, alkylenes, alkylene oxides, polyenes, lactic acid,
11 and glycolic acid. Further suitable coating materials include a hydrogel polymer, a histidine-
12 containing polymer, and a combination of a hydrogel polymer and a histidine-containing
13 polymer. Coating materials may also include combinations of biological materials such as a
14 polysaccharide, a polyaminoacid, a protein, a lipid, a nucleic acid, a glycerol, and a fatty acid.
15 Other biological materials for use as a coating material may be a heparin, heparin sulfate,
16 chondroitin sulfate, chitin, chitosan, cellulose, dextran, alginate, starch, carbohydrate, and
17 glycosaminoglycan. Proteins may include an extracellular matrix protein, proteoglycan,
18 glycoprotein, albumin, peptide, and gelatin. These materials may also be used in combination
19 with any suitable synthetic polymer material.

20 [0069] Inorganic coating materials may include any combination of a metal, a metal
21 alloy, and a ceramic. Examples of ceramic materials may include a hydroxyapatite, silicon
22 carbide, carboxylate, sulfonate, phosphate, ferrite, phosphonate, and oxides of Group IV
23 elements of the Periodic Table of Elements. These materials may form a composite coating
24 that also contains any biological or synthetic polymer. Where the magnetic particle is formed
25 from a magnetic material that is biocompatible, the surface of the particle itself operates as
26 the biocompatible coating.

27 [0070] Organic coatings or organic phases introduce substantially of carbon to the
28 nanoparticles by attaching a monolayer or multilayers of organic compounds to the surface of
29 the nanoparticles. Organic compounds can be organic groups, small molecules, monomers,
30 oligomers or polymers.

31 [0071] Hydrophilic coatings on the nanoparticles can be made as well. For example,
32 hydrophobic nanoparticles can be made suspendable in aqueous solutions by introducing

1 ionic or polar groups on the nanoparticle surface. Depending on the starting surface
2 properties of the particles, this can be accomplished, for example, by linking molecules to the
3 nanoparticle surface through chemisorption, to reactive groups on the particle surface that
4 may have been introduced in the synthesis process through covalent bonds, through
5 coordination bonds, ionic bonds, pi-bonds, or hydrophobic interactions.

6 [0072] The coating material may also serve to facilitate transport of the nanoparticle
7 into a cell, a process known as transfection. Such art-known coating materials, known as
8 transfection agents, include vectors, prions, polyaminoacids, cationic liposomes, amphiphiles,
9 and non- liposomal lipids or any combination thereof. A suitable vector may be a plasmid, a
10 virus, a phage, a virion, and a viral coat. The nanoparticle coating may be a composite of any
11 combination of transfection agent with organic and inorganic materials, such that the
12 particular combination may be tailored for a particular type of a healthy or diseased cell and a
13 specific location in a tissue or organ.

14 [0073] The metal oxide nanoparticles can be multi-functionalized. For example, the
15 nanoparticles can be modified with one portion of the molecule exhibiting affinity to the
16 particle or particle surface groups and another portion of the molecule having characteristics
17 that would make the conjugate hydrophilic. The portion of the molecule or another portion
18 could also render the conjugate biocompatible, a typical example would be a molecule
19 terminated with a polyethylene glycol (PEG) chain. This same portion of the molecule or
20 another portion could also introduce additional functionality on the conjugate surface for
21 conjugating additional materials onto the nanoparticle. For example, the functional coating
22 can further comprise a targeting ligand, wherein the targeting ligand targets the cell or tissue
23 of interest. The functional coating can comprise a material that promotes nanoparticle
24 aggregation within the cell or tissue of interest.

25 [0074] In some embodiments, the functional coating according to the present
26 disclosure comprises a first layer or stabilizer. The first layer and the stabilizer may further be
27 linked to an oleic acid (o) introduced by covalent bond or physical attachment.

28 [0075] In some embodiments, the functionally coated nanoparticles are further
29 embedded into a matrix.

30 [0076] As used herein, "matrix" refers to an essentially two and/or three-dimensional
31 environment capable of immobilizing, by embedding, entrapping or encapsulating at least
32 one nanoparticle for the purpose of supporting the functionality of the nanoparticle. The

1 relationship between the constituents of the matrix and the nanoparticle include, but is not
2 limited to, covalent, ionic, and van der Waals interactions and combinations thereof.

3 [0077] Various materials can be used for forming a matrix, for example, without
4 limitations, organic groups, organic compounds, inorganic compounds, polymers,
5 organometallic compounds, surfactants, biological organic material (such as amino acids,
6 proteins, lipids, DNA, enzymes, etc.), and the mixtures thereof. The matrix may also
7 comprise hydrogel polymers, such as polysaccharides including agarose, dextran,
8 carrageenan, alginic acid, starch, cellulose, or derivatives thereof.

9 [0078] The matrix can provide a structure to retain the nanoparticulate in a desired
10 distribution without interfering with proper functioning of the nanoparticles in the
11 application. Alternatively, the matrix may itself also provide some function for the
12 application. The matrix may, for example, have a function that is different than that of the
13 nanoparticles, have a function that compliments that of the nanoparticles, or have a function
14 that is the same as that of the nanoparticles. As yet another example, the matrix may be
15 selected for its surface modifying properties to beneficially modify the nanoparticles in a way
16 that is useful in some subsequent processing or use of the nanoparticles.

17 [0079] The coated nanoparticles embedded in a matrix can be in the form of
18 nanoparticles having a diameter ranging from 10 nm to 1000 nm, including but not limited to,
19 a size ranging from 15 nm to 500 nm, from 20 nm to 200 nm, from 50 nm to 250 nm, from
20 100 nm to 150 nm, or any combination of the lower and upper bounds of these ranges not
21 explicitly set forth herein. As used herein, nanoparticle diameter refers to the mean diameter
22 as measured by a dynamic light scattering system, such as the Zetasizer Nano range, available
23 commercially from Malvern Instruments, Ltd., Worcestershire, UK, or by using transmission
24 electron microscope, such as Hitachi H7000, available commercially from Hitachi, Tokyo,
25 Japan.

26 [0080] The nanoparticles may also be prepared in a form suitable for clinic use.
27 Thus, in some embodiments, the compositions of the present disclosure further comprise a
28 pharmaceutically acceptable vehicle in which the nanoparticles are mixed or dispersed.

29 [0081] A "pharmaceutically acceptable vehicle" used herein includes, but is not
30 limited to, any and all solvents, dispersion media, substrates, coatings, isotonic and
31 absorption agents, buffering agents, and the like, compatible with a desired administrations.
32 Particularly useful examples of such vehicles include, but are not limited to, water, saline,

1 buffers, surfactants, dispersing agents and dextrose solution. The volume of the
2 pharmaceutical vehicle may vary according to the specific tissue-stabilizing agents and
3 therapeutic agents selected and their respective solubilities with the vehicle. Moreover, other
4 products may be added to the vehicle as well, if desired, for example, to maximize tissue-
5 stabilization time, drug-delivery, preservation of life, or to optimize a particular method of
6 delivery.

7 **[0082]** The coated nanoparticles embedded in a matrix may further link to a functional
8 moiety. For example, the nanoparticle can comprise a detectable moiety for the tracking of
9 the nanoparticles homing towards a tissue or tumor. The term "detectable moiety" as used
10 herein refers to a label molecule (isotopic or non-isotopic) which is incorporated indirectly or
11 directly into nanoparticles of the present disclosure, wherein the label molecule facilitates the
12 detection of the nanoparticle in which it is incorporated. Thus, "detectable moiety" is used
13 synonymously with "label molecule". Label molecules, known to those skilled in the art as
14 being useful for detection, include without limitation, chemiluminescent and/or fluorescent
15 molecules, electrochemiluminescence, Raman, colorimetric, hybridization protection assay,
16 mass spectrometry, photoacoustic, magnetic, and radionuclides.

17 **[0083]** A detectable moiety also includes an imaging agent. Any suitable detectable
18 agent can be used to be incorporated with the nanoparticles to detect the nanoparticles
19 contained within a tissue or tumor. For example, an imaging agent may be a contrast agent,
20 which means an agent that when delivered to an animal or human subject can improve the
21 image obtained by a method such as magnetic resonance imaging (MRI). Such agents may
22 include, but are not limited to gadolinium, iron oxide, manganese and magnesium salts, and
23 the like that may be formulated into pharmaceutically acceptable compositions for
24 administering in vivo with limited and acceptable degrees of undesirable side effects.
25 Chelating agents containing paramagnetic metals for use in magnetic resonance imaging can
26 also be employed as agents. Typically, a chelating agent containing a paramagnetic metal is
27 associated with a coating on the nanoparticles. The chelating agent can be coupled directly to
28 one or more of components of the nanoparticle such as functional amino groups. Suitable
29 chelating agents include, but are not limited to, a variety of multi- dentate compounds
30 including EDTA, DPTA, DOTA, and the like.

31 **[0084]** An imaging agent may also include a labeling moiety that is useful for
32 providing an indication of the position of the label and adherents thereto, in a cell or tissue of

1 an animal or human subject, or a cell or tissue under *in vitro* conditions. Such agents may
2 include those that provide detectable signals such as fluorescence, luminescence,
3 radioactivity, or can be detected by such methods as MRI imaging, PET imaging and the like.

4 **[0085]** The functional moiety may also be a targeting moiety. The term “targeting
5 moiety” can refer to a molecule or molecules that are able to bind to and complex with a
6 biomarker. The term can also refer to a functional group that serves to target or direct an
7 imaging probe to a particular location, cell type, healthy or diseased tissue, or association. In
8 general, a “targeting moiety” can be directed against a biomarker.

9 **[0086]** In preferred embodiments, the compositions described herein are formulated
10 to be compatible with the intended route of administration, such as intratumoral, peritumoral,
11 or intravenous applications. The composition may also be made in a form suitable for implant
12 within the solid tumor using any suitable method known to those skilled in the art of
13 penetrating tumor tissue. Such techniques may include creating an opening into the tumor and
14 positioning the composition in the tumor. For example, suspensions, dispersions, or
15 emulsions may be used in a pharmaceutically acceptable vehicle for such administrations and
16 may include a sterile diluent, such as water for injection, saline solution, polyethylene
17 glycols, glycerine, propylene glycol or other synthetic solvents; anti-bacterial agents such as
18 benzyl alcohol or methylparabens; antioxidants such as ascorbic acid or sodium bisulfite;
19 buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such
20 as sodium chloride or dextrose. The pH may be adjusted with acids or bases, such as
21 hydrochloric acid or sodium hydroxide. Preparations may be enclosed in ampules, disposable
22 syringes or multiple dose vials made of glass or plastic.

23 **[0087]** The compositions can be included in a kit, container, pack or dispenser,
24 together with medical devices suitable for delivering the compositions intratumorally,
25 peritumorally, or intravenously. The compositions included in kits may be supplied in
26 containers of any sort such that the life of the different components may be preserved and
27 may not be adsorbed or altered by the materials of the container.

28 **[0088]** In its second aspect, the present disclosure provides methods of preparing a
29 multifunctional nanoparticle composition for the treatment of cancer by enhancing the anti-
30 tumor effectiveness of radiation directed to a tissue or a tumor, the method comprising the
31 steps of (1) preparing a metal oxide nanoparticle core; treating the metal oxide nanoparticle
32 core of step (1) with a first functional layer to form a single-coated functional metal oxide

nanoparticle; and (3) loading single-coated functional nanoparticles of step (2) in a matrix to form a bigger particle loaded with one or several functional metal oxide nanoparticles. The method may further comprise a step of treating the bigger particle loaded with coated functional metal oxide nanoparticle of step (3) with a matrix to form a functional metal oxide nanoparticle. These preparations are well-known in the art.

[0089] The compositions of the five formulations useful in certain disease treatment applications and the methods of making them are described below.

Formulation #1: A-MD NPs.

[0090] The formulation #1 comprises a MD NPs stabilized with poly(allylamine hydrochloride) (PAH) and is further crosslinked with bovine serum albumin (BSA).

[0091] The formulation #1 can be prepared by any method known in the art. For example, aliquot MD NPs stabilized with PAH is directly mixed with BSA in aqueous medium. Thus, the MD NPs are entrapped in a polyelectrolyte/BSA complex due to strong electrostatic interaction between the protein and the polymer. The formulation #1(A-MD NPs) have a diameter of about 20 nm to about 200 nm. Preferably, the nanoparticles of the formulation # 1 have a diameter of about 50 nm, with a negative charge of about -30 mV.

Formulation #2 – TER-MD NPs.

[0092] The formulation #2 comprises a MD NPs stabilized with poly(allylamine hydrochloride) (PAH) and loaded in a denatured protein matrix crosslinked with a graft terpolymer, such as poly(methacrylic acid)-polysorbate 80-starch (TER)..

[0093] The formulation #2 can be prepared by any method known in the art the MD NPs stabilized with PAH are loaded into a matrix composed of denatured BSA crosslinked with a graft poly(methacrylic acid)-polysorbate 80-starch (TER). BSA is denatured by heating and ultrasonication (100 Hz) at 80°C in the presence of the A-MD NPs followed by addition of activated TER polymer to crosslink the protein matrix through the covalent conjugation of the carboxylic groups of the TER polymer with amine groups of the BSA using

EDC/NHS chemistry. The nanoparticles of the formulation # 2 have about 50-250 nm in diameter and negative charge (-35 mV).

[0094] There are several advantages of the formulation #2. For example, the formulation # 2 has the ideal size and charge for *in vivo* applications (100-150 nm and -35 mV); popcorn-like shape allows for better tumor penetration; can be dissociated *in vivo* forming smaller particles; can be further functionalized with different molecules *via* the -OH or -COOH groups on the terpolymer, e.g. tumor targeting peptide/antibody, fluorescent and near infrared dyes etc.; has high colloidal stability under physiological conditions; is highly reactive towards endogenous hydrogen peroxide; produces fast and high oxygen generation; has no cytotoxicity (100% cancer cell viability); has no acute or chronic toxicity to mice (*via* intravenous injection); intratumoral or intravenous use; and has a potential to enter brain tumor through the PS 80 in the terpolymer.

Formulation #3 – PEG-TER-MD NPs:

[0095] The formulation #3 comprises a MD nanoparticle stabilized with PAH and loaded in a denatured protein matrix crosslinked with TER and a third layer of a melted lipid-PEG (polyoxyethylene stearate) coated on the crosslinked denatured TER-protein matrix.

[0096] One example of making the formulation #3 includes adding a melted lipid-PEG (polyoxyethylene stearate) during the nanoparticle preparation described above prior to adding the crosslinker (TER). In some embodiments, PEG has chain of about 2 kDa to about 10 kDa. The lipid-PEG forms a hydrophilic-like brush layer on the surface of the nanoparticle to improve the circulation time and accumulation in the tumor *via* intravenous injection. The nanoparticles of the formulation # 3 are about 50 nm to 250 nm in diameter, and preferably about 180 nm in diameter and with a negative charge of about -32 mV. Like the formulation #2, the formulation #3 also can increase blood circulation time and generate better tumor accumulation.

Formulation #4 – L-MD NPs:

[0097] The formulation #4 comprises a MD nanoparticle stabilized with PAH, a second layer of a hydrophobic molecule oleic acid covalently attached to the PAH, and a carrier of a solid lipid nanoparticle (L).

1 [0098] As an example of making the nanoparticles of the formulation # 4, the aqueous
2 MD NPs stabilized with PAH (pMD) are transferred to an organic phase attaching
3 hydrophobic oleic acid (amine groups of polyelectrolyte present on the surface of pMD NPs
4 covalently attached with carboxylic group of oleic acid using DDC chemistry). Hydrophobic
5 nanoparticles are then loaded into a solid lipid nanoparticle (L) composed of myristic acid
6 and PEG-lipid (polyoxyethylene stearate). This formulation consists of nanoparticles about
7 50-250 nm in diameter. Preferably, the nanoparticles are 180 nm in diameter and negatively
8 charged (about -25 mV).

9 [0099] There are several advantages of formulation #4. For example, the formulation
10 is comprised of endogenous lipids and a pharmaceutical excipient as the building material
11 and as such, is anticipated to be in a good position to quickly obtain regulatory approval;
12 preparation is simple; it is of a suitable size and charge for *in vivo* applications (e.g. 150 nm
13 and -30 mV); it results in NPs that are spherical; it has high colloidal stability under
14 physiological conditions; it can be further functionalized with different molecules
15 (polyethylene glycol (PEG), tumor targeting peptide, fluorescent and near infrared dyes etc.);
16 it has been shown to produce slow, continuous oxygen generation for prolonged modulation
17 of the tumor microenvironment; it has no cytotoxicity (100% cancer cell viability); it has no
18 acute or chronic toxicity to mice (via intravenous injection); intratumoral or intravenous use;
19 and it has prolonged blood circulation and fast and high tumor accumulation (1-4 h post
20 intravenous injection).

21
22 **Formulation #5 – PMA-MD NPs:**

23 [00100] The formulation #5 comprises a MD nanoparticle stabilized with PAH (pMD),
24 a second layer of oleic acid through covalent linkage with PHA, and a carrier of a grafted
25 amphiphilic polymer (PMA)

26 [00101] As an example of making the nanoparticles of the formulation # 5, the aqueous
27 pMD transferred to an organic phase as described in Formulation #4. The hydrophobic
28 nanoparticles are then emulsified with a grafted amphiphilic polymer (PMA), such as
29 poly(isobutylene-alt-maleic anhydride)-hexadecylamine. After these steps, the nanoparticles
30 of the formulation # 5 are formed with a diameter of about 50 nm to 250 nm. Preferably, the
31 nanoparticles are 130 nm in diameter and negative charge of about -30 mV.

1 [00102] There are several advantages of the advantages of the formulation #5. For
2 example, this formulation can be simply prepared with suitable size and charge for *in vivo*
3 applications (e.g. 150 nm and -30 mV); it is spherical; has high colloidal stability under
4 physiological conditions; can be further functionalized with different molecules
5 (polyethylene glycol (PEG), tumor targeting peptide, fluorescent and near infrared dyes etc.);
6 generates slow, continuous oxygen generation for prolonged modulation of the tumor
7 microenvironment; has no cytotoxicity (100% cancer cell viability); has no acute or chronic
8 toxicity to mice (via intravenous injection); has intratumoral or intravenous use; and has
9 prolonged blood circulation and fast and high tumor accumulation (1-4 h post intravenous
10 injection).

11 [00103] In sum, while all five formulations have been found to be useful to treat
12 cancer by enhancing the anti-tumor effectiveness of radiation therapy for cancer, they may
13 have different chemical, physical or therapeutic properties. For example, although the size
14 and surface chemistry of the nanoparticles can be tailored for intratumoral (i.t.) or
15 intravenous (i.v.) injections, the inventors further found that the formulations #2 to #5 are
16 highly stable in normal saline and medium (for example, in 10-50% FBS) and therefore are
17 particularly suitable for both intratumoral and intravenous applications. Also, although all
18 formulations #1 to #5 are able to generate oxygen by reaction with endogenous levels of
19 hydrogen peroxide, higher oxygen generation rates are obtained with hydrophilic
20 formulations, #1, #2, and #3. Moreover, compared with other formulations, formulations #4
21 and #5 show better tumor accumulation. Thus, one skilled in the art can determine and
22 choose the most suitable formulation depending on the need in a specific case.

23 [00104] In an aspect, the present disclosure provides methods of treating cancer
24 disease in a subject by administering an effective amount of a multifunctional nanoparticle
25 composition described above.

26 [00105] The methods are based on the inventors' discovery that the compositions
27 themselves have exhibited tumor inhibitory effect and antiangiogenic effect. Specifically,
28 when administered to a tumor, the compositions disclosed herein can reduce hypoxia and
29 acidosis of the tumor tissue microenvironment. The compositions can also down-regulate the
30 expression of the master regulators of tumor progression and aggressiveness including
31 hypoxia-inducible factor -1 alpha (HIF-1 α) and vascular endothelial growth factor (VEGF) in
32 the tumor tissue.

1 [00106] A "subject" used herein refers to a human or animal. Usually the animal is a
2 vertebrate such as a primate, rodent, domestic animal or game animal. Primates include
3 chimpanzees, cynomolgous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents
4 include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals
5 include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine
6 species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout,
7 catfish and salmon. Preferably, the subject is a mammal. The mammal can be a human, non-
8 human primate, mouse, rat, dog, cat, horse, or cow, but are not limited to these examples. A
9 subject can be male or female.

10 [00107] "Treatment of cancer" or "treating cancer" used herein refers to an activity that
11 prevents, alleviates, maintains or ameliorates any of the primary phenomena (initiation,
12 progression, metastasis) or secondary symptoms associated with the disease. For example,
13 the treatment may (1) kill cancer cells or reduce the size of the tumor, (2) inhibit tumor
14 growth or metastasis, (3) decrease tumor growth rate or metastasis rate, or (4) control the size
15 of the tumor or the development of metastasis. Preferably, the treatment can alleviate or
16 lessen the cancer.

17 [00108] What amount of the composition is effective depends on various factors and is
18 readily determinable within the skill of the art, such as by the attending physician based on
19 cancer type, subject data, observations and other clinical factors, including for example the
20 subject's size, body surface area, age, sex, the particular parvovirus to be administered, the
21 time and route of administration, the tumor type and characteristics, general health of the
22 subject, and other surgical and/or drug therapies to which the subject is being subjected for
23 the disease.

24 [00109] Administration of the composition may be effected in different ways by,
25 including, but not limited to, intravenous, intraperitoneal, subcutaneous, intramuscular,
26 topical or intradermal administration. The route of administration in a specific case, of
27 course, depends on various factors, such as the kind of therapy, the kind of tumor or disease,
28 and the kind of nanoparticles contained in the composition. In certain aspects, the tumors
29 described herein can be benign or malignant, and the treatments described herein can be for
30 both types of abnormal tissues.

31 [00110] In some embodiments, the administration can introduce the compositions into
32 target cells, tissues or organs of interest. This introduction can be achieved by using art-

1 known methods and commercially available injection or administration systems. For
2 example, the nanoparticles can be administered intravenously, intra-arterially, or locally to
3 achieve specific loading in and around the target tissue. A preferred route of administration is
4 intravenous or intratumoral administration.

5 **[00111]** The composition can be administered alone or in a combination therapy. For
6 example, the composition can be combined with treatments such as surgery, chemotherapy or
7 other adjuvant treatment modalities. Particularly, the inventors found that a combination of
8 the composition with ionizing radiation therapy has shown a significant decrease in tumor
9 growth rates, an increase in DNA double-strand breaks (DSBs) and tumor cell death
10 compared with ionizing radiation treatment alone. Thus, adding the composition(s) to
11 radiation therapy can be used to enhance the anti-tumor effectiveness of the ionizing radiation
12 and while modulating the tumor microenvironment as a result of the addition of the
13 composition(s). Accordingly, the method may further comprise an additional step of
14 applying ionizing radiation to the subject. Thus, the composition(s) enhancing a radiation
15 treatment or exposure directed to a tissue, cell or a tumor in a subject may act synergistically
16 with the radiation to cause apoptosis and necrosis of cancer cells. Radiation therapy can be
17 delivered with spatial accuracy using methods such as intensity-modulated radiation therapy
18 or stereotactic radiation therapy, which delivers the ionizing radiation mostly to the tumor
19 volume, which allows for localized treatment while sparing surrounding healthy tissues.
20 Radiation therapy can be delivered as single- and/or multi-fraction, and at all clinically
21 acceptable and practiced dose ranges. Radiation therapy can include conventional x-ray
22 irradiation and radioactive sources used clinically, including brachytherapy or other forms of
23 localized ionizing radiation treatment.

24 **[00112]** When used in a combination with radiation therapy, the composition(s) can be
25 administered simultaneously or non-simultaneously with radiation. Simultaneous
26 administration means that the composition(s) and the radiation are administered at the same
27 time or within a certain, short intervening period of time, such as within 1, 5 or 10 minutes.
28 Non-simultaneous administration means that the administration of the composition is at
29 various times relative to the application of radiation therapy. Preferably, the composition is
30 administered prior to the application of radiation. This time period between the
31 administration of the composition and the delivery of the radiation can vary from about 10
32 minutes to 7 days.

[00113] In some preferred embodiments, the composition is intravenously injected to the subject about 30 minutes to 24 hours prior to the radiation to allow the nanoparticles accumulation in the tumor. In other preferred embodiments, the composition is intratumorally injected to the subject about 5 minutes to about 3 hours, preferably about 30 minutes, prior to the radiation.

[00114] In a further aspect, the present disclosure provides a use of a multifunctional nanoparticle composition disclosed herein for the manufacture of a medicament for the treatment of cancer by enhancing the effectiveness of radiation directed to a tissue or a tumor in a subject.

[00115] The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present disclosure in any way. Indeed, various modifications of the disclosure in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and the following examples and fall within the scope of the appended claims.

EXAMPLES

[00116] ABBREVIATIONS

A-MD	Albumin-based MD NPs prepared by complexation of pMD NPs with bovine serum albumin (BSA) or other proteins
ANOVA	Analysis of variance
bMD	Bare MnO ₂ NPs
A-MD	Albumin-based MD NPs prepared by complexation of pMD NPs with bovine serum albumin (BSA) or other proteins
BSA	Bovine serum albumin
CT	Chemotherapy
DSB	Double strand break
DSWC	Dorsal window chamber
FBS	Fetal bovine serum
H&E	Haematoxylin and eosin
HIF	Hypoxia-inducible-factor
ICP	Inductively coupled plasma
i.t.	Intratumoral

1	i.v.	Intravenous
2	L-MD	oMD NPs are loaded in lipid (i.e., myristic acid) and PEG based NPs
3	MD	Manganese dioxide (MnO_2)
4	MSFI	Multi spectral fluorescence imaging
5	MEM	Minimal essential medium
6	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
7	NPs	Nanoparticles
8	oMD	pMD NPs are covalently conjugated with oleic acid
9	PA	Photoacoustic
10	PAH	Poly(allylamine hydrochloride)
11	PDT	Photodynamic therapy
12	PMA	Amphiphilic polymer (poly isobutylene alt maleic anhydride-
13	Hexadecylamine)	
14	PMA-MD	oMD NPs are loaded in amphiphilic polymer PMA and PEG based NPs.
15	pMD	MnO_2 NPs stabilized with a positively charged polyelectrolyte
16	ROS	Reactive oxygen species
17	RT	Radiotherapy
18	sO_2	Vascular saturated oxygen
19	TEM	Transmission electron microscopy
20	TER-MD	Terpolymer (TER) cross-linked denatured A-MD NPs with or without a
21		polyethylene glycol (PEG)-like brush on the surface of the NPs.
22	TME	Tumor microenvironment
23	TUNEL	Terminal deoxynucleotidyltransferaseUTP nick end labelling
24	VEGF	Vascular endothelial growth factor
25	H_2O_2	Hydrogen peroxide
26	MnO_2	Manganese dioxide
27		
28	Example 1: Synthesis and characterization of bare MnO_2 (bMD) NPs	
29	[00117]	A modified sonochemical reduction of permanganate with manganese ions
30		was used to prepare bare MD NPs (bMD). A potassium permanganate aqueous solution
31		(0.078 g in 10 mL of DDI water) containing a non-ionic surfactant Pluronic® F-68 (50 μL of
32		100 mg/mL solution) was kept under ultrasonic field for 30s by using an ultrasonic processor

1 probe operating at approximately 50 Hz (Heischer UP100H, Germany). Manganese acetate
2 aqueous solution (0.18 g in 1 mL) was added and the dark brown dispersion obtained was
3 sonicated for additional 30 s. Solid NPs were isolated by centrifugation (5,000 rpm for 10
4 min) and thoroughly washed with DDI water. The pellet was dispersed with 10 mL of DDI
5 water and an aliquot of Pluronic® F-68 solution was added to the dispersion (50 μ L, 100
6 mg/mL). Characterization of bMD NPs is shown in Fig. 1. The UV-Vis spectrum of the
7 golden brown colloidal dispersion obtained showed a broad peak at 360 nm, a characteristic
8 of colloidal MD NPs, indication of the formation of bMD NPs (Fig. 1a). Size distribution
9 (Fig. 1b) and zeta potential measurements revealed NPs at about 80-100 nm and negatively
10 charged (-18 mV). TEM images (Fig. 1c) revealed small like-flakes NPs with size
11 distribution about 80 nm. X-ray powder diffraction (XRD) analysis of the particles revealed
12 an amorphous structure of the bMD NPs (Fig. 1d).

13

14 **Example 2: Synthesis and characterization of polyelectrolyte-stabilized MnO₂NPs**
15 **(pMD)**

16 [00118] pMD NPs were prepared following a one-step polyelectrolyte-based route by
17 directly mixing potassium permanganate with a cationic polyelectrolyte aqueous solution
18 such as poly(allylamine hydrochloride) (PAH) or polyarginine (PARG). In brief: In a small
19 glass vial 18 mL KMnO₄ solution (3.5 mg/mL) was mixed with 2 mL of a
20 polyelectrolyte solution in DDI water (37.4 mg/mL for PAH and 18 mg/mL for PARG, with a
21 molar ratio 2:1 of polyelectrolyte's repeating unit). The mixture was left for 15 min at room
22 temperature until all permanganate was converted to MD, determined by absorbance
23 measurements. NPs were washed three times with doubly distilled (DDI) water using
24 ultracentrifugation (50k rpm for 1hr). This step led to small (10-15 nm) pMD NPs stabilized
25 with the polyelectrolyte. The absolute concentration of MD molecules in the NPs solution
26 was quantified by inductively coupled plasma (ICP) analysis to determine the concentration
27 of Mn²⁺ ions, and thereby the concentration of pMD in the emulsion. A typical preparation
28 produced a solution containing ~25 mM pMD. This synthesis procedure is rapid,
29 reproducible and gives stable pMD colloidal dispersions. Characterization of the pMD NPs is
30 shown in Fig. 2. The UV-Vis spectra of the KMnO₄ precursor solution and pMD NPs
31 prepared at various molar ratios between PAH and MD are compared in Fig. 2a. After the
32 reaction with PAH at ratios 2:1 and 3:1, the KMnO₄ peaks (315, 525 and 545 nm)

disappeared, and a new broad peak around 300 nm was originated for these samples, an indicative of the formation of pMD NPs. The new peak around 300 nm is attributed to the surface plasmon band of pMD NPs. Zeta potential analysis (Fig. 2b) and size distribution measurements (Figs. 2c-d) revealed NPs positively charged (+45 mV) at about 10-15 nm. The polyelectrolyte used served not only as a reducing reagent to reduce permanganate to MD, but also as a protective layer to stabilize as-formed NPs due to electrostatic repulsion.

Example 3: Preparation and characterization of MnO₂ NPs loaded into a protein/polyelectrolyte matrix (A-MD)

Preparation of A-MnO₂ NPs

[00119] For the synthesis of NPs, the inventors employed a one-step method to reduce manganese permanganate (KMnO₄) to MD NPs with cationic polyelectrolyte poly(allylamine hydrochloride) (PAH). This synthesis procedure is rapid, reproducible and gives stable MD colloidal dispersions with an average NP size distribution of 15 nm (Fig. 4a-b). In the present synthesis method, the inventors were able to decrease by 50% the amount of PAH normally used in polyelectrolyte-based NPs synthesis.²¹ The decrease in the amount of PAH utilized in the NP formulation is very important for *in vivo* applications, since cationic polyelectrolytes can show pronounced concentration-dependent cytotoxicity. The polyelectrolyte used here served not only as a reducing reagent to reduce KMnO₄ to MD, but also as a protective layer to stabilize as-formed NPs due to electrostatic repulsion (zeta potential +30 mV, Fig. 4c). In this formulation, pMD NPs were complexed with bovine serum albumin (BSA). BSA-MnO₂ (A-MD) NPs were prepared by directly mixing BSA with pMD NPs in aqueous medium. In brief, in a vial 4 mL of pMD NPs stock solution (25mM) was diluted with 6 mL of normal saline solution (0.9% NaCl in DDI water). This diluted pMD solution (10 mM) was mixed with 1.2 mL of BSA solution in normal saline (10 mg/mL). This step led to the formation of A-MD NPs (~50 nm), with several pMD NPs entrapped in a polyelectrolyte/BSA complex due to strong electrostatic interaction between the protein and the polymer. A-MD NPs were further diluted with cell medium or sterile saline for *in vitro* and *in vivo* studies, respectively. Protein labelling kits AnaTag™ HiLyte Fluor™ 594 (Texas Red) and AnaTag™ 750 (AnaspecInc, USA) were used to label albumin for the preparation of red fluorescent and near-infrared NPs, respectively. It was estimated 100% loading of the pMD NPs in the

polyelectrolyte/BSA complex. UV-Vis spectrophotometry analysis of the supernatant indicated the absence of free pMDNPs in the supernatant after ultracentrifugation (10k rpm, 10 min) of the NP emulsion. The A-MD NPs prepared were negatively charged (-25 mV) (Fig. 2b) and approximately 50 nm in size (Fig. 2c-d), and stable in alpha minimal essential medium (α MEM) cell medium and saline. TEM pictures (Fig. 3b) show several small pMD NPs within the protein/polymer complex.

[00120] To formulate MD NPs for biomedical *in vivo* applications the inventors took into account the issues of nanoparticle stability, size control and toxicity. For biological applications, NPs must be stable in cell culture medium or normal saline required for *in vitro* and *in vivo* studies, respectively. Polyelectrolyte-coated MnO₂ (pMD) nanoparticles are too small and positively charged, which can cause high instability in cell medium or saline and result in toxicity. To solve these problems, the inventors have conjugated small polyelectrolyte-coated MnO₂ nanoparticles with bovine serum albumin (BSA) and obtained particles of suitable size, charge, colloidal stability and biocompatibility for *in vitro* and *in vivo* applications, while maintaining the MD reactivity towards H₂O₂ for the production of oxygen and increase in pH. BSA can form stable non-covalent complexes with cationic polyelectrolytes,³¹ leading to lower NP toxicity.³² The prepared A-MD NPs were approximately 50 nm in size (Fig. 4b), negatively charged (-25 mV) (Fig. 4c) and stable in alpha minimal essential medium (α MEM) cell medium and saline (Fig 4d), making them suitable for *in vivo* applications. The albumin coating also provided the NPs with different surface charge and chemistry allowing the inventors to further functionalize the NP surface with protein-reactive fluorescent dyes such as Texas RedTM (excite 596/emit 617 nm) and amine-reactive near infrared dye (excite 754/emit 778 nm). These fluorescence-labelled NPs were utilized in our subsequent *in vitro* and *in vivo* studies.

Multifunctionality of A-MD NPs in culture medium

[00121] The inventors first investigated the multifunctionality of the A-MD NPs to generate O₂ and to increase the medium pH *in vitro* upon reaction with H₂O₂ at endogenous levels. The reaction between MD and H₂O₂ is a complex reaction leading to the decomposition of H₂O₂ and the production O₂ as summarized in Fig. 5a. Besides the production of O₂, the reaction causes an increase in the local pH by the consumption of H⁺ ions and the production of an intermediate Mn-oxo-hydroxide (MnOOH).²⁶ This

phenomenon can be particularly useful for the regulation of local pH in cancer cells and tumour tissue. Hence the inventors studied if A-MD NPs would generate measurable amounts of oxygen and increase pH at low concentrations of H_2O_2 found in the human body (i.e., 100 μM and up to 1 mM).³³ The inventors found that at a very low concentration ($\sim 45 \mu\text{M}$ of MD), the NPs were able to completely quench 1 mM H_2O_2 in cell medium within 40 minutes (Fig. 5b). The inventors further investigated the O_2 generating properties of the NPs using an in-house-made hypoxia-maintaining chamber coupled with both a commercially available oxygen probe and a pH microelectrode. Significant amounts of O_2 were produced (Fig. 5c) accompanied by an increase in the pH of physiological buffer (phosphate/saline buffer) by one pH unit from pH 6.8 to pH 7.8 (Fig. 5d) by the reaction of 45 μM of MD with 250 μM H_2O_2 . In an attempt to simulate *in vivo* conditions where H_2O_2 is continuously generated by tumor cells, the inventors measured the O_2 production by the NPs during the continuous addition of exogenous H_2O_2 (250 μM) into the chamber every 30 min. The inventors observed that a single dose of the NPs (90 μM MD) continuously generated O_2 for at least 6 cycles of 30 min each (Fig. 5e). These results demonstrated that H_2O_2 and protons can diffuse rapidly across the polyelectrolyte-albumin complex, access the reactive sites of the MnO_2 cores, produce O_2 and increase pH in a sustained manner under hypoxic conditions.

***In vitro* studies with cells**

[00122] The viability of human breast cancer MDA-MB 231 and murine breast cancer EMT6 exposed to various concentrations of MD NPs for 48 h was evaluated. In brief, plated cells (10 000 cells per well / 96 well plate) were incubated with different NP formulations dispersed in α -medium at desired concentrations (i.e., for 50 and 100 μM MD). Cells were incubated with NPs for 48 h and assayed following a standard MTT colorimetric assay protocol.

[00123] **Cellular uptake of NPs:** Murine EMT6 breast tumor cells (10^5 cells) were incubated for 1h with A-MD NPs (45 μM) at 37°C before microscopic analysis. Cell uptake of NPs by transmission electron microscopy (TEM) was performed using a H7000 TEM microscope (Hitachi, Japan), following standard methods for sample preparation. An EVOS fluorescence microscope (AMG, USA) was used to image live cells following incubation with red fluorescent dye labelled A-MD NPs. Cell nuclei were stained blue with HOESCHT 33342 (Invitrogen Molecular Probes, USA). The cellular uptake of NPs at various time points

1 was also evaluated. For this experiment, NPs were made fluorescent by loading of fluorescein
2 isothiocyanate (FITC) to the NPs as previously described. Platted cells (500,000 cells per well/
3 6 well plate) were incubated with FITC labelled A-MD NPs dispersed in culture medium at
4 the concentration 100 μ M MD. Cells were incubated with NPs for several time points: 0.5, 1,
5 2, 4 and 24 h. After incubation time cells were washed twice with PBS, trypsinized and
6 replated in 96-well plate (10, 000 cells per plate). Fluorescence was measured with a
7 microplate reader, ex: 485 nm and em: 520 nm.

8 [00124] These results are summarized in Fig. 6. Cell viability studies showed that all
9 formulations (protein, lipid and polymer based) are not toxic to the cancer cells at MD
10 concentrations used *in vitro* and *in vivo* (i.e., 100 μ M MD) (Fig 6a-c). It is known that the
11 aberrant metabolism of cancer cells leads to significantly elevated cellular concentrations of
12 H_2O_2 . If the NPs could be taken up by cancer cells, they could react quickly with endogenous
13 H_2O_2 produced by cancer cells under hypoxic stress, thus producing O_2 *in situ*. To test this
14 hypothesis, the inventors first examined the cellular uptake of the NPs by incubating EMT6
15 murine breast cancer cells with fluorescence-labelled A-MD NPs, and observed significant
16 cellular uptake of NPs within 60 min of incubation (Fig. 6d). This finding was confirmed by
17 transmission electron microscopy (TEM). TEM images (Fig. 6e) showed EMT6 cells *in vitro*
18 underwent membrane invagination and engulfment of the NPs and the NPs taken up by the
19 cell were distributed within the cell cytoplasm and vesicles after 1 h incubation. Different
20 formulations showed similar cellular uptake profile over the time for two different cancer cell
21 lines (Fig 6f-g). Cellular uptake of the NPs was observed within 60 min of incubation with
22 EMT6 cells (Fig. 6f).

26 Oxygen generation in the presence of hypoxic cancer cells

27 [00125] The inventors found that the NPs incubated with hypoxic breast cancer cells
28 could react quickly with endogenous H_2O_2 produced by the cells under hypoxic stress, thus
29 producing O_2 *in situ* (Fig. 6f). Significant amounts of O_2 (~6-fold increase of O_2 levels in the
30 medium) were detected within 2 min by reacting with H_2O_2 released by the cancer cells (Fig.
31 6f). These results indicate that the endogenous levels of H_2O_2 released by hypoxic cancer
32 cells *in vitro* is sufficient to react with the NPs and generate measurable O_2 without addition

of exogenous H_2O_2 to the culture medium. Moreover, at the concentration used for *in vitro* O_2 generation (45 μM MD), A-MD NPs showed relatively low cytotoxicity to EMT6 cancer cells (~80% cell viability) (Fig. 6g). Based on these data, the inventors hypothesize that elevated levels of H_2O_2 in solid tumors could serve as a reactant for O_2 production *in vivo*.

Effect of A-MD NPs on tumor oxygenation

[00126] The effect of A-MD NPs on oxygen saturation within orthotopic murine EMT6 cell breast tumors was assessed with a small animal photoacoustic (PA) imaging system following intratumoral (i.t.) injection of 50 μL of A-MD NP suspension in saline. A Vevo LAZR Photoacoustic Imaging System (VisualSonics Inc., Canada) with a 21 MHz centre frequency transducer (LZ-550, VisualSonics Inc., Canada) was used to measure vascular oxygen saturation (sO_2) *in situ* over time before and after i.t. treatment with A-MD NPs. Ultrasound was utilized to guide NP injection in order to administer treatments to the tumor. Animals were maintained below 7% oxygen atmosphere during the experiment and sO_2 measurements were assessed using standard multispectral photoacoustic imaging in the tumors *in vivo* using two excitation wavelengths (750 nm and 850 nm) for deoxygenated and oxygenated hemoglobin, respectively.

[00127] PA imaging measures vascular saturated O_2 (sO_2) by the differential optical absorption of oxygenated and deoxygenated hemoglobin at different wavelengths, which is directly correlated with changes in O_2 concentration in the blood⁴⁰. To ensure similar localization of the NPs in each tumor we used ultrasound image-guidance to inject the NPs into the tumor *in vivo*. Since the blood vessels maintain a saturated level of oxygen under normoxic conditions, the mice were maintained under 7% O_2 during the experiments to visualize the enhancement of oxygen production by the A-MD NPs. Vascular sO_2 was measured before and after i.t. injections of A-MD NPs suspension or saline only (control) and found that sO_2 increased promptly by approximately 45% as compared with control tumors (Fig. 7 a-c). It is important to point out that PA imaging of sO_2 depends on the presence of blood flow, which is lacking in the necrotic and avascular tumor core. Thus, the PA images revealed sO_2 was mainly generated within the tumor periphery (Fig. 7a). However, this does not imply that the NPs are unable to produce O_2 in the hypoxic region close to the tumor core, as the O_2 generating capacity of the NPs is limited only by the presence of H_2O_2 . Interestingly, the nearly immediate detection of O_2 at the tumor periphery suggests the rapid

1 distribution of the NPs within the tumor mass from the injection site (i.e., tumor center)
2 perhaps due to the interstitial pressure gradient with a higher pressure in the tumor core than
3 within the peripheral region⁴¹.

4 **[00128]** In this Example, the inventors used i.t. delivery of A-MD NPs for the
5 assessment of the effect of the NPs on tumor oxygenation *in vivo*. The reason for using i.t.
6 delivery is two-fold. Firstly, the local delivery method is better than systemic delivery (e.g.
7 intravenous injection) in terms of uniformity of NP dose delivered to each tumor owing to a
8 broad variation from tumor to tumor in morphology and NP penetration. Secondly, local
9 intratumoral delivery of therapeutics has been emerging as an effective treatment of many
10 types of localized operable and inoperable solid tumors (e. g. breast, colorectal, lung,
11 prostate, skin, head and neck and brain tumors) due to its advantages over systemic methods,
12 including dramatically higher local drug concentration, better therapeutic outcomes and
13 minimal systemic toxic side-effects.^{41, 42}

14
15 **pKa Calibration for SNARF in tissue like phantoms.**

16 **[00129]** SNARF-4F 5-(and-6)-carboxylic acid (Life Technologies, USA, #S23920) is a
17 dual imaging, pH-sensitive fluorophore that allows the measurement of pH values in solution
18 as well as in biological tissue. The typical fluorescence emission spectra of SNARF shift
19 from green-yellow to red when the pH changes from acidic to alkaline. The ratio between the
20 two fluorescence intensities, typically at 580nm and 640nm, provides quantitative
21 information about the pH values. For the quantitative measurement of pH using SNARF,
22 calibration curves must be obtained under the similar conditions at which the pH values are to
23 be determined as the pKa values of the dye are sensitive to the local environment. In the
24 present studies, the calibration of SNARF was performed using tissue-like phantoms prepared
25 in pH range 4-10, following an established protocol^{55, 56}.

26 **[00130]** In detail, buffers were prepared using the following buffering systems:
27 potassium hydrogen phthalate/hydrochloric acid from pH 3-4, potassium hydrogen
28 phthalate/sodium hydroxide from pH 4.5-6, potassium dihydrogen phosphate/sodium hydro
29 oxide from pH 6-8 and disodium hydrogen phosphate/hydrochloric acid from pH 8-10. pH
30 values for all buffers were measured using Acumet AB15 pH meter (Fisher Scientific, US).

31 **[00131]** Phantoms were prepared by heating 10% (wt/v) gelatin in deionised water at
32 50⁰C. Once gelatin melted, the temperature was reduced to 40 °C and haemoglobin (Sigma

#H2625) and intra lipid (Sigma #I141) were added to give final concentration of 42.5 μ M and 1% (wt/v), respectively. After few minutes of stirring, one part of gelatin solution was mixed with three parts of 1 mM SNARF solution prepared in different pH buffers (pH 4-10) with a pH increment of 0.5 unit. In a last step, 200 μ L of the final mixture was transferred to a 96-well microplate and placed in fridge for solidification. Biological phantom were prepared in triplicate for each pH value. Fluorescence images of the microplates were recorded using a Xenogen microscope (Xenogen IVIS Spectrum, Caliper Life Sciences Inc., USA) with two different filter channels; the excitation wavelength for each channel was 535nm whereas the emission intensities were recorded at 580nm (green channel) and 640nm (red channel).

[00132] For the calculation of the pKa values, the fluorescence intensities I_g (580 nm) and I_r (640 nm) were measured using Image-J program by drawing the region of interest (ROI) on the obtained image for each pH value (Fig. 8a). The ratio (R) of the intensities I_g/I_r was then calculated and plotted against the respective pH values (Fig. 8b), and the R curve was then fitted using the Boltzmann function (Fig. 8c). Fig. 8d shows the graph of $-\log(\dots)$ term versus pH, the ratio R was obtained from the Boltzmann fit in (c) and $I_{a(\lambda_2)}$ and $I_{b(\lambda_2)}$ are fluorescence intensities at 640nm (red channel) obtained from image in (a) at pH 4.5 and pH 8.5 using image-J program, $I_{a(\lambda_2)} = 41.92$ and $I_{b(\lambda_2)} = 63.11$. The intercept of linear fit of data points in (d) is pKa according to Eq. 2. The obtained pKa of SNARF was 6.39.

[00133] In the following equation (Eq.1):

$$pH = pK_a - \log \left(\frac{R - R_b}{R_a - R} \times \frac{I_{b(\lambda_2)}}{I_{a(\lambda_2)}} \right)$$

R is the measured ratio of fluorescence intensities at each pH value, R_a is the value of the R curve at acidic pH which is considered as the acidic titration endpoint, and R_b is the value of the R curve at alkaline pH which is considered as the basic titration endpoint. The parameter pH_{inf} is the point of inflection of the R curve, i.e. the pH value at which the slope of the curve is maximum, ΔpH is an indicator for the slope at the point of inflection. From the fitting of the R curve the fit parameters R_a , R_b , pH_{inf} and ΔpH were determined.

[00134] In the next step pKa value of SNARF was calculated using the following equation (Eq.2),

$$pH = pK_a - \log \left(\frac{R - R_b}{R_a - R} \times \frac{I_{b(\lambda_2)}}{I_{a(\lambda_2)}} \right) \quad \text{Eq. 2}$$

[00135] The $-\log(\dots)$ term in equation 2 was calculated for all pH values from pH4-10, the value R in $-\log(\dots)$ term correspond to each point in Boltzmann fitting curve, whereas parameter R_a and R_b were obtained from fitting of equation1. The two other parameters $I_{a(7.2)}$ and $I_{b(7.2)}$ in $-\log(\dots)$ term correspond to the fluorescence intensities of SNARF obtained from images using image-J program at 640nm (red channel) at acidic and alkaline region respectively for the fitted R curve (in the present study we took intensities at pH4.5 and pH 8.5). At the end, graph was plotted for $-\log(\dots)$ versus pH that would give a straight line (see Eq.1) and intercept of linear fit is pKa. The calculated pKa value was 6.39.

Effect of A-MD NPs on tumor pH

[00136] To measure intratumoral pH, the inventors employed complementary fluorescence imaging⁴³ and microneedle⁴⁴ methods. The inventors applied an established *ex vivo* tissue protocol for mapping tumor tissue pH which utilizes multispectral fluorescence imaging (MSFI) in conjunction with a pH-sensitive fluorescent dye (SNARF-4F) injected *in vivo* prior to animal sacrifice.⁴³ In this study, EMT6 tumor bearing animals were first injected i.t. with A-MDNPs, followed by intravenous injection of the SNARF-4F dye to stain for tumor pH. MSFI images of the intratumoral facets of dye-perfused tumors were then acquired *ex vivo* in tissue sections and correlated to local pH from the calibration curves obtained earlier with biological tissue-like phantoms. The tumor pH was also accessed *ex vivo* with a pH microneedle probe⁴³ immediately after the MSFI procedure. The inventors found that intratumoral injection of A-MDin orthotopic solid tumors led to higher intratumoral pH (Fig. 9). After a single i.t. injection of A-MDNPs, the tumor pH increased, after only 20 min, from 6.2 to 6.7 (as determined by MSFI) and from 6.7 to 7.3 (as determined by microneedle probe) (Fig. 9). The difference in pH values obtained with the two different methods can be attributed to interferences such as tissue autofluorescence and/or dye bleaching for MSFI images. Nonetheless, both methods revealed tumor pH values consistent with pH ranges reported in literature (e.g. pH 6.3 – 6.9, depending on the tumor model, cell line and measurement method).⁴⁵ The *ex vivo* tumor tissue pH measurements (Fig. 9b) revealed that A-MDNPs can quickly decrease tumor acidosis (i.e., within 30 min), most probably by quenching excessive protons produced by cancer cell glycolysis.

Prolonged regulation of tumor hypoxia, HIF-1 α and VEGF is related to extended tumor retention of A-MD NPs

[00137] The inventors injected near-infrared dye-labelled NPs into orthotopic EMT6 breast tumors. The A-MD NPs are expected to be cleared as MD NPs can be completely consumed by H₂O₂ (Fig. 10) and thereafter the remaining BSA/PAH complex is expected to undergo enzymatic degradation by specific proteases, via an already established mechanism for the fate of polyelectrolyte complexes taken by cells.^{46, 47} *In vivo* fluorescence imaging data (Fig. 11a) showed substantial diffusion of NPs within the tumor tissue almost immediately after the injection and prolonged retention of NPs within the tumors for at least 24 h, followed by gradual clearance from the tumors over 24-48 h.

[00138] The inventors then investigated the effect of A-MD NPs on tumor hypoxia *in vivo* within the 24 h period post NP-injection by immunohistochemistry to directly measure tissue hypoxia using a pimonidazole marker⁴⁸ and the expression of HIF-1 α and VEGF (Fig. 11b) using antibodies on *ex vivo* tissues. Unlike the *in vivo* PA experiments that measure hemoglobin-related vascular sO₂, immunohistochemistry of *ex vivo* tissues from animals injected with pimonidazole prior to sacrifice directly detects the presence of hypoxic tumor cells. The inventors found that tumors treated with A-MD NPs for 30 min, 60 min or 24 h showed 24, 45 and 37% less tissue hypoxia, respectively, as compared with the saline control (Fig. 11c), suggesting a time-dependent and sustained effect of NPs on tumor hypoxia. The same tumors also showed a 19, 21 and 10% decrease in the expression of HIF-1 α (Fig. 11d), and 7, 65 and 65% decrease in the expression of VEGF (Fig. 11e), after 30 min, 60 min and 24h treatment with A-MnO₂ NPs, respectively. HIF-1 α is a master regulator of the transcriptional response to acute and chronic hypoxia,^{48, 49} while VEGF is involved in cancer cell metabolism, angiogenesis, invasion, metastasis and apoptosis.⁵⁰ Over-expression of VEGF is a hallmark of tumor angiogenesis.⁵⁰ Based on the impact of angiogenesis on cancer progression and treatment, several anti-angiogenic agents including anti-VEGF molecules are now in clinical trials as a sole treatment or in combination with conventional cancer chemotherapy.⁴⁹ Thus downregulation of HIF-1 α and VEGF expression would be expected to improve tumor prognosis. The results presented above show that the effectiveness of A-MD NPs on the regulation of the TME is not limited to the transient increase of tumor oxygenation and pH; they also have an effect on the down-regulation of hypoxia-responsive protein expression that plays an important role in biological behavior and therapeutic

1 response of many types of cancers.⁷⁻⁸ Because the expression of HIF-1 α is transient
2 depending on the relative rate of synthesis (via an O₂-independent pathway) and degradation
3 (via an O₂-dependent pathway),⁵¹ the sustained *in situ* production of O₂ by A-MD NPs is
4 beneficial to prolonged regulation of TME especially impacting on the expression of
5 downstream proteins, such as VEGF-4.

6 [00139] The biodistribution and *ex vivo* images of the resected organs (Figs. 11f-g,
7 where lighter shade indicates higher fluorescence emission) showed that NPs (lipid and
8 polymer-based) reached and accumulated in the tumor site within 1 h post i.v. injection and
9 remained in the tumor during 24 h duration of the experiment.

11 **A-MD NPs enhanced anti-tumor effect of radiation**

12 [00140] Various studies have shown that hypoxic cells in solid tumors are two-to-three
13 times more resistant to a single dose of ionizing radiation than those with normal levels of
14 oxygen.^{3,4} To explore whether *in situ* oxygen production by A-MD NPs can enhance RT, the
15 inventors conducted preliminary studies in an *in vivo* orthotopic murine breast tumor model.
16 EMT6 tumors were treated with A-MD NPs or saline 30 min prior to irradiation. A
17 significant tumor growth delay was observed in mice treated with the combination of A-MD
18 NPs and RT (Fig. 12a) compared to the control groups. The average tumor volume in the A-
19 MD NPs + RT group at day 5 remained at $\approx 78 \text{ mm}^3$ while the RT alone group (treated with
20 saline + RT) reached an average tumor volume of $\approx 231 \text{ mm}^3$ at the end of day 5 after
21 treatment. Tumor weight was also significantly lower in the A-MD NPs + RT group (Fig.
22 12b). Interestingly, a decreased tumor growth was observed in the A-MD NP alone treated
23 group compared to the saline group (non-irradiated controls) (Fig. 12a). This moderate
24 antitumor effect may be attributable to the manipulation of the TME by the A-MD NP
25 formulation which reduces VEGF levels by 65%. A more in-depth study will be conducted in
26 the future to further investigate this observation.

27 [00141] To confirm that the effect of A-MD NP on the enhancement of radiation
28 response was due to tumor cell cytotoxicity, the percentage of tumor apoptotic and necrotic
29 areas was determined. Tumors treated with A-MD NP + RT showed a significantly higher
30 tumor cell death (71%) compared to the saline + RT treated group (40%) (Fig. 12c). The
31 inventors further evaluated radiation-induced DNA double strand breaks (DNA DSBs) in
32 tumor tissues by using gamma H2AX staining to stain for DNA DSBs. The DNA DSB is

considered the most lethal type of damage induced by ionizing radiation and is a major indicator of the efficacy of treatment.⁵² Combined treatment with A-MD NPs and irradiation resulted in increased DNA damage (71%) compared to the saline control with irradiation (28%) in the EMT6 solid tumor (Fig. 12d). A window chamber mouse model bearing tumor⁴⁴ was employed to determine the induction of DNA DSB after treatment with radiation combined with A-MD NPs or saline (Figs. 12e-f). Spatially localized focal x-ray irradiation was performed on half of the tissue in the transparent window chamber allowing the inventors to determine the relative effect of treatment in the same mouse. Increased DNA DSBs were observed when the tumor was treated with both A-MD NP and RT versus radiation alone. Oxygen close to DNA is known to react with radiation produced radicals in DNA “fixing” them in a state that is difficult for the intrinsic cellular DNA repair mechanisms to deal with.⁶ Therefore, it is likely that oxygen generated by reaction of A-MD NP with H₂O₂ in tumor tissue facilitated the oxygenation effect causing more tumor cell death upon radiation thereby leading to an enhanced delay in tumor growth.

[00142] The inventors have demonstrated a completely new and innovative application of A-MD NPs for the modulation of the TME. The intratumoral treatment of murine breast tumors with A-MD NPs resulted in simultaneous attenuation of hypoxia and acidosis in solid tumors *in vivo*. Moreover, for the first time a bioinorganic nanoparticle system has been demonstrated to promote down-regulation of crucial tumor progression-related factors, i.e., HIF-1 α and VEGF. In addition the inventors have demonstrated the application of A-MD NPs for enhancement of radiation induced tumor growth delay and cancer cell death. This work suggests a potential of the A-MD NP system to regulate multiple biological attributes of the TME simultaneously and to improve cancer response to radiation treatment. The *in vivo* results obtained in the present work encourage continuing efforts for the optimization and application of MD NPs in combination with other cancer treatments such as chemotherapy and photodynamic therapy (PDT).

[00143] METHODS

[00144] **Nanoparticle synthesis:** MD NPs were prepared by directly mixing the aqueous solutions of KMnO₄ and poly(allylamine hydrochloride) (PAH, 15kDa). Briefly, 18 mL of KMnO₄ solution (3.5 mg mL⁻¹) was mixed with 2 mL of PAH solution (37.4 mg mL⁻¹), the mixture was left for 15 min at room temperature until all permanganate was converted to MnO₂. NP formation was confirmed by recording UV-Vis absorption spectrum. NPs were

1 washed three times with doubly distilled (DDI) water using ultracentrifugation (50k rpm for
 2 1hr). This step led to small (~15 nm) MD NPs stabilized with PAH. At the final step, BSA
 3 was added to the MD NP solution at a BSA/NP ratio 2.5 % (wt/wt), and NaCl was added to
 4 make the solution normal saline (0.9% NaCl). This step led to the formation of A-MD NPs
 5 (~50 nm), with several MD NPs entrapped in a PHA/BSA complex due to strong electrostatic
 6 interaction between the protein and the polymer. A typical preparation led to a $\approx 0.7\%$ A-MD
 7 NPs solution, corresponding to ≈ 1.1 mM MD as determined by inductively coupled plasma
 8 (ICP) analysis. A-MD NPs were further diluted with cell medium or sterile saline for *in vitro*
 9 and *in vivo* studies, respectively. Protein labelling kits AnaTag™ HiLyte Fluor™ 594 (Texas
 10 Red) and AnaTag™ 750 (Anaspec Inc, USA) were used to label albumin for the preparation
 11 of red fluorescent and near-infrared NPs, respectively.

12 **[00145] Cell lines, tumor models and treatments:** *In vitro:* A murine EMT6 breast
 13 cancer cell line was utilized and cultured following standard cell culture procedures.⁴⁵ For all
 14 *in vitro* experiments, cells in α MEM medium (10^5 cells per mL) were treated with A-MDNPs
 15 for 1h. Cell viability was measured using a standard MTT colorimetric assay protocol.⁴⁵ *In*
 16 *vivo:* All procedures strictly complied with the ethical and legal requirements under Ontario's
 17 Animals for Research Act and the Federal Canadian Council on Animal Care guidelines for
 18 the care and use of laboratory animals and were approved by the University Animal Care
 19 Committee of the University of Toronto. Solid tumors of EMT6 breast cancer cells (10^6)
 20 were grown orthotopically in Balb/c mice and animals were randomly allocated for all
 21 treatments (n=3/group). For *in vivo* experiments, tumors were injected with 50 μ L of A-MD
 22 NPs solution in saline (0.2 mM MD), which made the MD concentration ≈ 45 μ M in a ≈ 200
 23 mm³ tumor. Controls were injected with equivalent volume of sterile saline.

24 **[00146] Quenching of H₂O₂ by nanoparticles:** For the quenching experiments, A-MD
 25 NPs (90 μ M) in cell medium containing 10% FBS at 37°C and H₂O₂ (1 mM) was added to
 26 initiate the reaction. The residual concentration of H₂O₂ was determined over time using a
 27 PeroXOquant assay kit (Pierce, USA), at 37°C. Cell medium with 10% FBS was used as a
 28 vehicle control.

29 **[00147] *In vitro* oxygen and pH measurements:** O₂ generated by A-MD NPs and pH
 30 changes were measured in a semi-sealed chamber coupled with a MI-730 micro-oxygen
 31 electrode and a MI-407 pH + MI 402 reference microelectrodes (Microelectrodes Inc, USA),
 32 at 37°C. A-MD NPs were dispersed in α MEM cell medium containing 10% FBS to give

1 various MD concentrations (10 – 90 μ M). The system was made hypoxic by bubbling with
2 N₂. Endogenous level of H₂O₂ (250 μ M) was injected into the chamber to initiate O₂
3 generation. *For experiments with hypoxic cells:* Murine breast cancer EMT6 cells (10⁵ cells
4 per mL) were suspended and stirred in α MEM medium in glass vials plugged with rubber
5 stoppers and pierced with two hypodermic needles for gassing. The cell suspension was made
6 hypoxic by introducing a mixture of 95% N₂ and 5% CO₂ for 20 min at 37°C. A-MD NPs (45
7 μ M) were then injected and the oxygen levels monitored over time. *For all experiments:* pH
8 or O₂ were monitored every 60 s using an Oakton pH 1100 (Termo Fisher Scientific Inc,
9 USA) coupled with O₂-ADPT Oxygen Adapter (Microelectrodes Inc, USA) for O₂
10 measurements. All electrodes were calibrated according to manufacturer's instructions.
11 α MEM medium with 10% FBS, with or without cells was used as control.

12 **[00148] Cellular uptake of NPs:** Murine EMT6 breast tumor cells (10⁵ cells) were
13 incubated for 1h with A-MD NPs (45 μ M) at 37°C before microscopic analysis. Cell uptake
14 of NPs by transmission electron microscopy (TEM) was performed using a H7000 TEM
15 microscope (Hitachi, Japan), following standard methods for sample preparation.⁴⁵ An EVOS
16 fluorescence microscope (AMG, USA) was used to image live cells following incubation
17 with red fluorescent dye labelled A-MD NPs. Cell nuclei were stained blue with HOESCHT
18 33342 (Invitrogen Molecular Probes, USA).

19 **[00149] Tumor retention of NPs:** AXenogen IVIS Spectrum Imaging System
20 (Caliper Life Sciences Inc., USA) was used to image tumor bearing animals over time
21 following i.t. treatment with near-infrared labelled A-MD NPs. At each time point, a bright
22 field image was acquired and fluorescence-labelled A-MD NPs were imaged at 754 nm
23 excitation and 778 nm emission. Image fluorescence was quantified by equalizing the
24 fluorescence intensity range across all images.

25 **[00150] Tumor pH measurements:** ApH-sensitive fluorophore SNARF-4F (Life
26 technologies S23920, NY USA) was used for *ex vivo* tumor pH imaging following an
27 established protocol.³⁴ Tumor bearing mice were injected i.t. with NPs in saline followed by
28 i.v. injection of the dye (1 nmol of SNARF-4F in 200 μ L of sterile saline). Animals were
29 sacrificed 20 min following injections, tumor tissue was immediately harvested, cut in half
30 and imaged with Xenogen IVIS Spectrum (Caliper Life Sciences Inc., USA). For control
31 experiment sterile saline was injected i.t. followed by intravenous (i.v.) injection of SNARF,
32 tumors were imaged *ex vivo* using the same conditions. All the necessary calibration curves

of dye were performed following published protocols^{55, 56} and biological tissue-like phantoms were prepared following standard procedures⁴³. Tumor pH was also measured using a MI-407 pH + MI 402 reference microelectrodes following a standard protocol⁴⁵ (Microelectrodes Inc, USA).

[00151] Tumor oxygenation measurements: A Vevo LAZR Photoacoustic Imaging System (VisualSonics Inc., Canada) with a 21 MHz centre frequency transducer (LZ-550, VisualSonics Inc., Canada) was used to measure vascular oxygen saturation (sO_2) *in situ* over time before and after i.t. treatment with A-MD NPs. Ultrasound was utilized to guide NP injection in order to administer treatments to the tumor. Animals were maintained below 7% oxygen atmosphere during the experiment and sO_2 measurements were assessed using standard multispectral photoacoustic imaging in the tumors *in vivo* using two excitation wavelengths (750 nm and 850 nm) for deoxygenated and oxygenated hemoglobin, respectively.

[00152] Immunohistochemistry detection of tumor hypoxia: The hypoxia marker pimonidazole hydrochloride (HypoxyprobeTM-1 plus kit, HypoxyprobeInc, USA) was used for *ex vivo* tissue staining of hypoxia following the protocol provided with the kit. Rabbit polyclonal HIF-1 α antibody (dilution 1:100, Novus Biologicals, Catalog number: NB100-134) and Rabbit anti-VEGF (dilution 1: 100, Thermo Scientific, Catalog number: ab-222-P) were used for the staining of HIF-1 α and VEGF, respectively. Briefly, tumor-bearing mice (n=3/group) were treated i.t. with A-MD NPs or saline (control). After pre-determined times animals were sacrificed and tumor tissues were harvested and fixed with 10% neutral buffered formalin solution (Sigma Aldrich, USA) for histological analysis. Tumor tissue preparation and analysis were performed by the CMHD Pathology Core laboratory at Mount Sinai Hospital, Toronto. Slides were scanned with a NanoZoomer 2.0 RS whole slide scanner (Hamamatsu, Japan) and images were analysed with Visiopharm 4.4.4.0 software.

[00153] *In vivo* ionizing radiation treatment: Solid tumor of EMT6 murine breast cancer cells were grown orthotopically in Balb/c mice. Mice were divided into 4 groups (n=3/group): 1) Saline, 2) Saline + RT, 3) A-MD NP, 4) A-MD NP + RT. Treatments were initiated when the tumors reached an approximate volume of 100 mm³. The mice were restrained in a specially designed acrylic box, and the tumors were irradiated locally with 10 Gy, 30 min after i.t administration of saline and A-MD NP. The tumor size was measured as a function of time with vernier calipers in two dimensions and tumor volumes were

calculated by the formula $V = [(length) \times (width)^2]/2$. At the end of experiment, the animals were sacrificed and the tumor masses were excised and weighed. Tumor tissue was also formalin fixed and stained with terminal deoxynucleotidyltransferase/UTP nick end labelling (TUNEL) and haematoxylin and eosin (H&E) to determine percent apoptosis and necrosis.

[00154] In another set of experiments, mice were sacrificed 24 hours after radiation treatment. Tumor tissue was excised, formalin fixed, sectioned and gamma H2AX measured to evaluate DNA DSBs. Slides were scanned with a NanoZoomer 2.0 RS whole slide scanner (Hamamatsu, Japan) and images were analysed with Visiopharm 4.4.4.0 software.

[00155] The enhancement of DNA DSBs due to the combination of RT + A-MD NP was also evaluated in a dorsal skin-fold window chamber (DSWC) EMT6 mouse model.⁴⁴ Treatments (Saline and A-MD NP) were injected i.t. and only half of the chamber was irradiated at 10 Gy. Irradiating only half of the chamber allowed the inventors to determine the effect of treatment alone in the same mice. 24 hours post irradiation tumor tissue was excised and stained with gamma H2AX staining to evaluate DNA DSBs. Slides were scanned and images were analysed with Visiopharm 4.4.4.0 software.

[00156] **Statistical Analysis:** Data are presented as mean $\pm$ standard error of the mean for results obtained from three independent trials unless otherwise indicated. Student's t-test or analysis of variance (ANOVA) followed by Tukey t-test (OriginPro8[®]) were utilized to determine statistical significance between two or more groups, respectively. p-values < 0.05 were considered statistically significant.

Example 4: Preparation and characterization terpolymer crosslinked denatured albumin-complexed MD NPs with or without PEG (TER-MD)

[00157] A-MD NPs were denatured and crosslinked with a terpolymer (TER) (poly(methacrylic acid)-polysorbate 80-g-starch), prepared as previously described⁵⁶. BSA was denatured by heating and ultrasonication of A-MD NPs (80°C, 100 Hz), to which activated TER polymer was added to crosslink the protein matrix through covalent reaction of the carboxylic groups of the TER polymer with the amine groups of the protein. In brief, TER polymer (50 mg) was dissolved with 1 mL of DDI water and 15 mg of both N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were added. The solution was stirred for 30 min at room temperature for activation of the carboxylic groups of the TER polymer. Meanwhile, in a 14

1 mL polypropylene tube (falcon tube) 10 mL of polyelectrolyte pMD NPs solution (10 mM) was mixed with 1.2 mL of BSA solution in normal saline (10 mg/mL) and 50 μ L of Pluronic® F-68 solution (100 mM in DDI water). The solution in a falcon tube was transferred to a water bath at 80°C and kept under stirring and sonicated for 60 sec by using an ultrasonic processor probe operating at 100 Hz (Heischer UP100H, Germany). The mixture was removed from the hot water, to which 500 μ L of activated TER polymer solution was added and the dispersion was sonicated for another 5 min. To obtain TER-MD NPs with a PEG corona, a mixture of melted lipid-PEG (polyoxyethylene stearate) of two different chain lengths (e.g. 8mg of 5kDa and 4 mg of 2kDa) was added prior to adding the crosslinker, i.e., activated terpolymer. The obtained brown dispersion was washed with DDI water using ultracentrifugation (14k rpm for 20min) for three times. The obtained pellets were then dispersed with 1 mL of normal saline solution by ultrasonication. About 85-90% of the pMD NPs was loaded in the TER crosslinked denatured BSA matrix, as determined by measuring remaining pMD in the supernatant after washing using UV-Vis spectrophotometry. A typical preparation resulted in a $\approx$ 7 mM TER-MD NP emulsion. The schematic summarizing the preparation of the TER-MD NPs is shown in Fig. 13. Obtained NPs were negatively charged (-35 mV) with average size around 150 nm (Fig. 14a). TEM pictures revealed particles with a popcorn-like shape, with several pMD-BSA complex nanoparticles entrapped in one TER-MD NP (Fig. 14b).

Example 5: Covalent conjugation of oleic acid to the surface of MD nanoparticles (transfer to hydrophobic phase of MD NPs)

[00158] Hydrophilic, pMD NPs were transferred to a hydrophobic phase by covalently linking the hydrophobic molecule oleic acid to the surface of pMD NPs. For this purpose amine groups of poly(allylamine hydrochloride) (PAH) polyelectrolyte present on the surface of pMD NPs were covalently attached with carboxylic group of oleic acid using N,N'-Dicyclohexylcarbodiimide (DCC) carbodiimide reagent chemistry for cross-linking. Briefly, 150 mg of oleic acid (0.5 mole) was dissolved in 5 mL of dimethylformamide (DMF) and in a separate vial 110 mg of DCC (0.5mmole) was dissolved in 5 mL of DMF. Once DCC solution was cleared it was poured in oleic acid-DMF solution under stirring. The mixture was left under stirring for 15 minutes, after the required time, 500 μ L of pMD NP aqueous solution (30 mM based on ICP analysis) was added slowly under vigorous stirring. After 5–

1 10 minutes big aggregates were observed, at this stage 5 mL of chloroform was added and a
2 clear dispersion of oMD NPs was obtained (Fig. 15a). The solution was left under stirring
3 over night for complete reaction of oleic acid with pMDNPs. Next day, for oMD NPs
4 separation, reaction solution was mixed with acetone in a volume ratio of 1:3 (reaction
5 mixture: acetone), after addition of acetone big aggregates of oMD NPs were observed which
6 were then separated by centrifugation at 4k rpm. The precipitate was re-dissolved in
7 chloroform and once again washed using the acetone to remove by products of reaction.
8 Finally, precipitate was re-dissolved in chloroform and stored at 4°C. Oleic acid conjugate-
9 pMDNPs (oMD) were stable and no aggregates were observed even after months (Fig. 15b).
10 The obtained hydrophobic oMD NPs were characterized using the UV- absorbance
11 spectrometer, an absorbance peak was observed with a sharp absorption maximum at 280 nm
12 which corresponds to the oleic acid (Fig. 15c).

13

14 **Example 6: Preparation and characterization of PEG-lipid matrix (L-MD)**
15 **nanoparticles loaded with oMD nanoparticle**

16 [00159] The oMD NPs loaded lipid based nanoparticles were prepared using myristic
17 acid, polyoxyethylene stearate and polyvinyl alcohol. A schematic diagram illustrating the L-
18 MD formulation is shown in Fig.16, which can be generalized as a two-step process as
19 follows: step one - pMD NPs are transferred to an organic phase by covalent conjugation
20 with oleic acid to make oMD NPs; and step two - oMD NPs are loaded into a lipid and PEG
21 based matrix. Briefly, a mixture of 10 mg of myristic acid, 2 mg of
22 polyoxyethylene(40)Stearate (Myrj 52) and 3 mg of polyoxyethylene (100) Stearate (Myrj
23 59) were dissolved in 50 µl chloroform, the solution was then poured into 100 µL of oMD
24 NPs suspension (containing 15 mM MD based on ICP analysis). In a 15 mL conical tube 1
25 mL of 0.25 wt % polyvinyl alcohol (PVA) was heated at 55 °C using a water bath, after 5
26 min the mixture containing myristic acid, Myrj 59, Myrj 52 and oMD NPs was emulsified
27 and sonicated for another 5 min at 100% peak amplitude and 5 mm probe depth using a
28 Hielscher UP100H probe ultrasonicator (Hielscher USA, Inc., Ringwood, NJ, USA). After 5
29 mins., the suspension was immediately transferred to 0.5 mL of ice-cold 0.25 wt % polyvinyl
30 alcohol solution under stirring and gently purged with N₂ for 30 min to remove chloroform.
31 Finally, the emulsion was left under low vacuum to confirm the complete removal of organic
32 solvent. To confirm the loading of oMD NPs, obtained L-MD NPs were centrifuged at

1 14000rpm for 1 hour and trace of oMDnanoparticles in the supernatant was determined by
2 UV-absorption spectrum. There was no absorbance peak detected, confirming ~100% loading
3 of oMD NPs into the lipid nanoparticles.

4 **[00160]** For *in vitro* studies, fluorescein amine labelled myristic acid was used for L-
5 MD NPs preparation. For this purpose fluorescein amine dye was covalently linked with
6 myristic acid using the N,N' Dicyclohexylcarbodiimide (DCC) carbodiimide reagent
7 chemistry for cross-linking carboxylic acid of myristic acid with primary amine of
8 fluorescein. The reaction was performed by mixing the myristic acid, fluoresceinamine and
9 DCC at a molar ratio 1:0.5:1 (myristic acid:fluoresceinamine:DCC). Briefly, 100 mg of
10 myristic acid dissolved in chloroform was mixed with 80 mg of DCC dissolved in chloroform
11 under stirring, after 5 min 70 mg of fluoresceinamine dissolved in 1 mL of tetrahydrofuran
12 (THF) was added and the solution was left under stirring overnight. The reaction mixture was
13 protected from light to avoid bleaching of the fluorescent dye. The mixture was then washed
14 with DDI water following the extraction technique; the organic phase containing the
15 fluorescein-labelled myristic acid was separated from the aqueous phase using a separation
16 funnel. The washing step was repeated until no trace of free fluorescein was seen and clear
17 DDI water layer was observed. At the end of washing, the organic layer containing the dye-
18 labeled myristic acid was collected and dried under vacuum for 24 hours. In the last step, the
19 obtained powder was lyophilized over night to remove any traces of water.

20 **[00161]** For *in vivo* studies, hydrophobic near infra-red (NIR) dye Indocyanine green
21 (ICG) was co-loaded with oMD NPs in the lipid matrix. 20 μ L of 50mM ICG in methanol
22 was mixed with myristic acid, oMD NPs, Myrj 59 and Myrj52 and emulsified with 0.25 wt %
23 PVA, the L-MD NPs were then prepared using the same method as described above.

24 **[00162]** The obtained L-MD NPs were characterized using zeta sizer to confirm the
25 size distribution and surface charge of nanoparticles (Fig. 17b). The results revealed a size
26 distribution of 50-250 nm with negative surface charge of -26 mV. L-MD NPs were also
27 analyzed using transmission electron microscope (TEM) to confirm the size distribution and
28 morphology of particles (Fig. 17a). TEM analysis revealed particles with spherical shape with
29 smooth surface morphology. Results obtained from TEM analysis were in well agreement
30 with zeta size measurements. The stability of L-MD NPs was also examined in α -medium
31 with different FBS concentration (10-50%) and in pH 7.4 weak buffer (i.e. PBS) in the

1 presence of H₂O₂ (Fig. 17c). These results showed the L-MD NPs were highly stable under
2 physiological conditions.

3
4 **Example 7: Preparation and characterization of amphiphilic polymer matrix loaded**
5 **with oMD NPs (PMA-MD NPs)**

6 **[00163]** An amphiphilic polymer (PMA) with hydrophilic back bone and hydrophobic
7 side chains was synthesized by reacting poly(isobutylene-alt-maleic anhydride) (~6,000 Da)
8 with hexadecylamine. In a typical reaction, 75% of anhydride rings of the polymer were
9 linked with hexadecylamine through amide linkage between anhydride and NH₂ groups of
10 polymer and hexadecylamine respectively. As molecular weight of a single monomer of
11 poly(isobutylene-alt-maleic anhydride) was 154.14 g/mole (C₄H₂O₃ · C₄H₈) the total number
12 of monomer units for 6000Da polymer molecule were 39. In general, 1.028g of
13 poly(isobutylene-alt-maleic anhydride) (0.17 mmol = 6.6 mmol monomers) was placed in
14 round flask, in a separate glass vial 1.21 g of hexadecylamine (5 mmol) was dissolved with
15 30 mL of anhydrous tetrahydrofuran at 50°C and then quickly poured to and vigorously
16 mixed with the polymer powder. The cloudy mixture was sonicated for several seconds (e.g.
17 30 s) and then kept at 60 °C under vigorous stirring. The initial cloudy solution turned clear
18 after 10 minutes confirming the reaction of maleic anhydride rings with NH₂ groups. In
19 order to increase the reaction efficiency between maleic anhydride and primary amine, after 1
20 hour, the reaction mixture was concentrated roughly up to one fifth of the original volume by
21 evaporating the solvent using a rotavapor system. The concentrated solution was left stirring
22 overnight at 50 °C. The solvent was slowly evaporated until the polymer was completely dry
23 (pale yellow solid). Finally, the resulting polymer was re-dissolved in anhydrous chloroform
24 giving the final concentration of 200 mg/mL.

25 **[00164]** Hydrophobic oMD NPs were loaded in amphiphilic polymer PMA using oil in
26 water emulsion method. A schematic diagram illustration the step for NPs formulation is
27 shown in Fig 7. Briefly, 50 µL of PMA (200 µg/mL), 50 µL of oMD NPs (30 mM MD
28 based on ICP analysis) dissolved in chloroform, 3 mg of Myr59 and 1 mg of Myrj 52
29 dissolved in 50 µL of chloroform were mixed and emulsified in 1 mL of 0.25 wt % polyvinyl
30 alcohol at 48 °C. The emulsion was sonicated for 5 min using a probe sonicator at 100%
31 peak amplitude and 5 mm probe depth using a Hielscher UP100H probe ultrasonicator
32 (Hielscher USA, Inc., Ringwood, NJ, USA). After required time, the mixture was transferred

1 to ice cold 0.25 wt % polyvinyl alcohol solution under stirring and gently purged with N₂ for
2 30 min to remove chloroform. Finally the PMA-MD NPs suspension was left under low
3 vacuum to facilitate complete removal of organic solvent. To confirm the loading efficiency
4 of oMDNPs, obtained PMA-MD particles were centrifuged at 14k rpm for 1 hour and trace of
5 nanoparticles in the supernatant was determined by UV-absorption spectrum. There was no
6 absorbance peak detected, confirming ~100% loading of oMD NPs in the PMA-MD
7 nanoparticles.

8 **[00165]** For *in vitro* studies, fluorescein amine modified PMA was used to synthesize
9 PMA-MD NPs. For *in vivo* studies, hydrophobic near infra-red (NIR) dye Indocyanine green
10 (ICG) was co-loaded along with oMDNPs in the core of PMA-MD NPs. For this purpose 20
11 µL of 50 mM ICG in methanol was mixed with oMD NPs, PMA, Myrj 59, Myrj52 and
12 emulsified with 0.25 wt % PVA, the PMA-MD NPs were then obtained using the same
13 protocol as described above.

14 **[00166]** The obtained particles were characterized using zeta sizer to measure the
15 particle size and surface charge. The results confirmed particle size of 50-250nm with
16 negative surface charge of -27mV (Fig. 18a). The particles were also analyzed under
17 transmission electron microscope (TEM), to examine the particle size and morphology of
18 NPs (Fig. 18b). The TEM analysis revealed smooth and spherical morphology of the PMA-
19 MD NPs. The colloidal stability of the PMA-MD NPs was examined in α-medium with
20 different FBS concentration (10-50%) and in pH 7.4 weak buffer (i.e. PBS) in the presence of
21 H₂O₂ (Fig. 18c). The results showed that the PMA-MD NPs were highly stable under
22 physiological conditions.

24 **Example 8: *In vitro* oxygen generation and pH change measurements without cells**

25 **[00167]** Oxygen (O₂) generated by MD containing NPs and pH changes was measured
26 in a semi-sealed chamber coupled with a MI-730 micro-oxygen electrode and a MI-407 pH +
27 MI 402 reference microelectrodes (Microelectrodes Inc, USA), at 37°C. In brief, in a small
28 glass vial a pre-determined aliquot of NPs was added to 3 mL of saline or αMEM cell
29 medium containing 10% FBS to give various MD concentrations (10-150 µM). The vial was
30 sealed with parafilm and pierced with the probes and two hypodermic needles for gassing, as
31 represented in the schematic in Fig. 19. The vial was placed in a water bath with temperature
32 set to 37°C and bubbled with N₂ for 30 min to remove dissolved O₂. When oxygen levels

reached concentration close to zero (as indicated by the O₂ probe), 7.5 µL of a H₂O₂ solution in DDI water (100 mM) was injected to the solution to initiate O₂ generation. Oxygen generation and/or pH changes were monitored every 60 s using an Oakton pH 1100 (Thermo Fisher Scientific Inc., USA) coupled with O₂-ADPT Oxygen Adapter (Microelectrodes Inc., USA) for O₂ measurements. Electrodes were calibrated according to manufacturer's instructions. Saline or αMEM medium with 10% FBS was used as control.

[00168] The oxygen generation curves of different MD-containing NP formulations are compared in Fig. 20. All formulations (albumin, lipid and polymer-based) generated measurable amounts of oxygen at endogenous concentrations of H₂O₂. Significant amounts of O₂ (up to 0.20 mM within 30 min) was produced by the reaction of 100 µM of MD with 250 µM H₂O₂. The results showed that the kinetics of the MD reaction towards H₂O₂ and rates of oxygen generation can be controlled by the carrier. More hydrophobic PMA-MD and L-MD NPs loaded with MDNPs showed slower oxygen generation than the more hydrophilic albumin-containing A-MD and TER-MD NPs. These observations suggest that the MD NP formulations can be engineered for fast oxygen generation (e.g. A-MD and TER-MD) or for slow, prolonged oxygen generation (e.g. lipid-based PMA-MD and L-MD).

Example 9: Multifunctionality of A- MD nanoparticles in culture medium

[00169] The multifunctionality of the A-MD NPs to generate O₂ and to increase the medium pH *in vitro* upon reaction with H₂O₂ at endogenous levels was examined. The reaction between MD and H₂O₂ is a complex reaction leading to the decomposition of H₂O₂ and the production of O₂ as summarized in Fig. 5a. Besides the production of O₂, the reaction causes an increase in the local pH by the consumption of H⁺ ions and the production of an intermediate Mn-oxo-hydroxide (MnOOH)²⁶. This phenomenon can be particularly useful for the regulation of local pH in cancer cells and tumour tissue. Hence we studied if A-MD NPs would generate measurable amounts of oxygen and increase pH at low concentrations of H₂O₂ found in the human body (i.e., 100 µM and up to 1 mM)³³. It was observed that at a very low concentration (~45 µM of MD), the NPs were able to completely quench 1 mM H₂O₂ in cell medium within 40 minutes (Fig. 5b). Further investigation of the O₂ generating properties of the NPs used an in-house-made hypoxia-maintaining chamber coupled with both a commercially available oxygen probe and a pH microelectrode 9. Significant amounts of O₂ was produced (Fig. 5c) accompanied by an increase in the pH of physiological buffer

(phosphate/saline buffer) by one pH unit from pH 6.8 to pH 7.8 (Fig. 5d) by the reaction of 45 μM of MD with 250 μM H_2O_2 . In an attempt to simulate *in vivo* conditions where H_2O_2 is continuously generated by tumor cells, we measured the O_2 production by the NPs during the continuous addition of exogenous H_2O_2 (250 μM) into the chamber every 30 min. We observed that a single dose of the NPs (90 μM MD) continuously generated O_2 for at least 6 cycles of 30 min each (Fig. 5e). These results demonstrated that H_2O_2 and protons can diffuse rapidly across the polyelectrolyte-albumin complex, access the reactive sites of the MD cores, produce O_2 and increase pH in a sustained manner under hypoxic conditions.

Example 10: *In vitro* oxygen generation in presence of hypoxic cancer cells

[00170] O_2 generated by MD-containing NPs was measured in a semi-sealed chamber coupled with a MI-730 micro-oxygen electrode (Microelectrodes Inc, USA), at 37°C. In brief, in a small glass vial murine breast cancer EMT6 cells (10^5 cells per mL) were suspended and stirred in αMEM medium. The vial was plugged with rubber stoppers and pierced with the oxygen probe and two hypodermic needles for gassing, as represented in the schematic in Fig. 21a. The cell suspension was made hypoxic by introducing a mixture of 95% N_2 and 5% CO_2 for 20 min at 37°C. When oxygen levels reached concentration close to zero (as indicated by the O_2 probe), a small aliquot of the NPs was injected to make the MD concentration in the medium 100 μM and the oxygen levels monitored every 60 s using an Oakton pH 1100 (Thermo Fisher Scientific Inc, USA) coupled with O_2 -ADPT Oxygen Adapter (Microelectrodes Inc, USA). Electrode was calibrated according to manufacturer's instructions. αMEM medium with 10% FBS, with or without cells was used as control.

[00171] Fig. 21b shows that protein-based MD (A-MD) NPs incubated with hypoxic breast cancer cells reacted quickly with endogenous H_2O_2 produced by the cells under hypoxic stress, thus producing O_2 . Significant amounts of O_2 (~6-fold increase of O_2 levels in the medium) were detected within 2 min by reacting with H_2O_2 released by the cancer cells. These results indicate that the endogenous levels of H_2O_2 released by hypoxic cancer cells *in vitro* is sufficient to react with the NPs and generate measurable O_2 without addition of exogenous H_2O_2 to the culture medium.

Example 11: Cytotoxicity and cellular uptake of various MnO_2 NP formulations

1 [00172] The viability of human breast cancer MDA-MB 231 and murine breast cancer
2 EMT6 exposed to various concentrations of MD NPs for 48 h was evaluated. In brief, plated
3 cells (10 000 cells per well / 96 well plate) were incubated with different NP formulations
4 dispersed in α -medium at desired concentrations (i.e., for 50 and 100 μ M MnO_2). Cells were
5 incubated with NPs for 48 h Murine EMT6 breast tumor cells (10^5 cells) were incubated for
6 1h with MD NPs (45 μ M) at 37°C before microscopic analysis. Cell uptake of NPs by
7 transmission electron microscopy (TEM) was performed using a H7000 TEM microscope
8 (Hitachi, Japan), following standard methods for sample preparation. An EVOS fluorescence
9 microscope (AMG, USA) was used to image live cells following incubation with red
10 fluorescent dye labelled A-MD NPs. Cell nuclei were stained blue with HOESCHT 33342
11 (Invitrogen Molecular Probes, USA). The cellular uptake of NPs at various time points was
12 also evaluated. For this experiment, NPs were made fluorescent by conjugating fluorescein
13 isothiocyanate (FITC) to the NPs as previously described. Platted cells (500,000 cells per well/
14 6 well plate) were incubated with FITC labelled MD NPs dispersed in culture medium at the
15 concentration 100 μ M MD. Cells were incubated with NPs for several time points: 0.5, 1, 2, 4
16 and 24 h. After incubation time cells were washed twice with PBS, trypsinized and replated
17 in 96 well plate (10, 000 cells per plate). Fluorescence was measured with a microplate
18 reader, ex: 485 nm and em: 520 nm. These results are summarized in Fig. 6. Cell viability
19 studies showed that all formulations (A-MD, L-MD, TER-MD and PMA-MD NPs) are not
20 toxic to the cancer cells at MD concentrations used *in vitro* and *in vivo* (i.e., 100 μ M MD)
21 (Figs. 6a-c). It is known that the aberrant metabolism of cancer cells leads to significantly
22 elevated cellular concentrations of H_2O_2 . If the NPs could be taken up by cancer cells, they
23 could react quickly with endogenous H_2O_2 produced by cancer cells under hypoxic stress,
24 thus producing O_2 *in situ*. To test this hypothesis, the cellular uptake of the NPs was
25 determined by incubating EMT6 murine breast cancer cells with fluorescence-labelled A-
26 MD NPs, and observed significant cellular uptake of NPs within 60 min of incubation (Fig.
27 6d). This finding was confirmed by transmission electron microscopy (TEM). TEM images
28 (Fig. 6e) showed EMT6 cells *in vitro* underwent membrane invagination and engulfment of
29 the NPs and the NPs taken up by the cell were distributed within the cell cytoplasm and
30 vesicles after 1 h incubation. Different formulations showed similar cellular uptake profile
31 over the time for two different cancer cell lines (Figs. 6 f-g). Cellular uptake of the NPs was
32 observed within 60 min of incubation with EMT6 cells (Fig. 6f).

1

2 Example 12: Animal tumor models for *in vivo* studies

3 **[00173]** For *in vivo* studies, transplantable mouse mammary tumor cells EMT6 were
4 grown in eight-week old female Balb/c mice (Jackson Laboratory, Bar Harbor, Maine, USA).
5 For this purpose 1×10^6 EMT6 cells suspended in 30 μL of cell culture medium (αMEM ,
6 10% FBS) were inoculated in the mammary fat pad of mice. Briefly, for the breast tumor
7 model mice were anaesthetized with 1% isoflurane and injected with a pain killer
8 (buprenorphine, 0.05mg/kg), a 5mm incision was made in the skin over the lateral thorax,
9 exposing the mammary fat pad. A 30 μL volume of 1×10^6 EMT6 cells in cell culture medium
10 (αMEM , 10% FBS) was injected into the mammary fat pad using a 27-gauge needle.
11 Exposing the fat pad ensured the injection of cells into the fat pad and not into the
12 subcutaneous space. The incision was closed with sutures and surgical glue and the mice
13 were monitored during recovery until sternal recumbency was observed, at which point the
14 mice were returned to a clean cage. Mice were monitored daily for 2 days after tumor
15 implantation, and tumor growth was monitored weekly until reaching the desirable size,
16 approximately 100 mm^3 for irradiation experiments (about 2 weeks) and 300-400 mm^3 for
17 biodistribution studies (about 3-4 weeks). Tumors were implanted in one or two sides of the
18 animal, depending on the experiment. All animal experiments were performed in accordance
19 with guidelines and regulation of animal Care Committee at University Health Network
20 (AUP 2407). The animal care committee operates under the standards of animal care set forth
21 by the Canadian Council of Animal Care.

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23 Example 13: *In vivo* biodistribution of different MnO_2 NPs formulations

24 **[00174]** The *in vivo* biodistribution and tumor accumulation capacity of different
25 formulations was determined using EMT-6 grafted Balb/c mice following tumor growth to
26 volumes of 300 – 400 mm^3 . Once tumor reached the required size, 100 μL of formulation
27 such as TER-MD, L-MD or PMA-MD (1mM MD based on ICP analysis) was intravenously
28 injected through the tail vein of the animals. The NIR-labelled formulation were synthesized
29 by co-loading the near infra-red dye ICG (in case of L-MD and PMA-MD), whereas in case
30 of TER polymer NPs ICG was linked to the particles surface through electrostatic interaction.
31 After the injection, biodistribution and tumor uptake characteristics of the NPs were
32 monitored non-invasively for up to 24 hours using the Xenogen IVIS Spectrum Imaging

System (Caliper Life Sciences Inc., USA). During imaging, mice were anaesthetized with isoflurane, and whole body fluorescence images were acquired using 745 nm excitation and 820 nm emission filters. For all of the recorded images, the fluorescence intensity was equalized. For *ex vivo* biodistribution studies at 4 and 24 hours after injection, the animals were euthanized and the organs were collected. *Ex vivo* fluorescence images of whole organ was obtained using the Xenogen IVIS Spectrum Imaging System. The biodistribution and *ex vivo* images of the resected organs (Figs. 15 a-b) showed that NPs (lipid- and polymer-based) reached and accumulated in the tumor site within 1 h post i.v. injection and remained in the tumor during 24 h duration of the experiment.

Example 14: Effect of L-MD and TER-MD nanoparticle formulations on enhancement of radiation therapy

[00175] Solid tumors of EMT6 murine breast cancer cells were grown orthotopically in Balb/c mice. Cells were implanted on both sides and treatments were initiated when the tumors reached an approximate volume of 100 mm³. Mice were divided into 6 groups (n=3/group): 1) Saline, 2) Saline + RT, 3) TER-MD NPs, 4) TER-MD NPs + RT, 5) L-MD NPs, 6) L-MD NPs + RT. Tumors on both sides were treated with 50 µL intratumoral (i.t.) injection of different MD NP formulations (1mM MD in saline) or saline (control). The mice were restrained in a specially designed acrylic box, and the tumors from only one side were irradiated locally with 10 Gy, 30min post i.t. injections. In one experiment, mice were sacrificed 24 h post-treatment and tumors were resected, formalin fixed and analyzed for: histopathology analysis (H&E), apoptosis (using terminal deoxynucleotidyltransferase UTP nick end labelling (TUNEL), cell proliferation (Ki-67), and DNA double-strand breaks (DNA DSB) (anti-gamma-H2AX antibody).

[00176] In another experiment, the tumor size was measured as a function of time with vernier calipers in two dimensions and tumor volumes were calculated by the formula $V = \frac{[(\text{length}) \times (\text{width})^2]}{2}$. At the end of experiment (i.e., 5 days post-treatments), the animals were sacrificed and the tumor masses were excised and weighed. Tumor tissue was also formalin fixed and stained for: histopathology analysis (H&E). Tumor tissue preparation and analysis were performed by the CMHD Pathology Core laboratory at Mount Sinai Hospital, Toronto. Slides were scanned with a NanoZoomer 2.0 RS whole slide scanner (Hamamatsu, Japan) and images were analysed with Visiopharm 4.4.4.0 software.

1 [00177] A significant change in tumor volume was observed in mice treated with the
2 combination of NPs and RT compared to saline control groups. The combination of TER-MD
3 NPs + RT and L-MD NPs + RT led to tumor regression of -31% and -87% of initial tumor
4 volume, respectively (Fig. 23a). Moreover, L-MD NPs alone led to a tumor volume
5 regression of about -44% (Fig. 23b). Tumor weight was also significantly lower in the NPs +
6 RT groups (Fig. 24a). Decreased tumor weight was observed in the L-MD NP alone treated
7 group compared to saline and TER-MD groups (irradiated and non-irradiated). Visual
8 analysis of resected tumors also showed smaller, less vascularized and less inflammatory
9 tumors in the NP + RT treated groups than saline group (Figs. 24 b-c).

10 [00178] Histopathology analysis of resected tumor tissue revealed that the combination
11 of NPs with irradiation led to significant increase in tumor necrosis as compared to the
12 control group (Fig. 25). Tumors treated with L-MD or TER-MD NPs + single irradiation
13 dose (10 Gy) showed massive tumor necrosis (80-85% and 90-95% of the total tumor area,
14 respectively), while saline + RT group showed only 50-60% tumor necrosis 5 days post-
15 treatment. For L-MD + RT treated group, only the fibrous capsule of the tumor was left 5
16 days post-treatment, indication of almost total regression of the tumor. Also, treatment with
17 L-MD NPs alone led to similar tumor necrosis as saline + RT group, about 60% of the total
18 tumor area.

19 [00179] The percentage of tumor apoptotic areas was also determined by TUNEL
20 staining. Tumors treated with L-MD NPs + RT showed a four-fold increase in cell apoptosis
21 compared to the control (saline + RT) group. Moreover, tumors treated with L-MD NPs alone
22 showed comparable cell death to the saline + RT group.

23 [00180] The immediate effect of the NP formulations on the irradiation-induced cell
24 kill was determined by measuring radiation-induced DNA damage 24 h post-treatment by
25 using gamma H2AX staining to stain for DNA DSBs. These results showed that combined
26 treatment with TER-MD NPs and irradiation resulted in increased DNA damage (35%)
27 compared to the saline control with irradiation (20%) in the EMT6 solid tumor (Fig. 26a).
28 This enhanced effect can be attributed to the fast oxygen generation properties of the
29 hydrophilic formulation.

30 [00181] The effect of the treatments on the cell proliferation was determined by
31 staining using the cell proliferation marker Ki-67. In all groups, irradiation treatment
32 consistently led to decreased cell proliferation compared to respective non-irradiated groups

(Fig. 26b). However, the combination of NP treatment and RT led to an even higher decrease in cell proliferation. Treatment with TER-MD NPs decreased cell proliferation from 20% to 5% 24 h post-irradiation, while saline-treated group went from 18% to 11% cell proliferation 24 h post-irradiation. Interestingly, decreased cell proliferation was observed following treatment with L-MD NPs alone when compared to the saline control (15%).

Example 15: Safety of MD nanoparticles formulations

[00182] EMT6 murine breast cancer cells were grown orthotopically in Balb/c mice. Mice were divided into 3 groups (n=3/group): 1) Saline, 2) TER-MD NPs and 3) L-MD NPs. Treatments were initiated when the tumors reached an approximate volume of 300 mm³. The mice received daily 100 µL intravenous injections of the treatments (1 mM MD in saline or saline) for 7 consecutive days. At the end of experiment, the animals were sacrificed and the organs (lungs, heart, kidneys, spleen and liver) were resected, formalin fixed and H&E for histopathology analysis.

[00183] The safety of the NPs to healthy tissue was evaluated through histological analysis of the organs. There was no histological evidence of accumulated toxicity in the different organs associated with i.v. administration of TER-MD and L-MD NPs once daily for 7 days, demonstrating the excellent *in vivo* biocompatibility of these NP formulations.

Example 16: MD NPs as radiosensitizers in prostate cancer cell lines and *in vivo* human prostate cancer model

[00184] *In vitro* studies using the human PC-3 prostate cancer cell line were conducted. PC-3 cells were cultured in alpha-MEM media supplemented with 10% FBS. The cells were plated in 96-well plate (1000 cells/ well) and incubated for 6 hours at a temperature of 37°C and 5% CO₂. Cells in hypoxia group were then incubated in hypoxic work station (HypOxystation H35, HypOxygen, MD) at 37°C and 0.2% O₂ for 18 hours; cells in normoxia group were left in the 5% CO₂ incubator for a total of 24 hours. On the second day, the cells were exposed to MD NPs (100 µM, suspended in saline) either in hypoxia chamber or in normoxia for 1 hour, followed by irradiation (X-Rad 320, Precision X-ray Inc.). The cells were washed with PBS and fresh media was added to the wells 1 hour after irradiation. MTT assay was performed according to the established protocol 24 hours following irradiation.

1 [00185] In normoxia, PC-3 exhibited moderate radiosensitivity as reported in
2 literature; on the other hand, exposure to hypoxia (0.2% O₂ for 18 hours) resulted in
3 radioresistance (up to 5 Gy) (Fig. 27a). In all irradiated groups, cells that were treated with
4 MD NPs showed lower viability compared to the cells without the MD NPs, demonstrating
5 the radiosensitizing effect of the MD NPs. There were no significant differences in cellular
6 response to TER-MD and L-MD (Fig. 27a). Notably, radioresistant (hypoxic) cells exposed
7 to MD NPs treatment were sensitive to irradiation (60 % cell viability with 5 Gy irradiation),
8 suggesting that the MD NPs could overcome cellular radioresistance induced by hypoxia.
9 Data were normalized to the control group (0 Gy, no nanoparticles) for both normoxia and
10 hypoxia group.

11 [00186] Human PC-3 prostate tumors were established by subcutaneous injection of 2
12 $\times 10^6$ PC-3 cells at abdominal side of SCID mice. After about four weeks, when tumor
13 volume reached $\sim 100 \text{ mm}^3$, the mice were divided into 2 groups: 1) Saline + RT and 2) L-
14 MD NP + RT and the treatments were initiated. The mice were restrained in a specially
15 designed box of acrylic polymer, and the tumors were irradiated locally at 10 Gy, 30 min
16 after i.t administration 50 μL of saline (control) or 1 mM MD NPs in saline. The tumor size
17 was measured with a Vernier caliper in two dimensions and tumor volumes were calculated
18 by the formula $V = [(\text{length}) \times (\text{width})^2]/2$. At the end of experiment, the animals were
19 sacrificed and the tumor masses were excised and weighed. Tumor tissue was fixed in
20 formalin and H&E stained to determine percent necrosis.

21 [00187] Histopathology analysis of resected tumor tissue revealed that the combination
22 of the L-MD NPs with irradiation led to significant increase in tumor necrosis as compared to
23 the control group. Tumors treated with L-MD NPs + RT at a single dose of 10 Gy showed
24 significant tumor necrosis, while the tumors in the saline + RT group showed no histological
25 evidence of necrosis 5 days post-treatment (Fig. 27b).

26 [00188] The *in vivo* biodistribution and tumor accumulation ability of L-MD
27 nanoparticles was determined using human PC3-grafted SCID male mice. In detail, once the
28 tumor reached a volume of 400- 500 mm^3 , 100 μL of L-MD nanoparticles (1mM MnO₂ based
29 on ICP analysis) synthesized by co-loading the hydrophobic near infrared dye indocyanine
30 green was injected intravenously. Mice were anaesthetized with isoflurane and at different
31 time points, whole body fluorescence images were acquired using 745 nm excitation and 820
32 nm emission filter under Xenogen IVIS Spectrum Imaging System (Caliper Life Sciences

1 Inc., USA). Biodistribution of L-MD nanoparticles using *ex vivo* analysis was also
2 determined. For *ex vivo* biodistribution studies, animals were euthanized and different organs
3 collected 24 hours post-nanoparticle injection. *Ex vivo* fluorescence images of organs were
4 then obtained using the Xenogen IVIS Spectrum Imaging System using 745nm excitation and
5 820 emission filter. From whole body real-time imaging, tumor uptake of L-MD
6 nanoparticles was easily determined, and 24 hours post-injection, tumor was easily delineated
7 from the surrounding healthy tissue (Fig. 27c). The *ex vivo* optical data of excised tissue
8 showed a significant uptake of L-MD nanoparticles by tumor 24 hours post-particle
9 administration (Fig. 27d). In Figs. 27c and 27d, lighter shade indicates higher fluorescence
10 emission.

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CLAIMS

We claim:

1. A multifunctional nanoparticle composition comprising a coated nanoparticle embedded in a matrix, the coated nanoparticle comprising a metal oxide nanoparticle and a functional coating on the surface of the metal oxide nanoparticle.
2. The composition of claim 1, wherein the matrix is selected from the group consisting of organic groups, inorganic compounds, proteins, nucleic acids, polymers, lipids and the mixtures thereof.
3. The composition of claim 1, wherein the coated nanoparticle embedded in the matrix is nanoparticulate.
4. The composition of claim 1, wherein the coated nanoparticle embedded in a matrix further comprises a functional moiety.
5. The composition of claim 4, wherein the functional moiety is selected from the group consisting of a targeting moiety, a detectable moiety, a radioisotope, a radionuclide, a labeling agent, an imaging agent and the mixtures thereof.
6. The composition of claim 1, wherein the metal oxide nanoparticle is a manganese dioxide nanoparticle (MD NP).
7. The composition of claim 1, wherein the functional coating is selected from the group consisting of a biocompatibility coating, a colloidal coating, an organic coating, an inorganic coating, a hydrophilic coating, and the mixtures thereof.
8. The composition of claim 1, wherein the functional coating comprises a first layer.
9. The composition of claim 8, wherein the first layer is poly(allylamine hydrochloride) (PAH).
10. The composition of claim 9, wherein the coated nanoparticle is loaded into the matrix.

11. The composition of claim 10, wherein the matrix comprises bovine serum albumin (BSA).
12. The composition of claim 11, wherein the multifunctional nanoparticle composition is A-MD NP (Formulation #1).
13. The composition of claim 12, wherein the A-MD NP is about 20 nm to about 1000 nm in diameter.
14. The composition of claim 10, wherein the matrix comprises a graft terpolymer (TER).
15. The composition of claim 14, wherein the TER is poly(methacrylic acid)-polysorbate 80-starch.
16. The composition of claim 15, wherein the TER is further crosslinked with a denatured BSA.
17. The composition of claim 14, wherein the multifunctional nanoparticle composition is TER-MD NP (Formulation #2).
18. The composition of claim 17, wherein the TER-MD NP is about 50 nm to about 1000 nm in diameter.
19. The composition of claim 10, wherein the matrix comprises a lipid-PEG (polyoxyethylene stearate).
20. The composition of claim 19, wherein the lipid-PEG comprises a chain of about 2kDa to about 10kDa.
21. The composition of claim 20, wherein the multifunctional nanoparticle composition is a PEG-TER-MD NP (Formulation #3).
22. The composition of claim 21, wherein the PEG-TER-MD NP is about 50 nm to about 1000 nm in diameter.
23. The composition of claim 10, wherein the matrix comprises an organic group.

24. The composition of claim 23, wherein the organic group is a carboxylic group of oleic acid.
25. The composition of claim 24, wherein the matrix comprises a lipid (L).
26. The composition of claim 24, wherein the lipid matrix comprises a myristic acid and a PEG-lipid.
27. The composition of claim 26, wherein the multifunctional nanoparticle composition is L-MD NP (Formulation #4).
28. The composition of claim 27, wherein the L-MD NP is about 50 nm to about 1000 nm in diameter.
29. The composition of claim 10, wherein the matrix comprises a grafted amphiphilic polymer (PMA).
30. The composition of claim 29, wherein the PMA is a ploy(isobutylene-alt-maleic anhydride-hexadecylamine).
31. The composition of claim 30, wherein the multifunctional nanoparticle composition is PMA-MD NP (Formulation #5).
32. The composition of claim 31, wherein the PMA-MD NP is about 50 nm to about 1000 nm in diameter.
33. The composition of claim 1, wherein the multifunctional nanoparticle composition is negative charged.
34. The composition of claim 33, wherein the negative charge is about -25 to about -40 mV.
35. The composition of claim 1, wherein the coated nanoparticle embedded in the matrix has a mean particle size of 10 nm to 1000 nm.
36. A method of preparing a multifunctional nanoparticle composition for the treatment of cancer or the enhancement of radiation directed to a tissue or a tumor, the method comprising the steps of

- (1) preparing a metal oxide nanoparticle,
- (2) treating the metal oxide nanoparticle of step (1) with a first functional layer to form a single-coated functional metal oxide nanoparticle; and
- (3) loading one or more single-coated functional metal oxide nanoparticles of step (2) in a matrix to form a particle loaded with one or more single-coated functional metal oxide nanoparticles.

37. The method of claim 36 further comprising a step of treating the particle loaded with one or more single-coated functional metal oxide nanoparticle of step (3) with a functional moiety to form a functional loaded particle.

38. The method of claim 36, wherein the metal-oxide nanoparticle is a manganese dioxide nanoparticle (MD NP).

39. The method of claim 36, wherein the first functional layer is selected from the group consisting of a biocompatibility coating, a colloidal coating, an organic coating, an inorganic coating, a hydrophilic coating, and the mixtures thereof.

40. The method of claim 36, wherein the matrix is selected from the group consisting of organic groups, proteins, nucleic acids, polymers, lipids and the mixtures thereof.

41. The method of claim 36, wherein the first layer is poly(allylamine hydrochloride) (PAH).

42. The method of claim 36, wherein the matrix comprises a carboxylic group.

43. The method of claim 42, wherein the carboxylic group is a carboxylic group of oleic acid.

44. The method of claim 36, wherein the matrix comprises an albumin.

45. The method of claim 44, wherein the albumin is bovine serum albumin (BSA).

46. The method of claim 36, wherein the matrix comprises a polymer.

47. The method of claim 43, wherein the polymer is graft terpolymer (TER).

48. The method of claim 47, wherein the polymer is a carboxylic group.
49. The method of claim 36, wherein the matrix comprises a lipid.
50. The method of claim 49, wherein the lipid is a melted lipid-PEG (polyoxyethylene stearate).
51. The method of claim 50, wherein the PEG comprises a chain of about 2 kDa to about 10 kDa.
52. The method of claim 50, wherein the lipid-based nanoparticle (L-MD NP) comprises a myristic acid and a PEG-lipid (polyoxyethylene stearate).
53. The method of claim 47, wherein the polymer is a grafted amphiphilic polymer (PMA).
54. The method of claim 36, wherein the multifunctional nanoparticle composition is a A-MD NP (Formulation #1).
55. The method of claim 54, wherein the A-MD NP is about 20 nm to about 1000 nm in diameter.
56. The method of claim 36, wherein the multifunctional nanoparticle composition is TER-MD NP (Formulation #2).
57. The method of claim 56, wherein the TER-MD NP is about 50-1000 nm in diameter.
58. The method of claim 36, wherein the multifunctional nanoparticle composition is PEG-TER-MD NP (Formulation #3).
59. The method of claim 58, wherein the PEG-TER-MD NP is about 50 nm to about 1000 nm in diameter.
60. The method of claim 36, wherein the multifunctional nanoparticle composition is L-MD NP (Formulation #4).
61. The method of claim 60, wherein the L-MD NP is about 50-1000 nm in diameter.

62. The method of claim 36, wherein the multifunctional nanoparticle composition is PMA-MD NP (Formulation #5).

63. The method of claim 62, wherein the PMA-MD NP is about 50 nm to about 1000 nm in diameter.

64. The method of claim 36 further comprising a step of preparing the multifunctional nanoparticle composition in a form suitable for intratumoral injection, peritumoral injections, or intravenous injection.

65. A method of treating cancer disease in a subject comprising the steps of:

administering an effective amount of a multifunctional nanoparticle composition according to any one of claims 1-35 to the subject,

applying ionizing radiation to the subject,

wherein the cancer disease is treated.

66. The method of claim 65, wherein the composition includes a pharmaceutically acceptable vehicle.

67. The method of claim 65, wherein the administration is intratumoral, peritumoral, or intravenous injection.

68. The method of claim 67, wherein the step (1) of administering the composition is prior to the step (2) of applying the radiation.

69. The method of claim 68, wherein the composition is intravenously injected to the subject about 1 to about 24 hours prior to the radiation.

70. The method of claim 68, wherein the composition is intratumorally injected to the subject about 5 minutes to about 3 hours prior to the radiation.

71. A method of enhancing a radiation directed to a tissue or a tumor in a subject comprising the steps of:

(1) administering an effective amount of a multifunctional nanoparticle composition according to any one of claims 1-35 to the subject, and

(2) applying radiation to the subject,

wherein the radiation is enhanced in the subject.

72. The method of claim 71, wherein the administration of the composition is intratumoral, peritumoral, or intravenous injection.

73. The method of claim 71, wherein the step (1) of administering the composition is prior to the step (2) of applying the radiation.

74. The method of claim 73, wherein the composition is intravenously injected to the subject about 1 hour to about 24 hours prior to the radiation.

75. The method of claim 73, wherein the composition is intratumorally injected to the subject about 5 minutes to 3 hours prior to the radiation.

76. Use of a multifunctional nanoparticle composition according to any one of claims 1-35 for the manufacture of a medicament for the treatment of cancer in a subject.

77. Use of a multifunctional nanoparticle composition according to any one of claims 1-35 for the manufacture of a medicament for the enhancement of a radiation directed to a tissue or a tumor in a subject.

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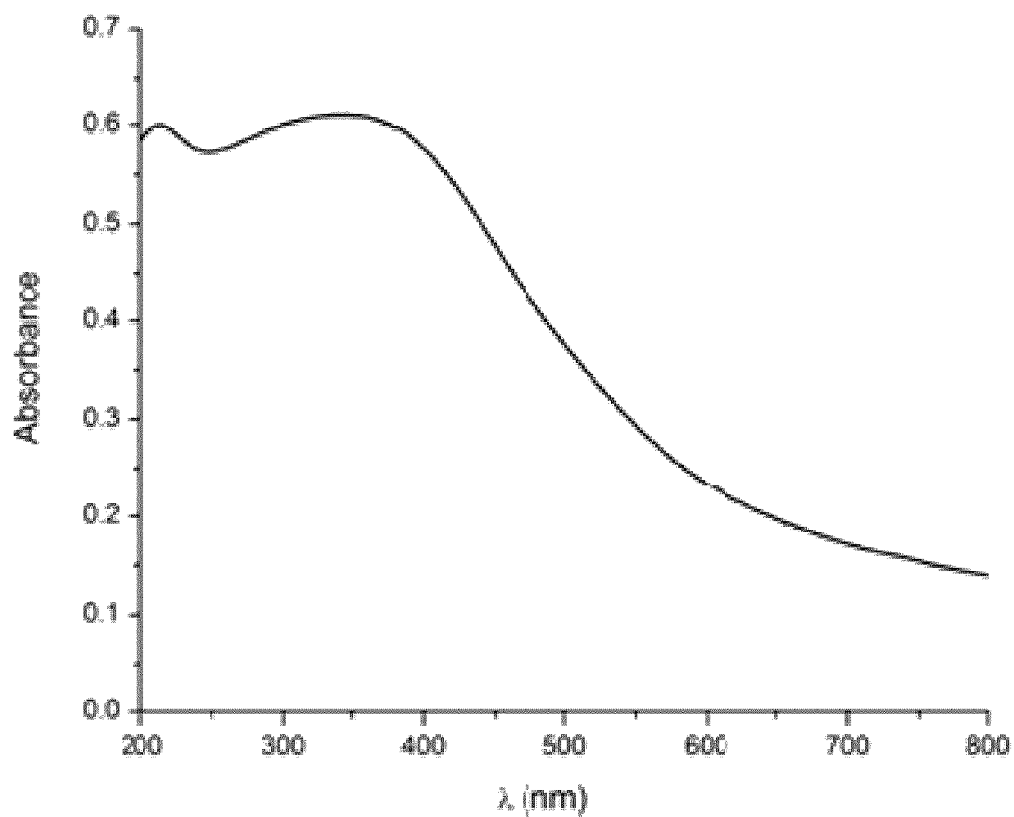


Fig. 1a

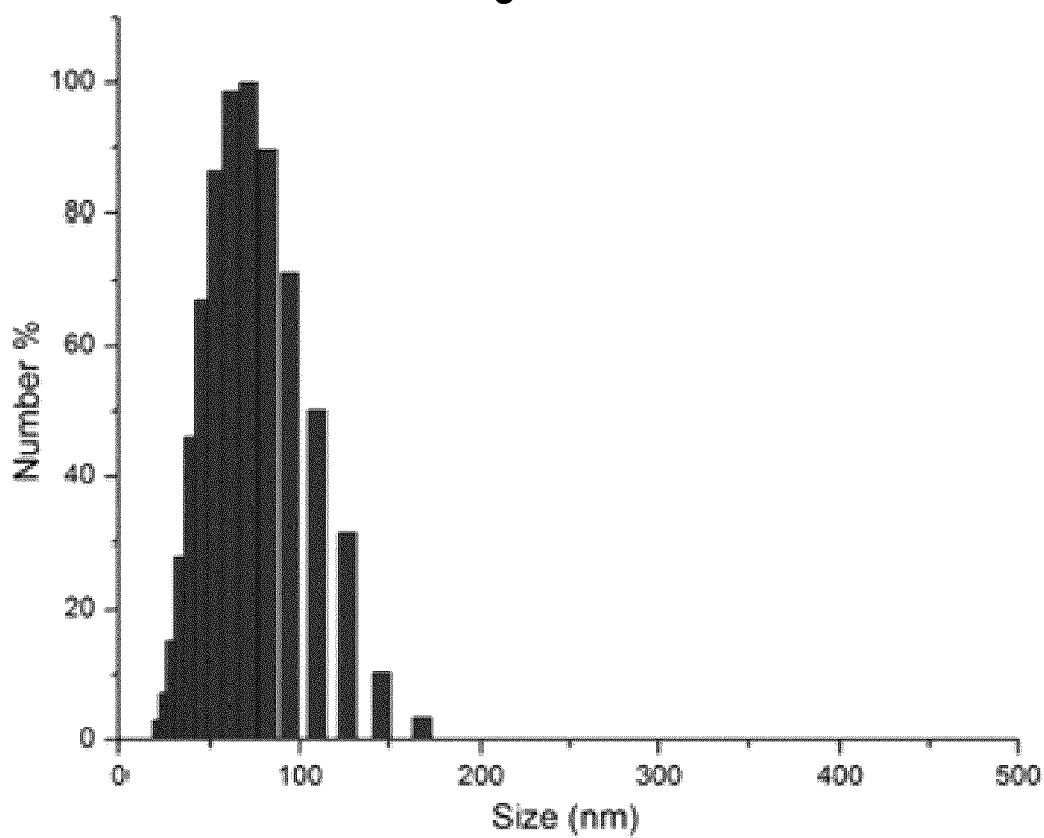


Fig. 1b

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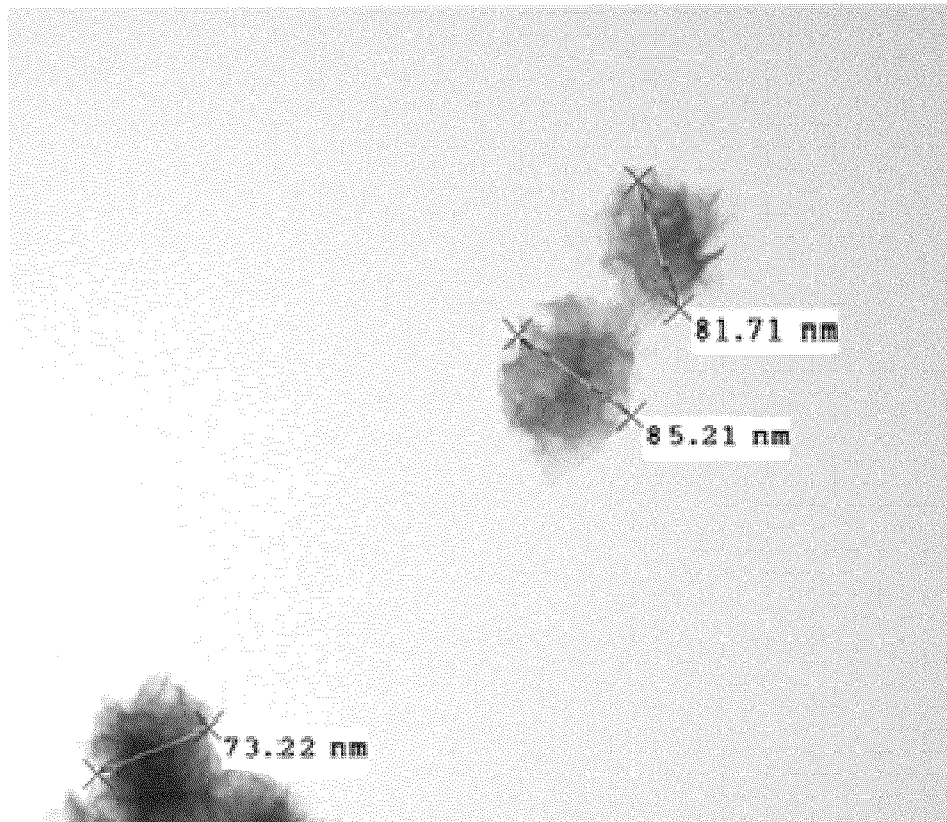


Fig. 1c

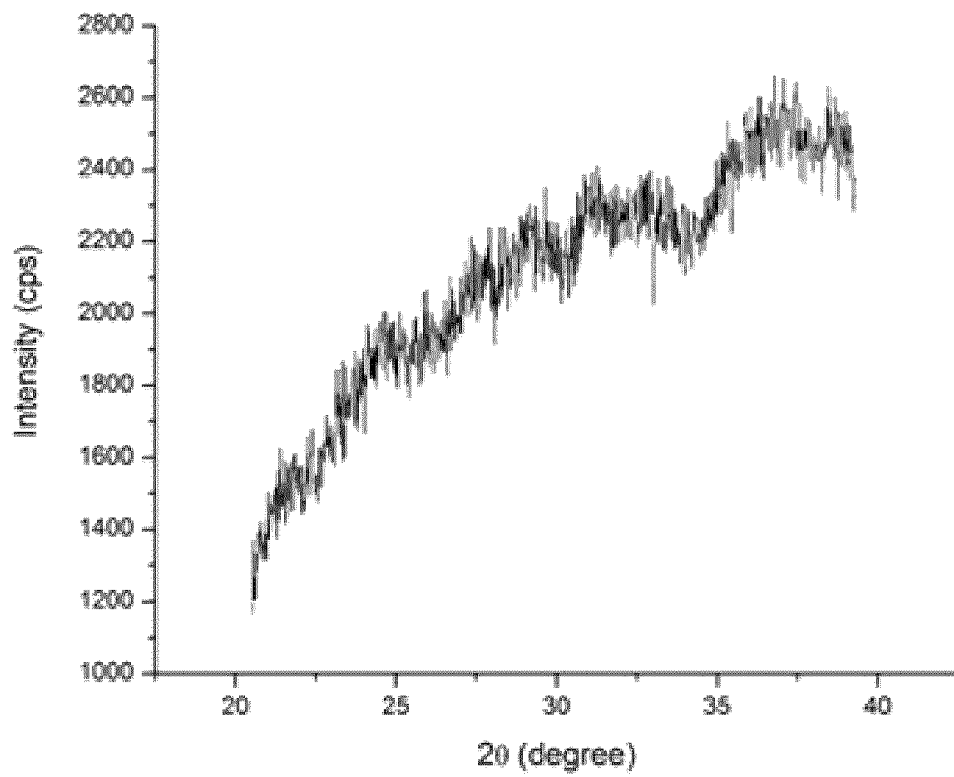


Fig. 1d

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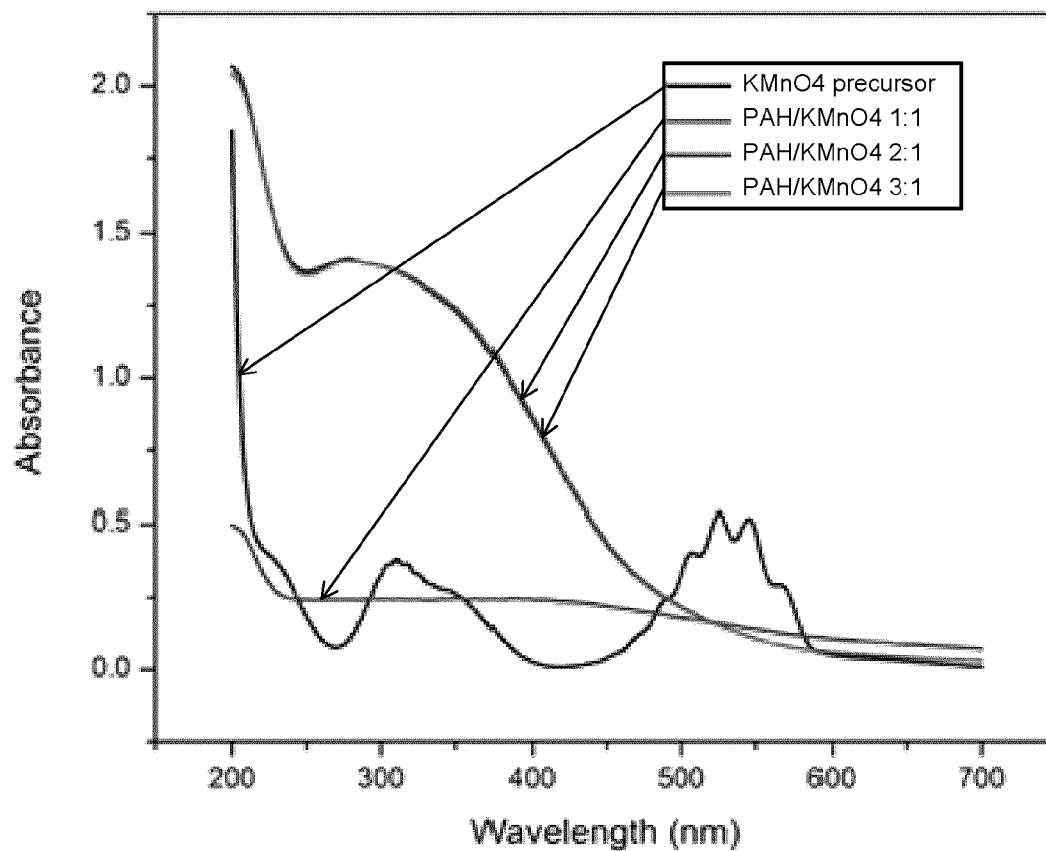


Fig. 2a

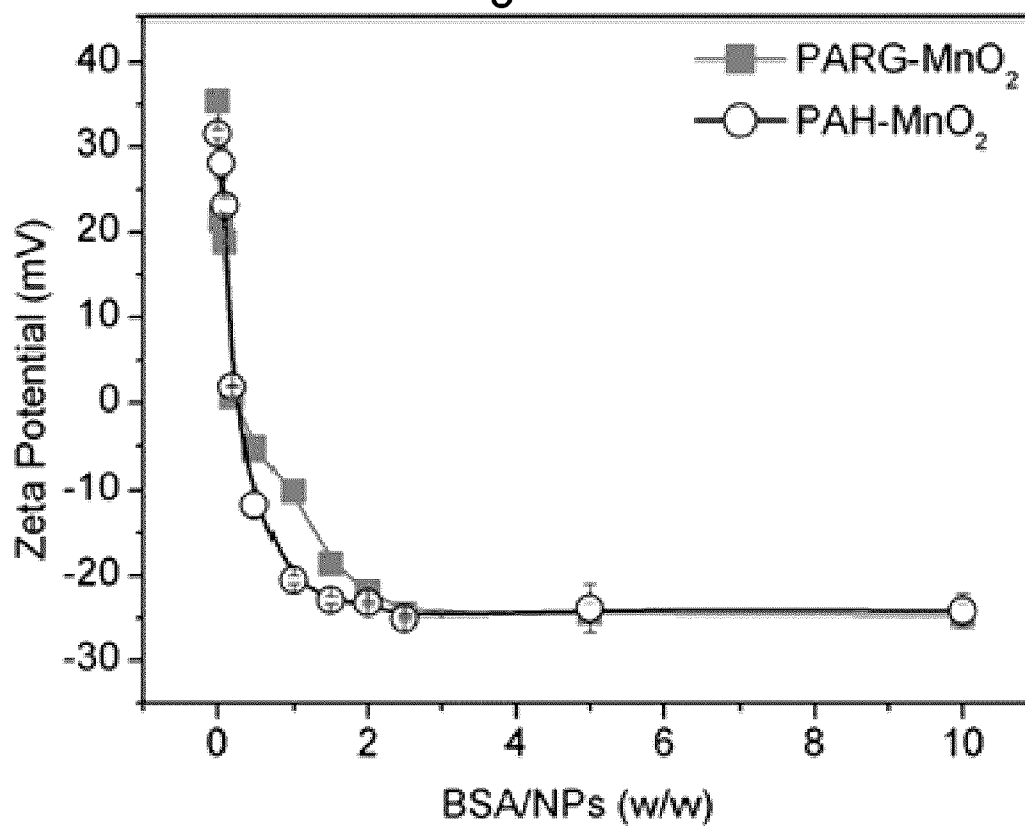


Fig. 2b

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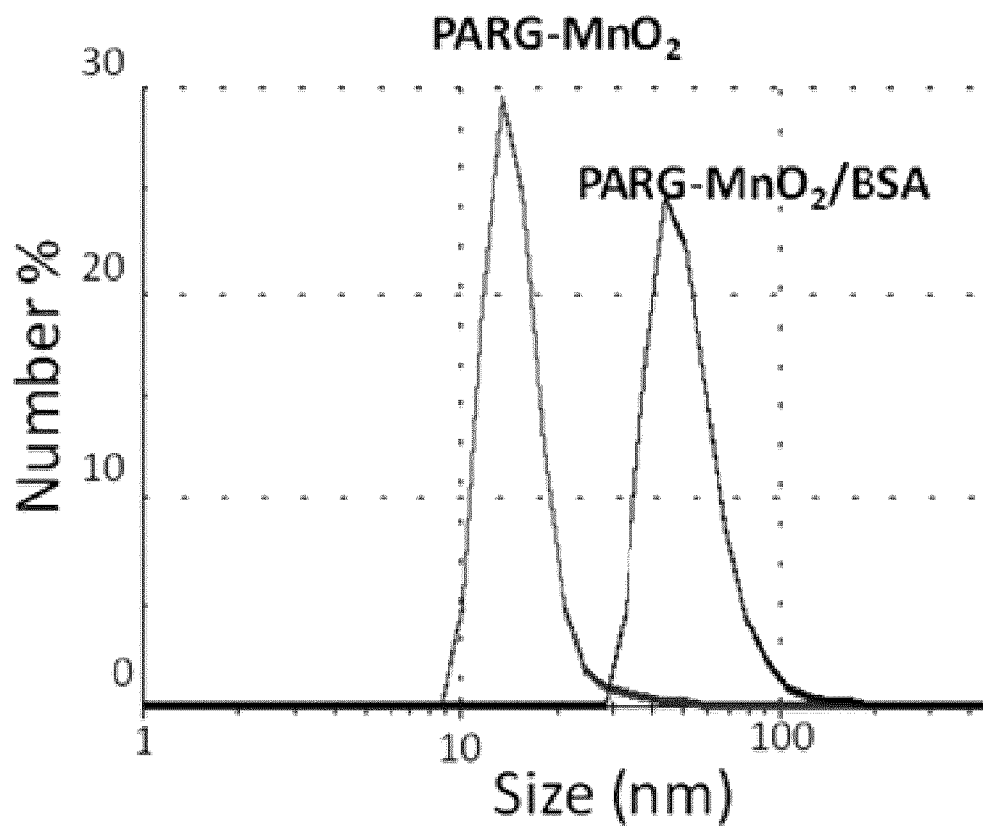


Fig. 2c

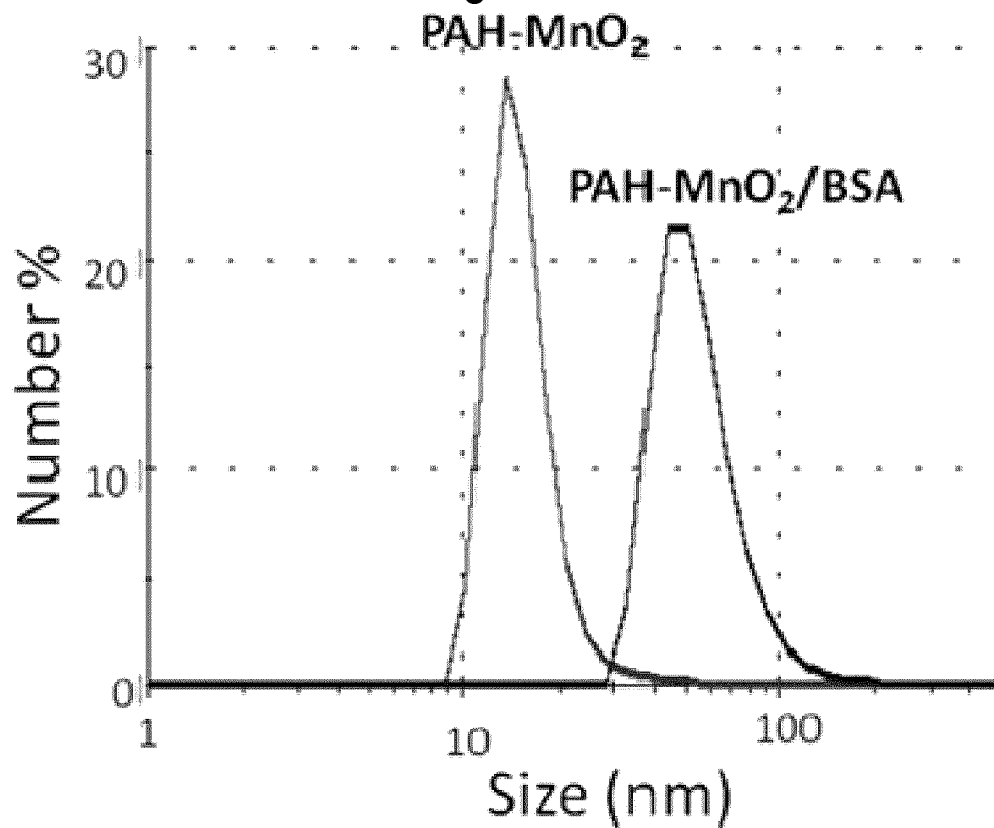


Fig. 2d

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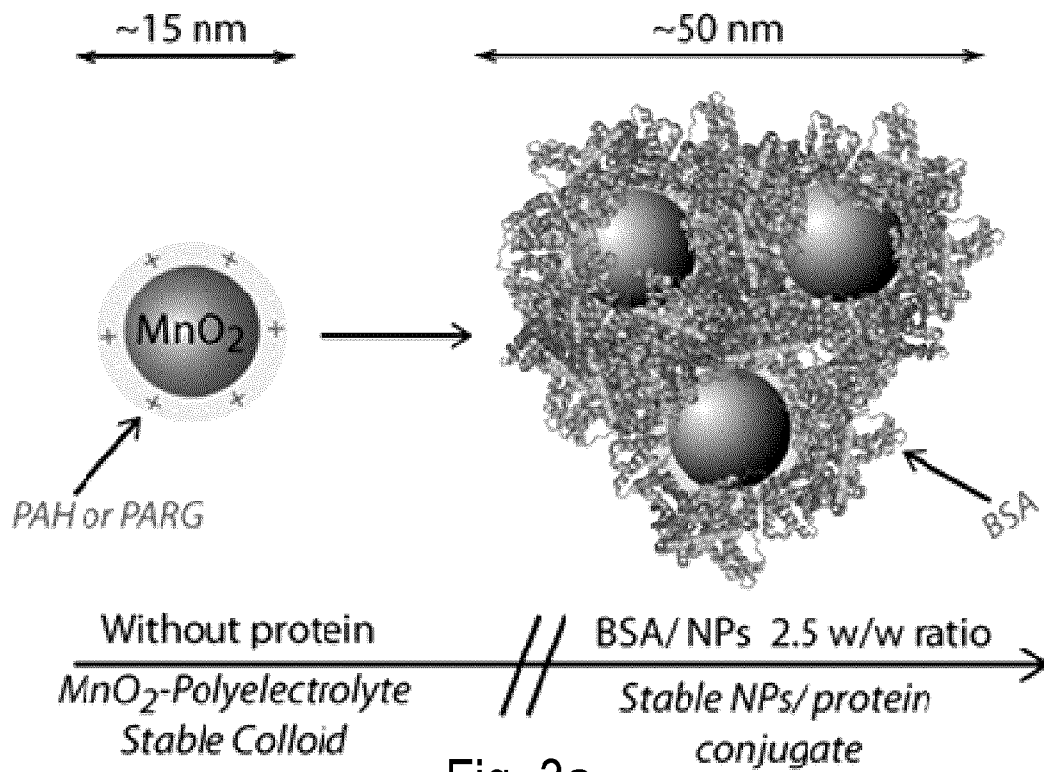


Fig. 3a

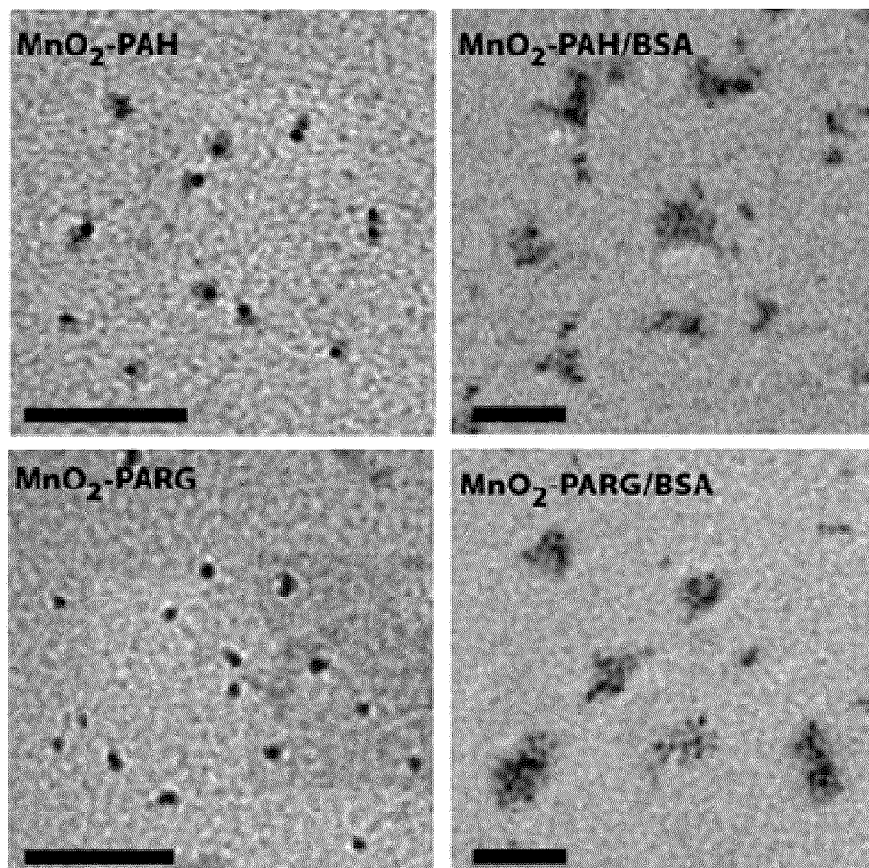


Fig. 3b

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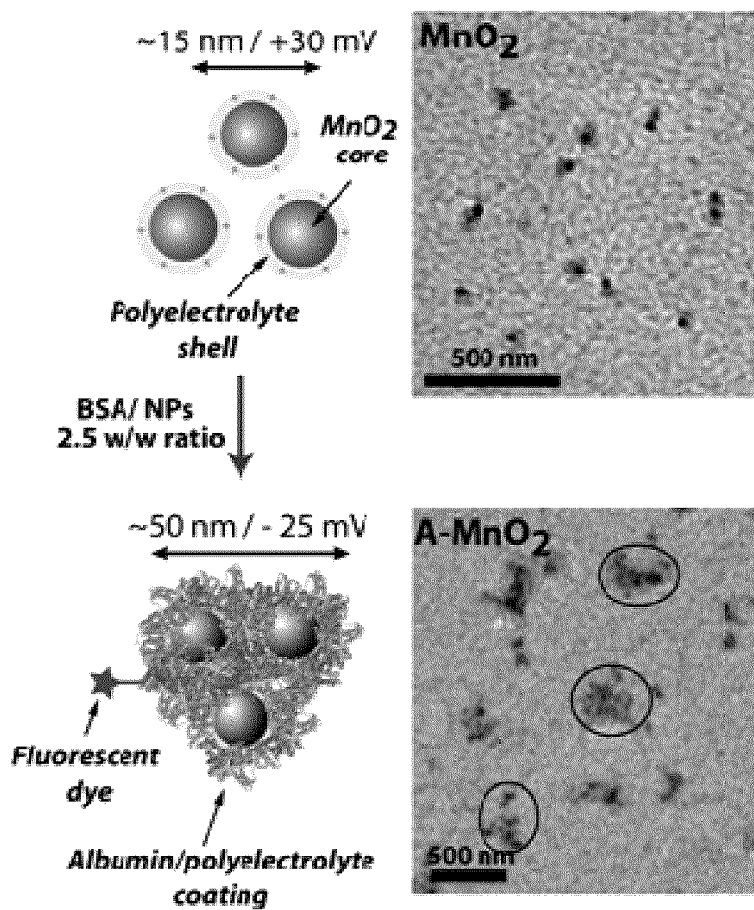


Fig. 4a

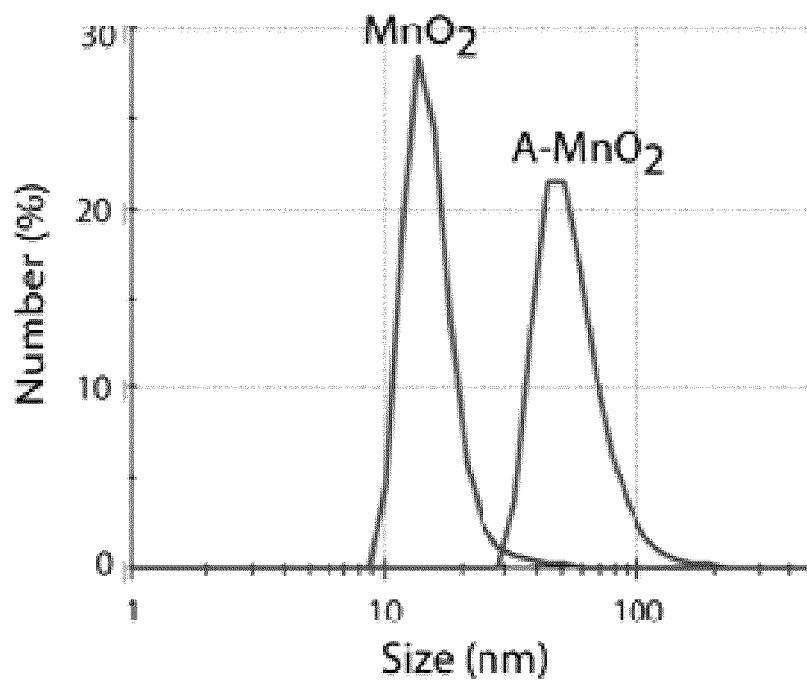


Fig. 4b

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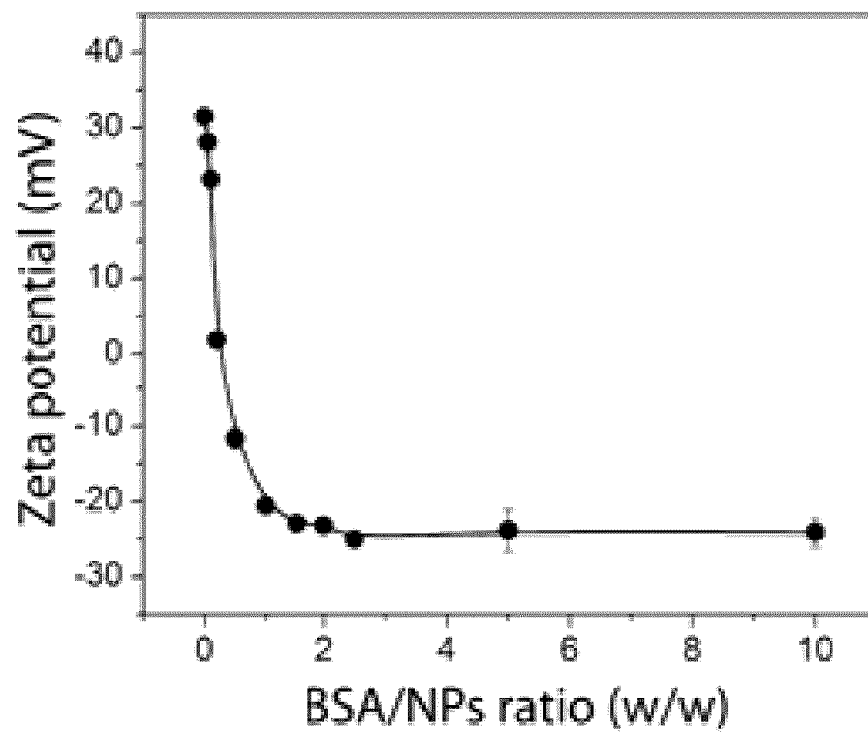


Fig. 4c

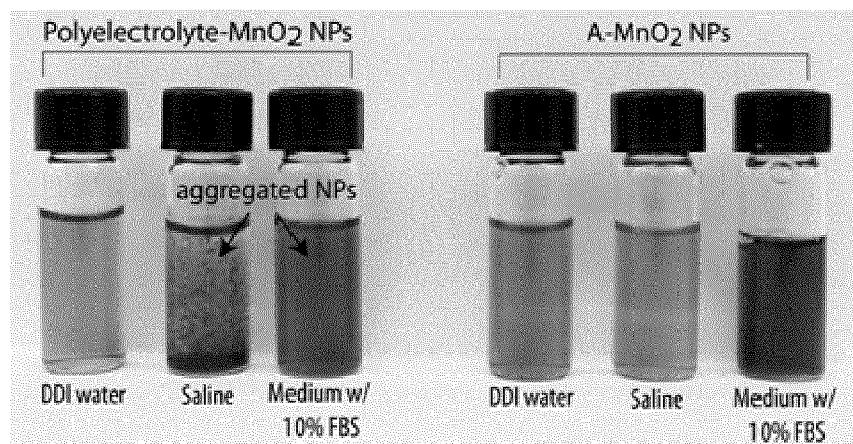


Fig. 4d

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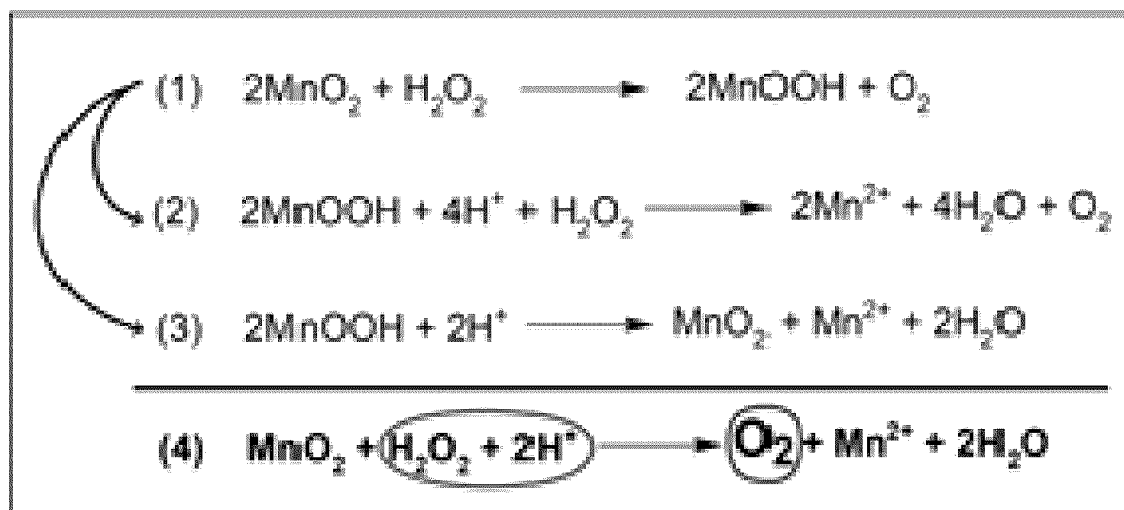


Fig. 5a

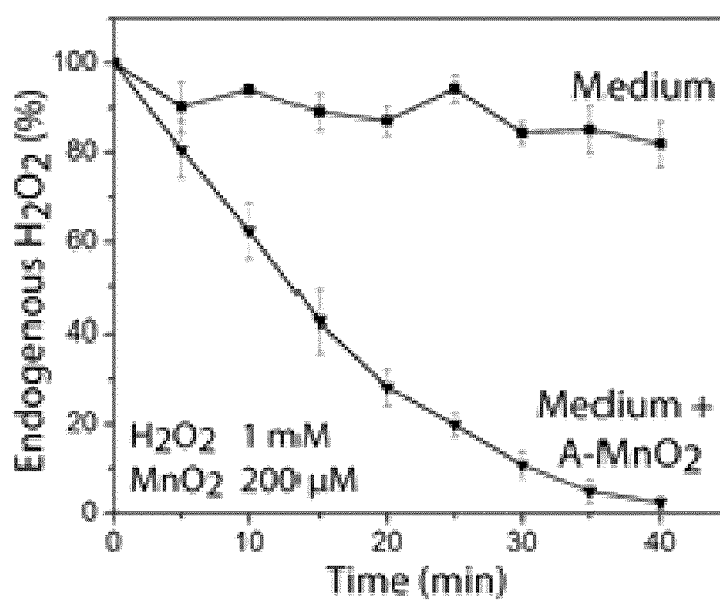


Fig. 5b

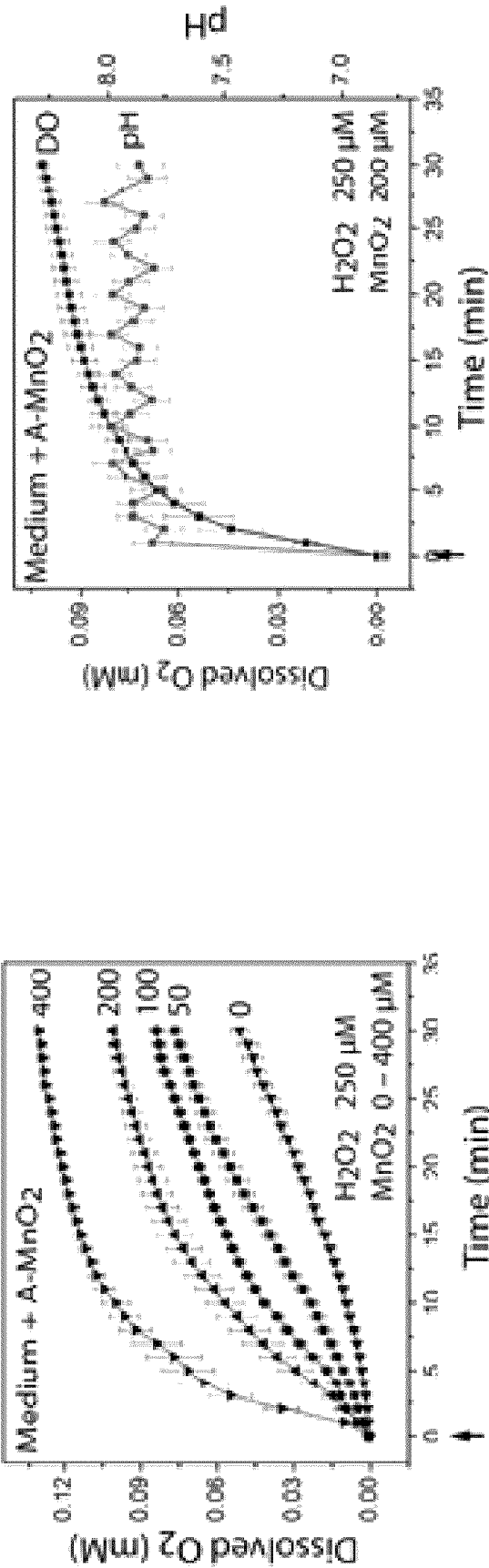


Fig. 5c

Fig. 5d

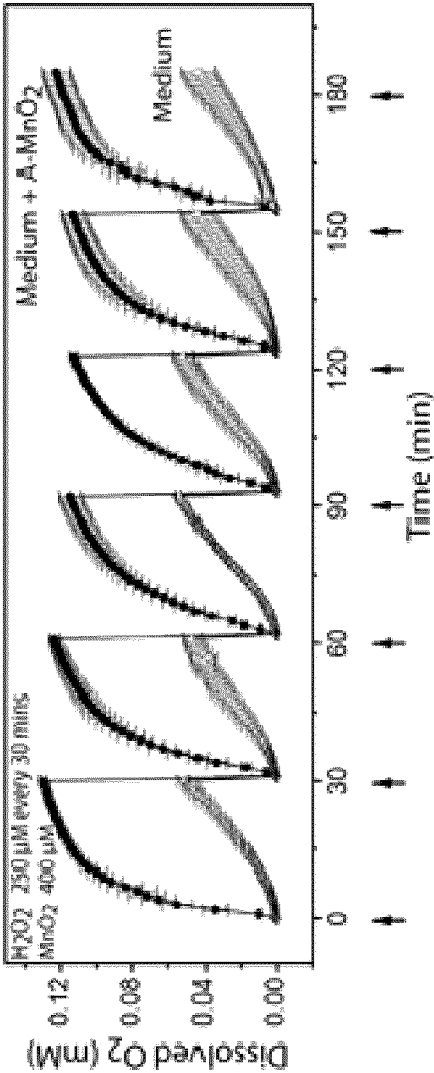


Fig. 5e

10/42

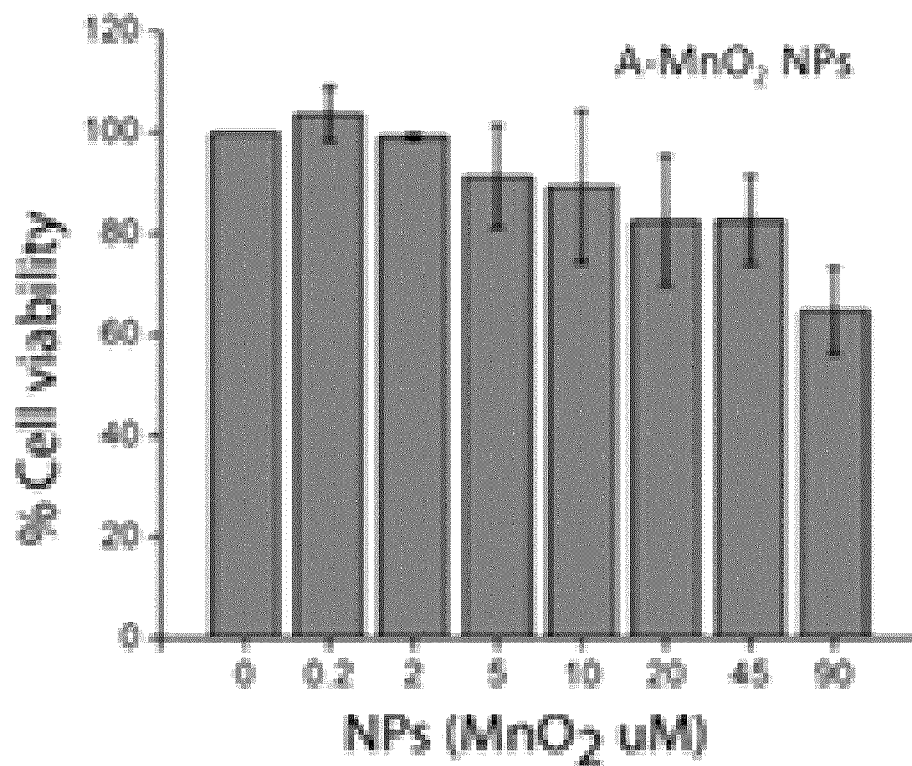


Fig. 6a

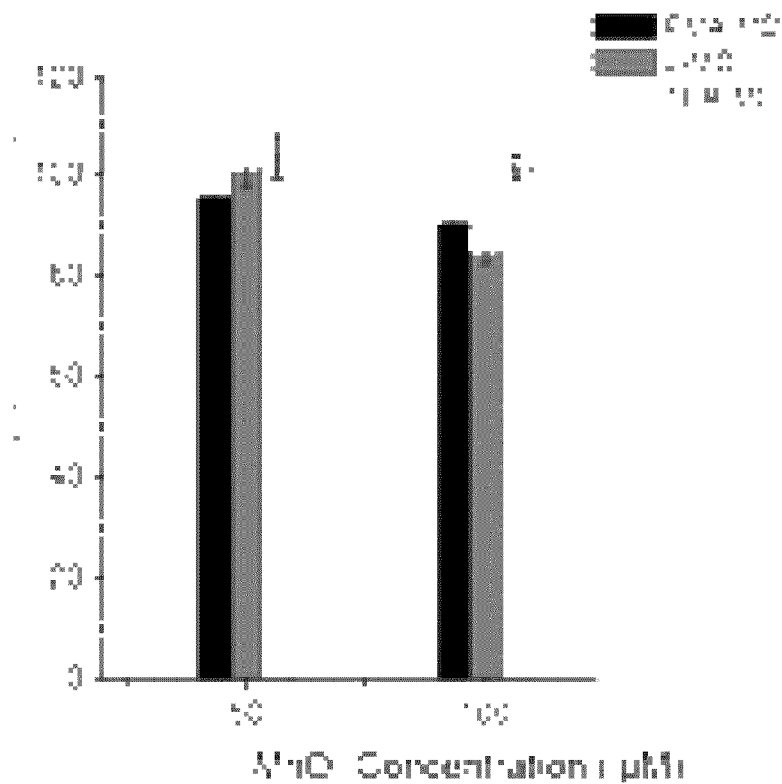


Fig. 6b

11/42

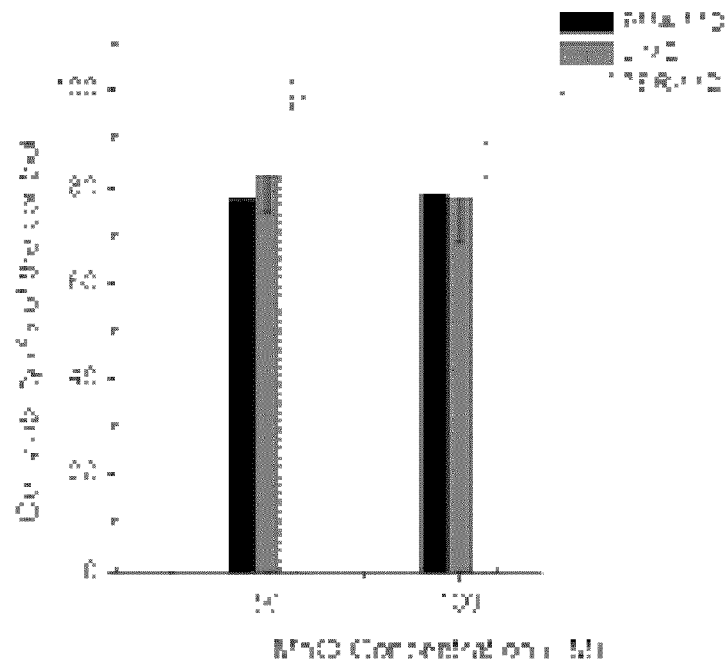


Fig. 6c

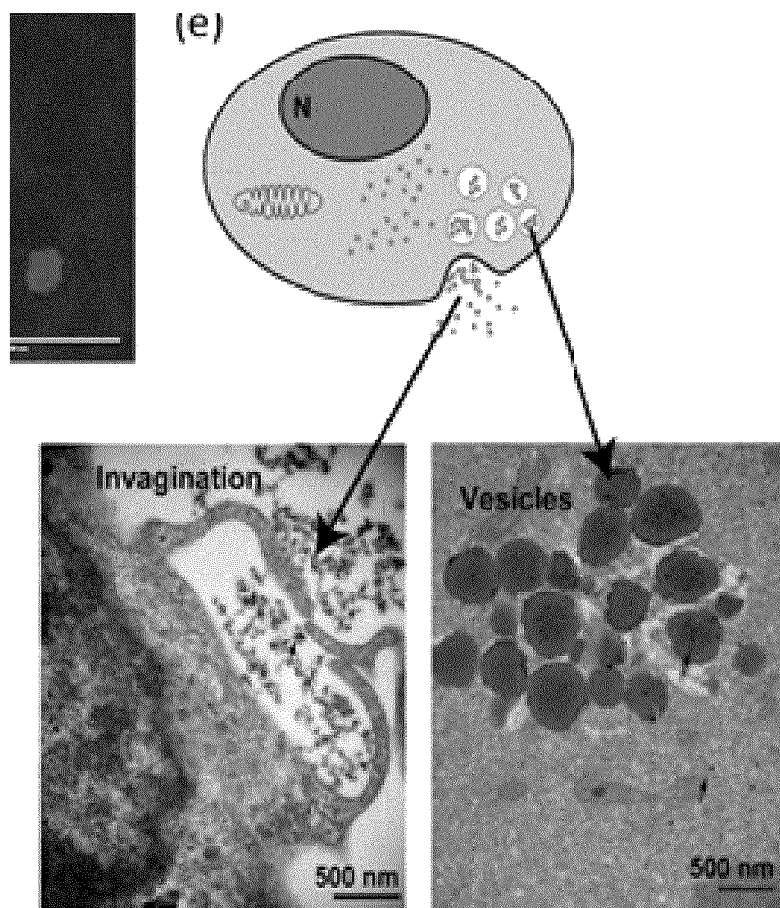


Fig. 6e

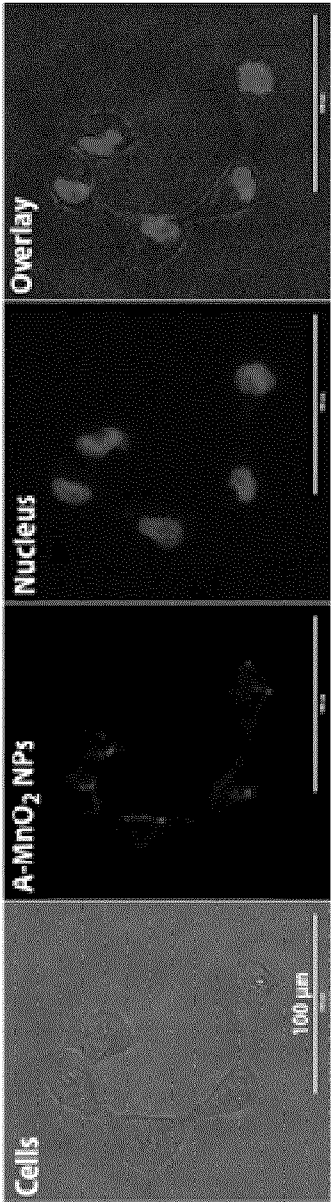


Fig. 6d

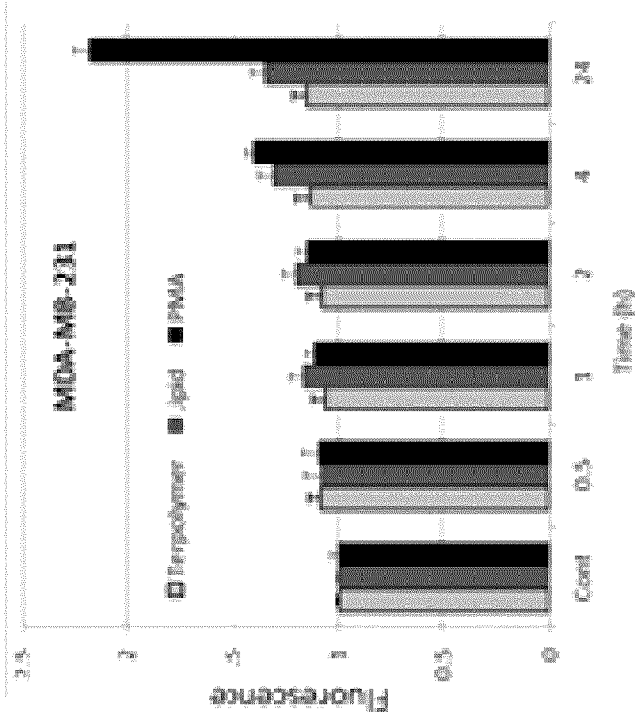


Fig. 6f

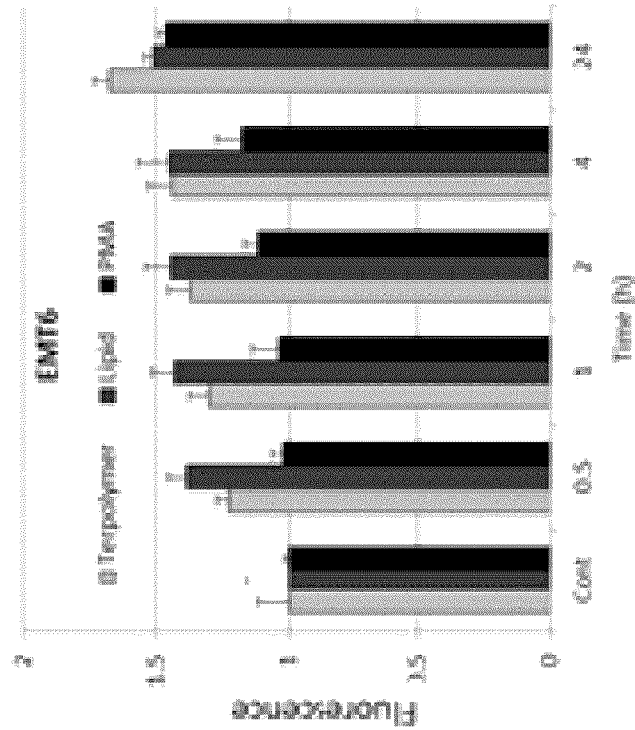


Fig. 6g

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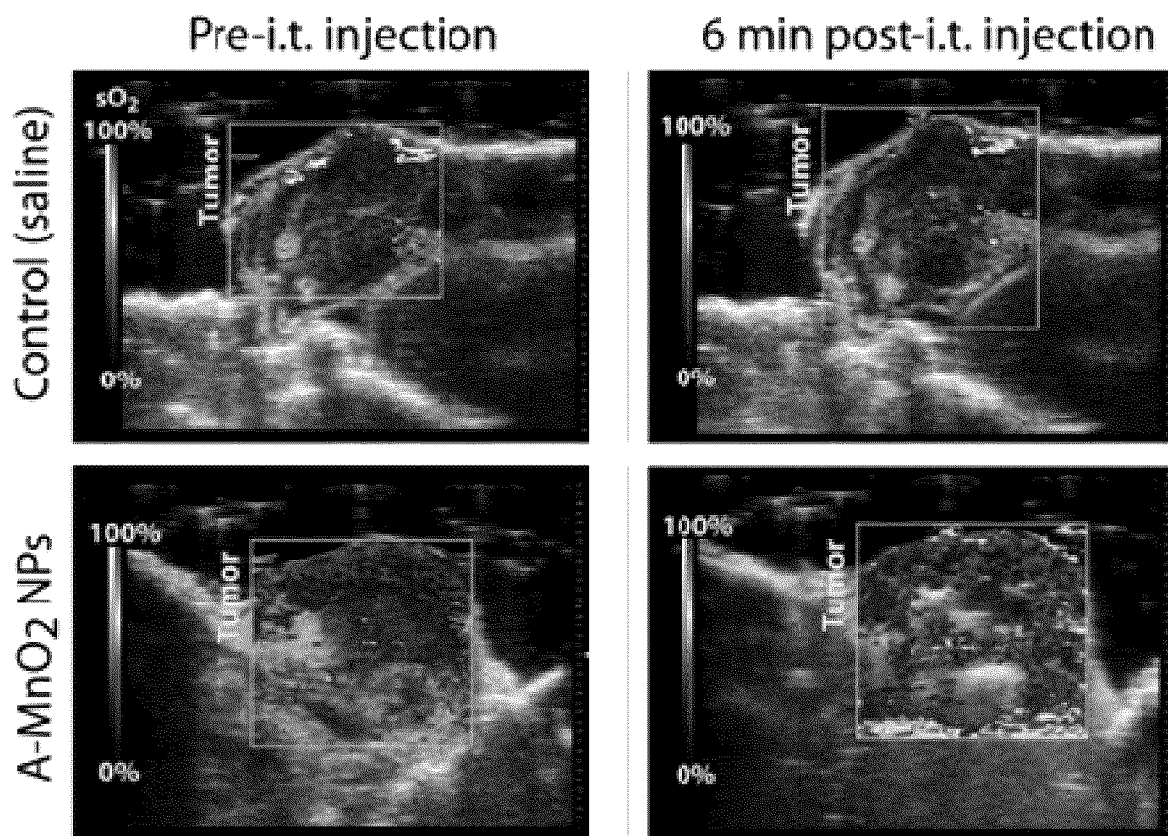


Fig. 7a

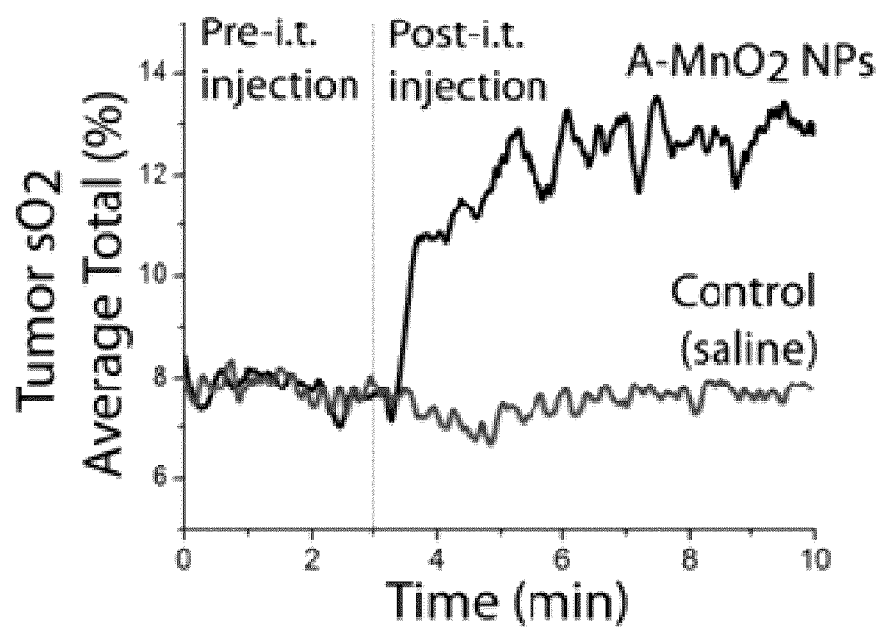


Fig. 7b

14/42

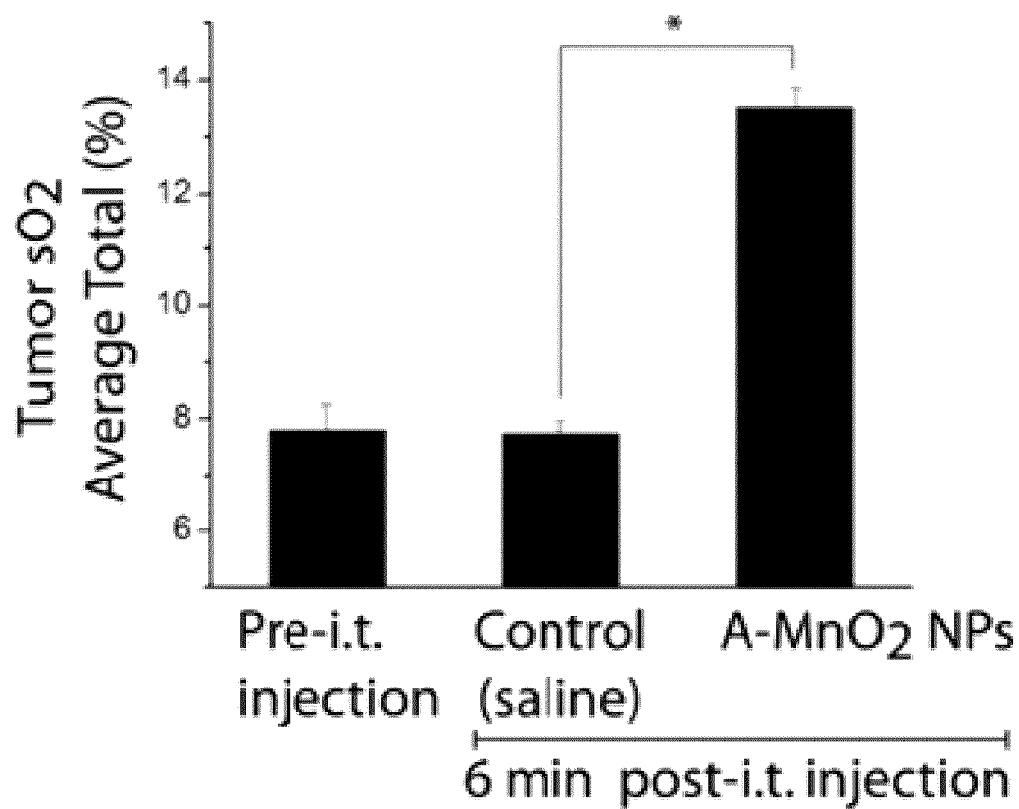


Fig. 7c

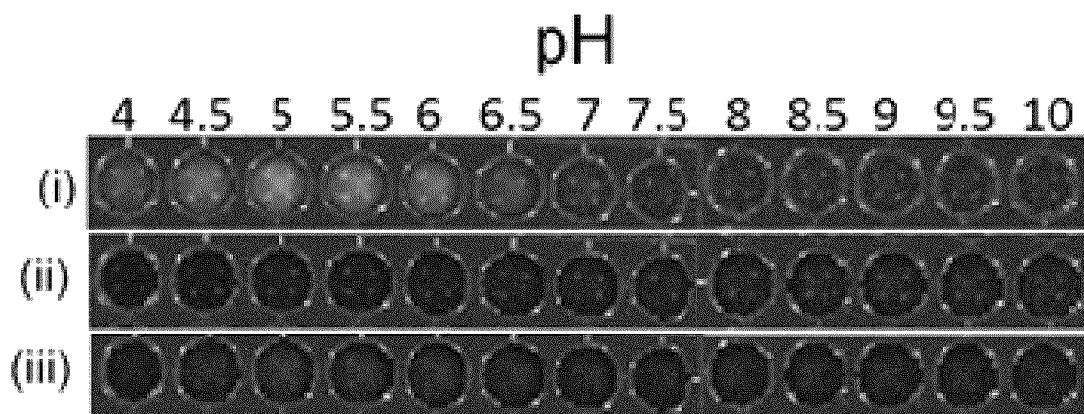


Fig. 8a

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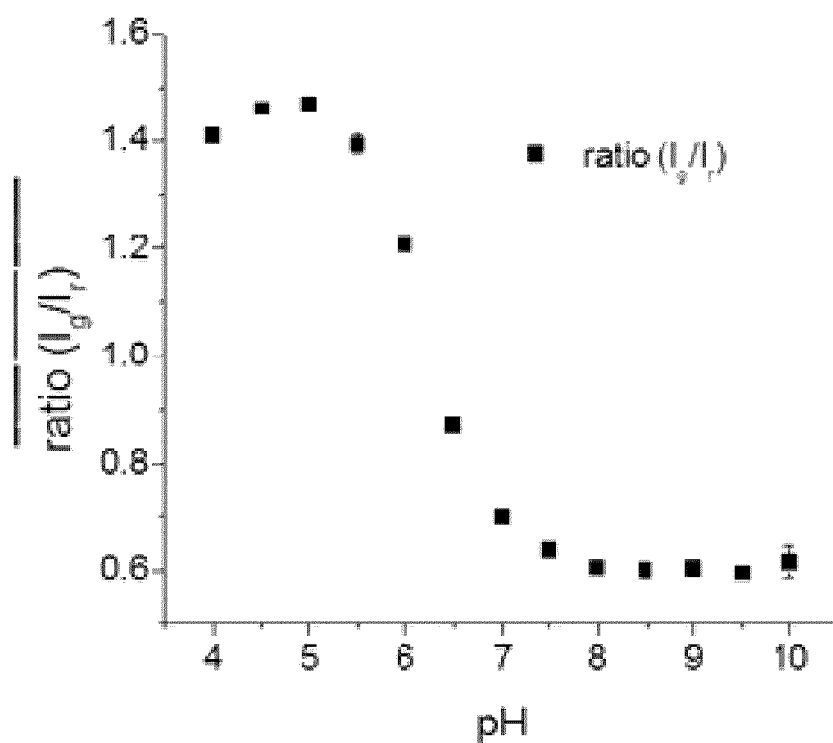


Fig. 8b

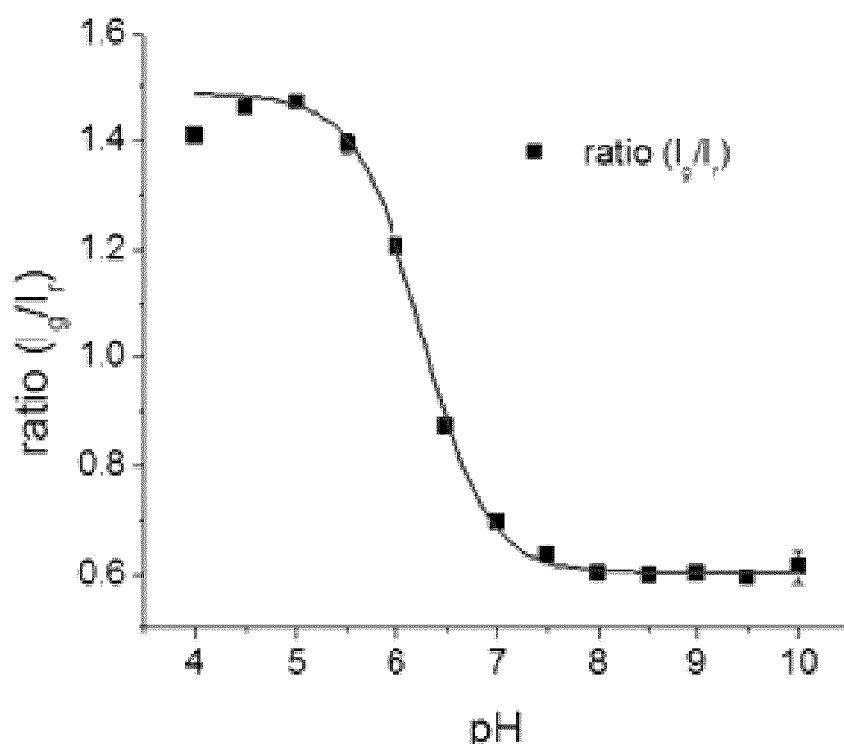


Fig. 8c

16/42

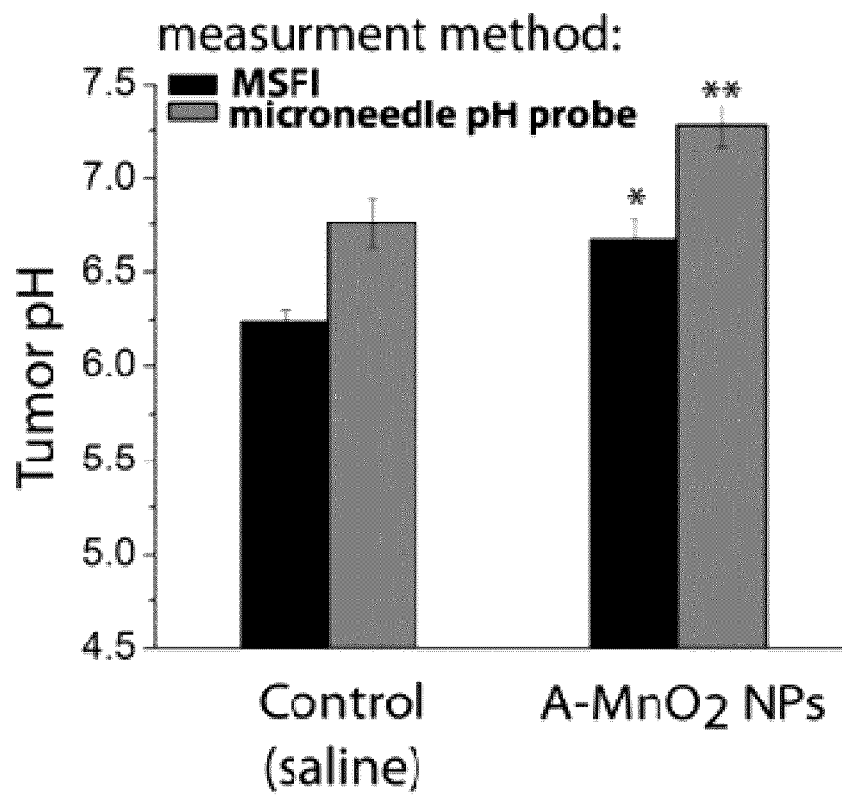
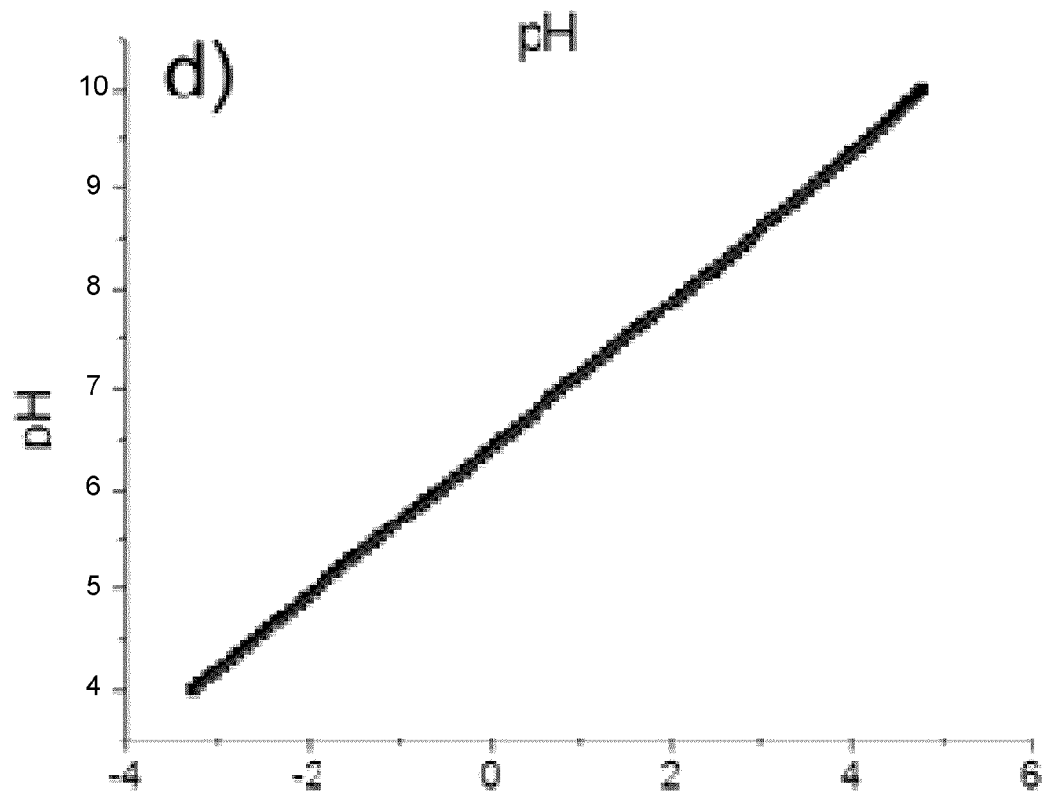


Fig. 9b

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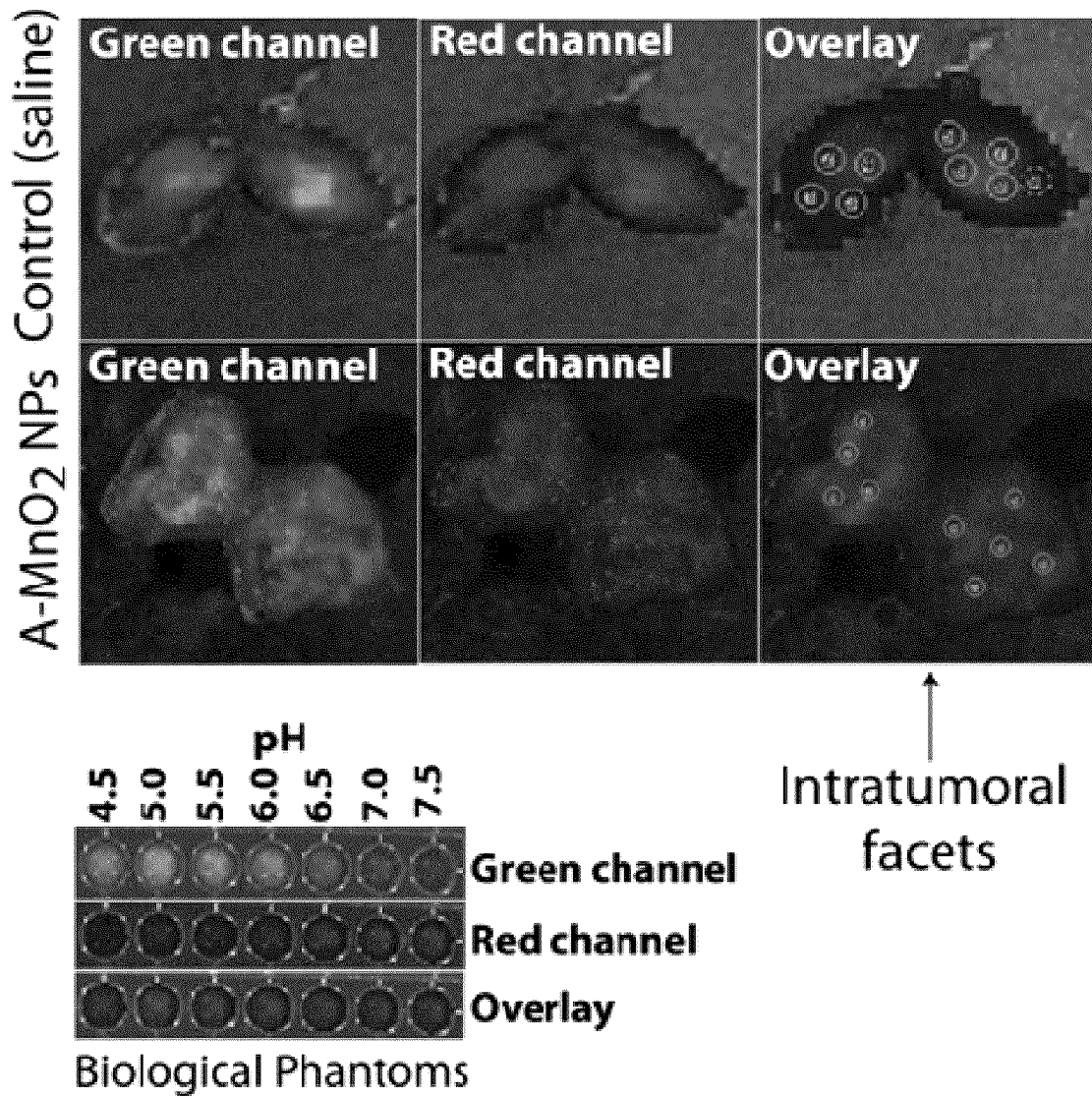


Fig. 9a

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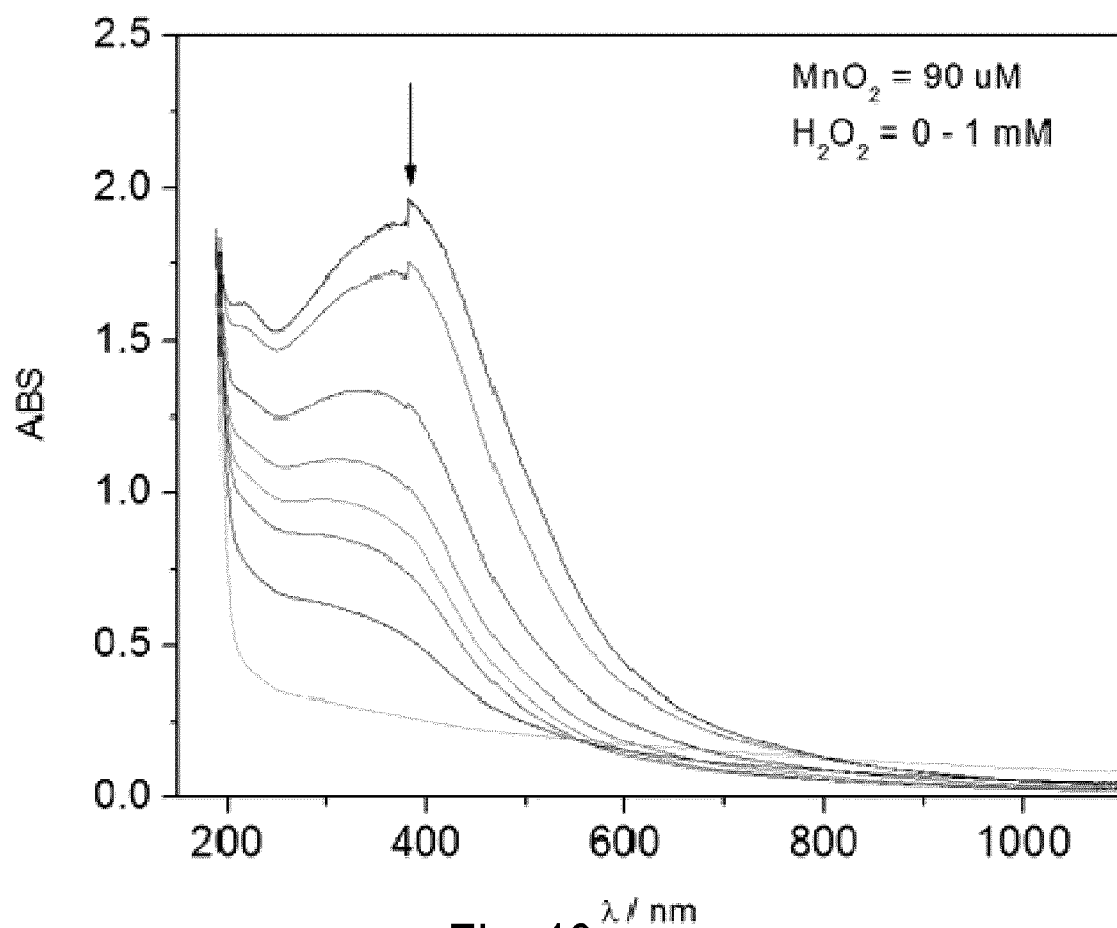


Fig. 10

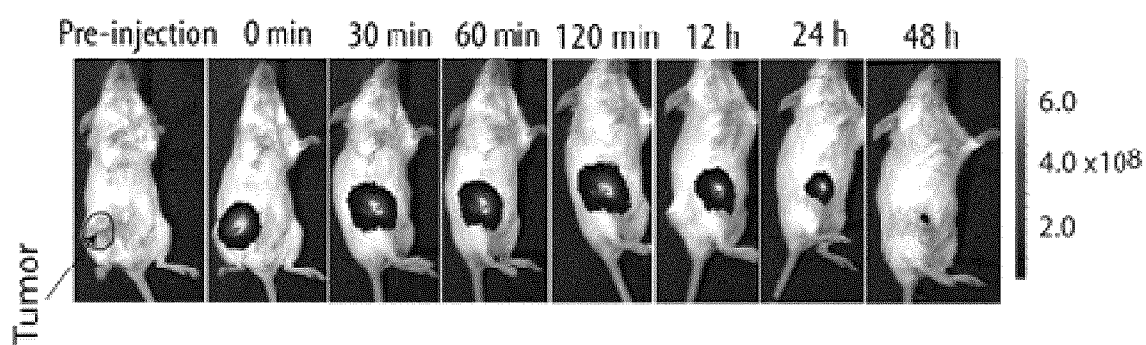


Fig. 11a

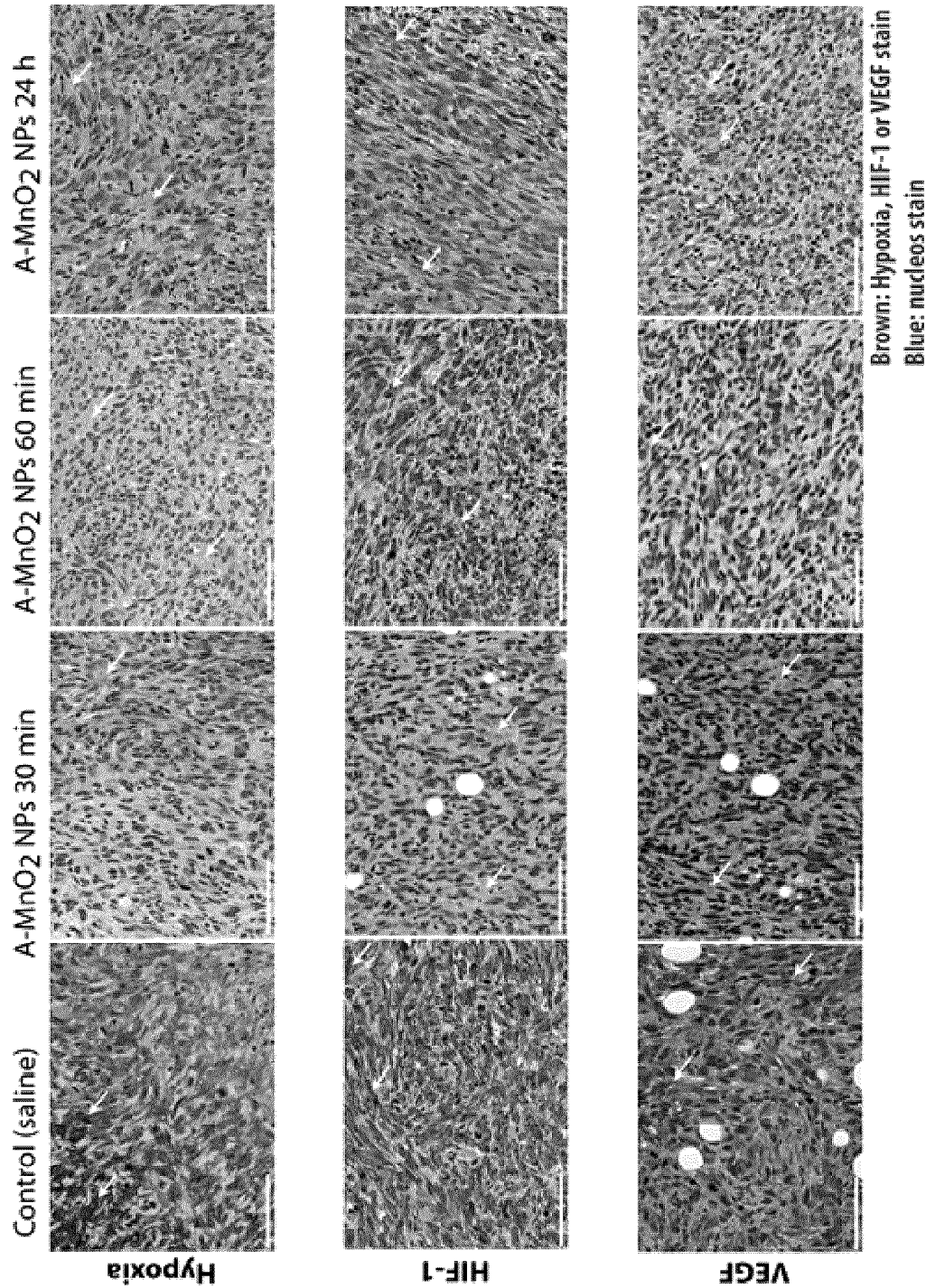


Fig. 11b

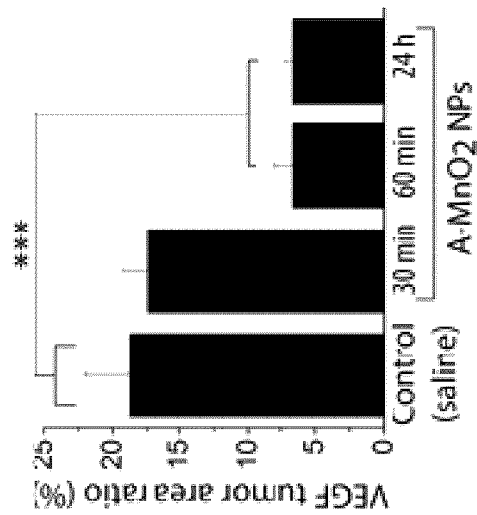


Fig. 11e

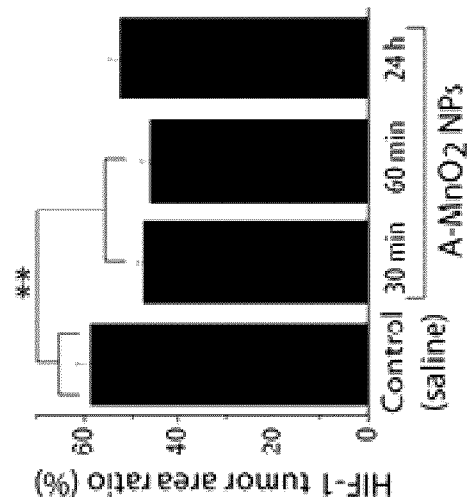


Fig. 11d

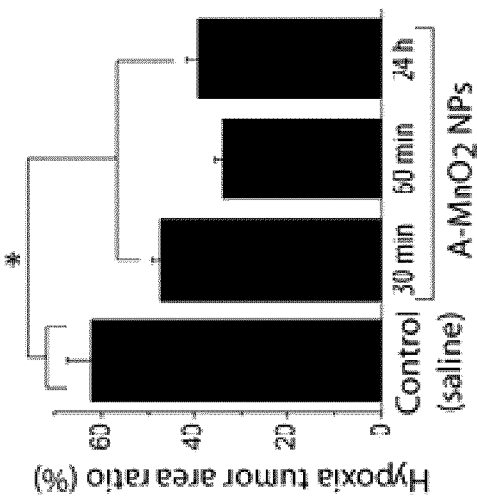


Fig. 11c

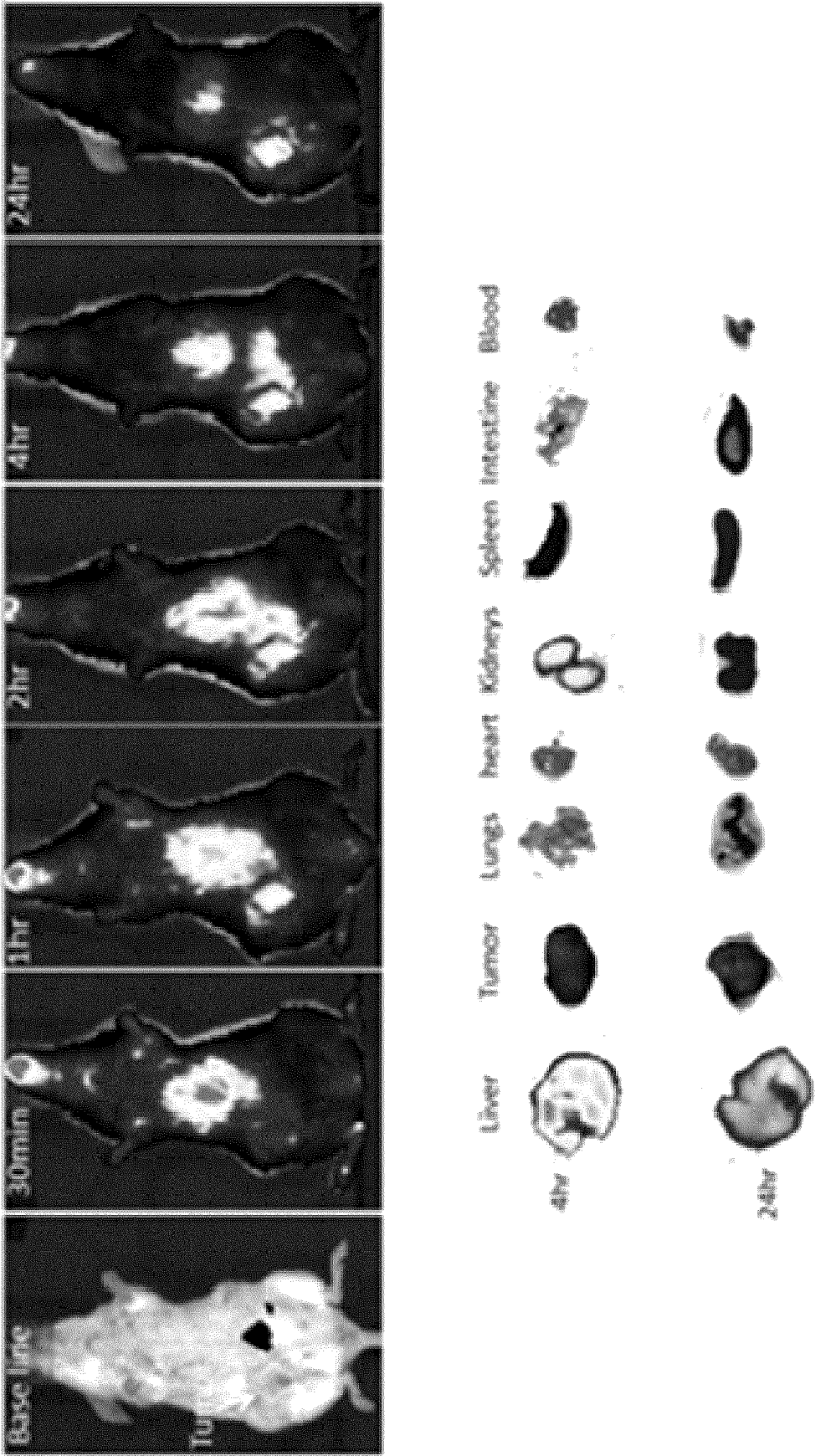


Fig. 11f

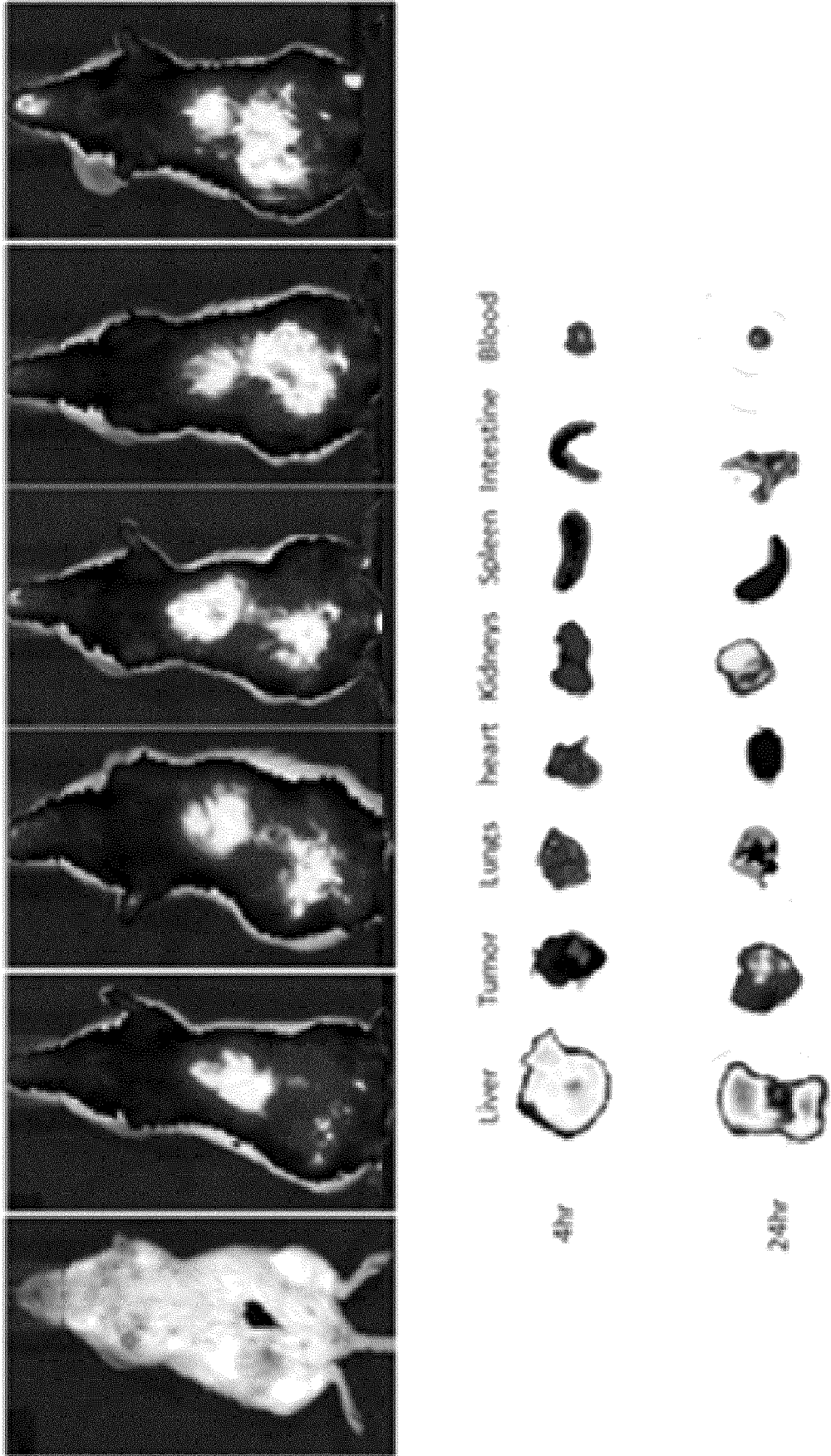


Fig. 11g

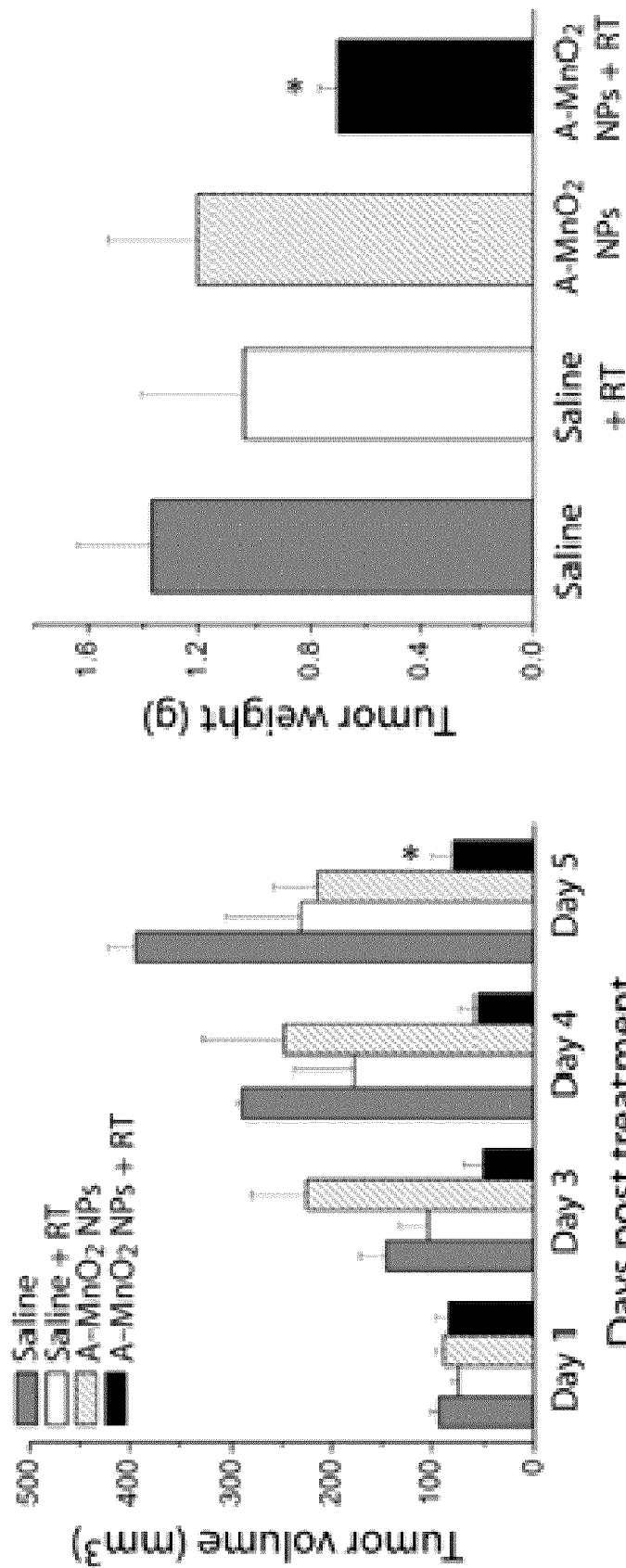
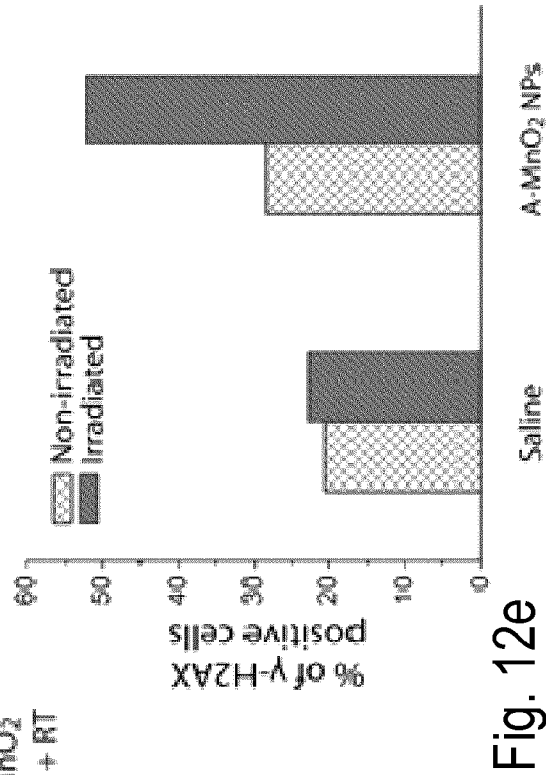
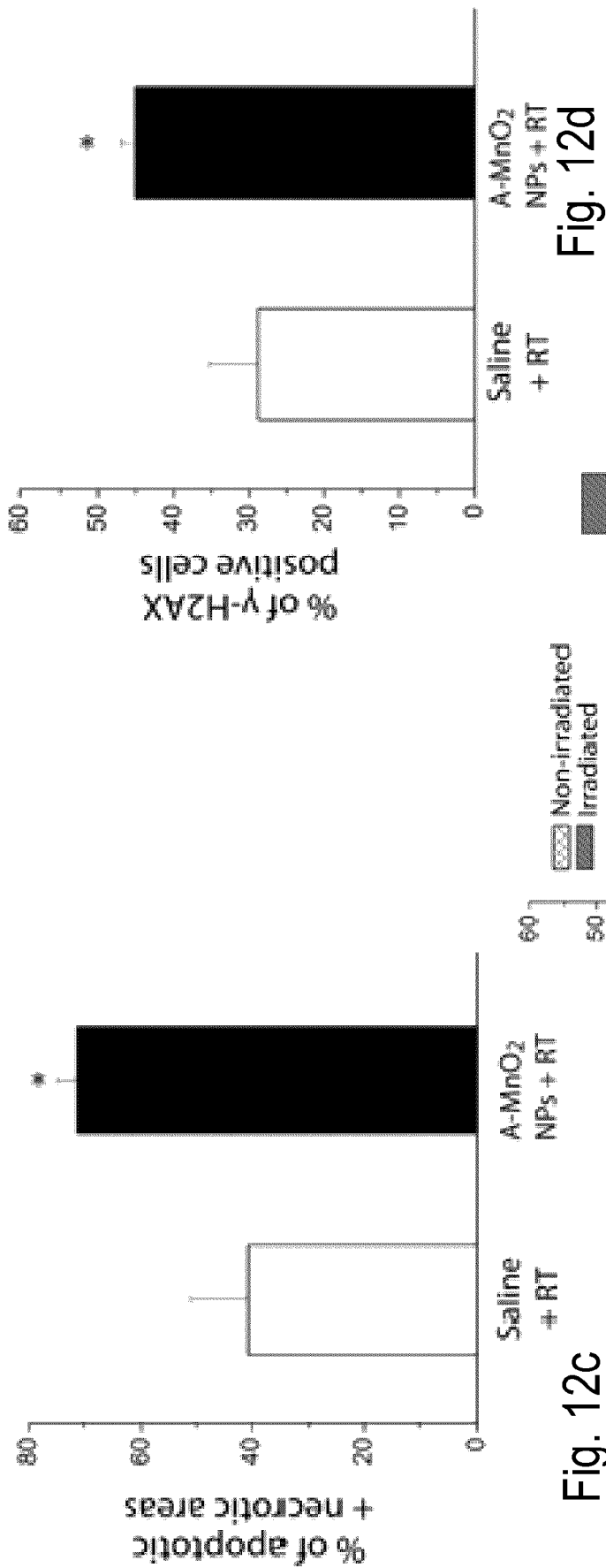


Fig. 12a

Fig. 12b



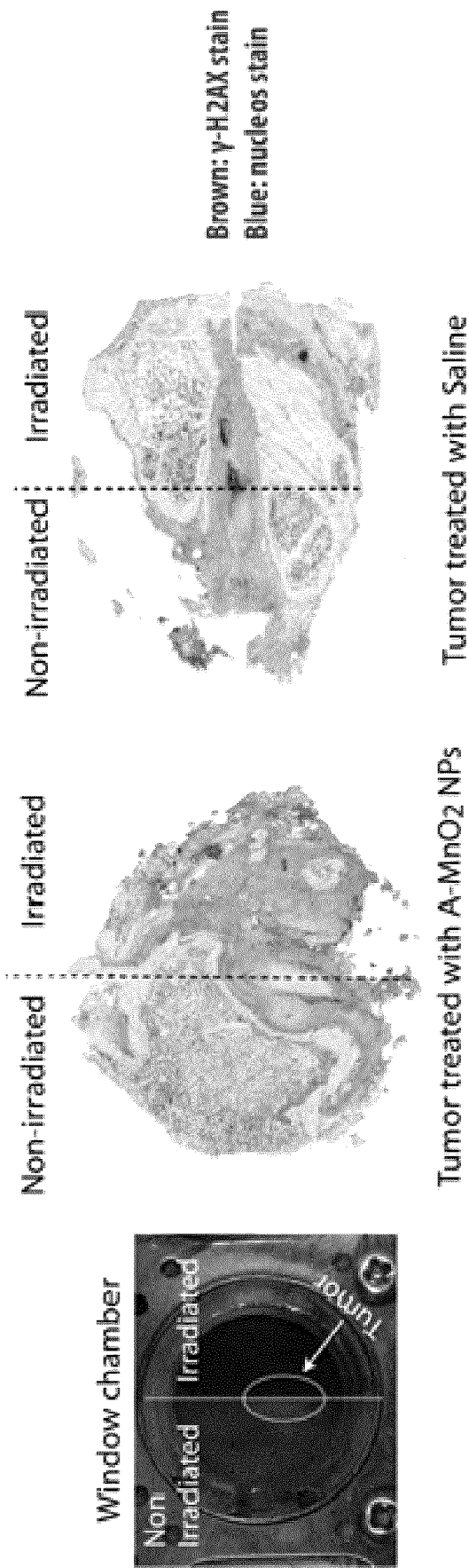


Fig. 12f

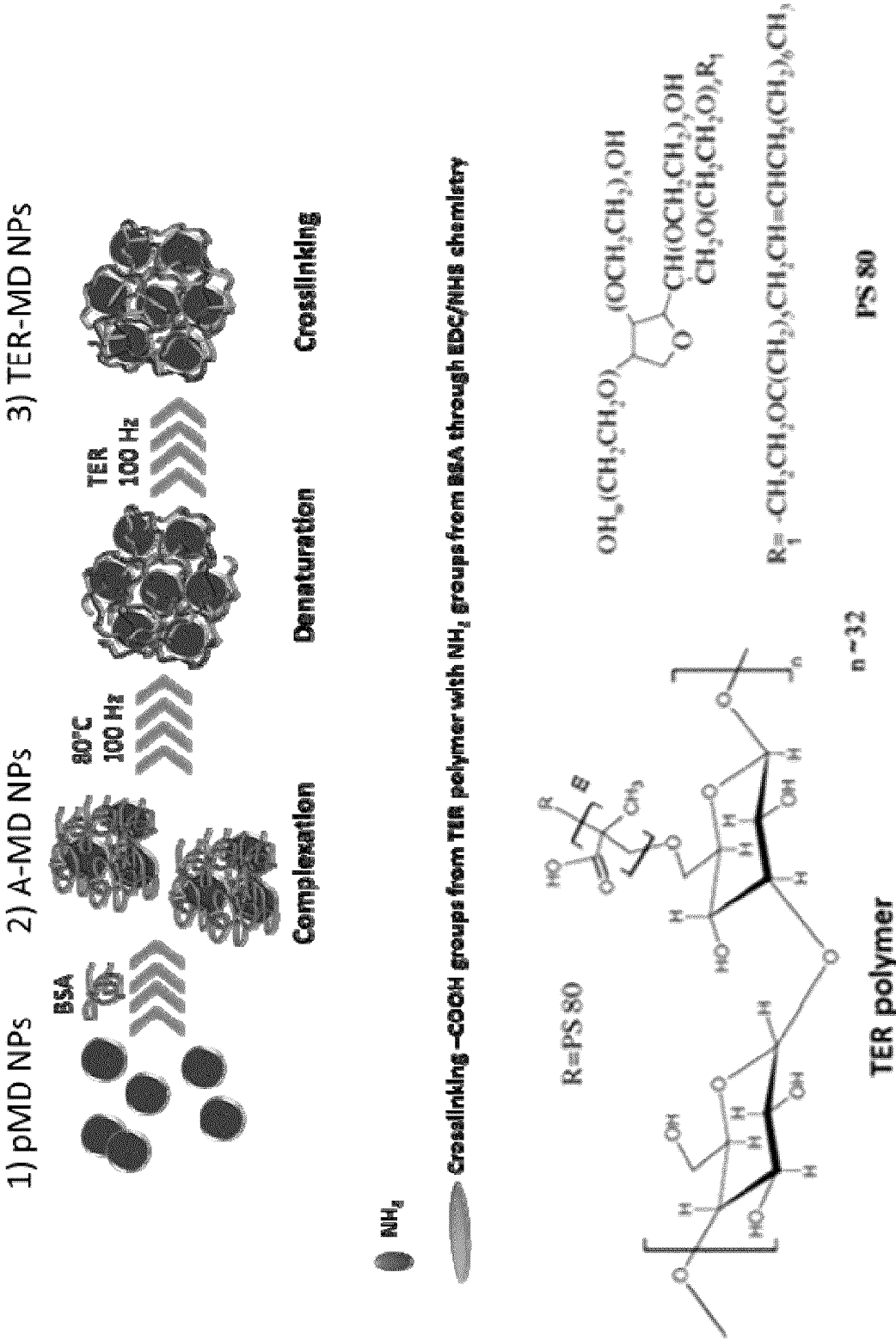


Fig. 13

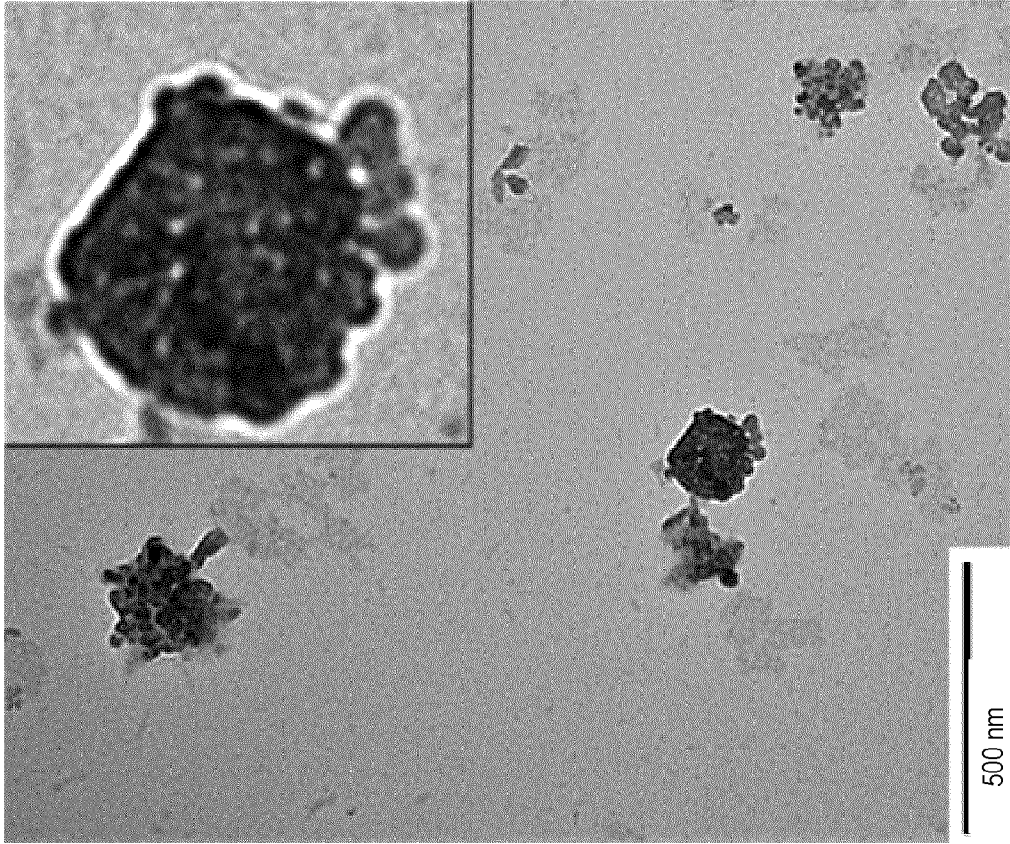


Fig. 14b

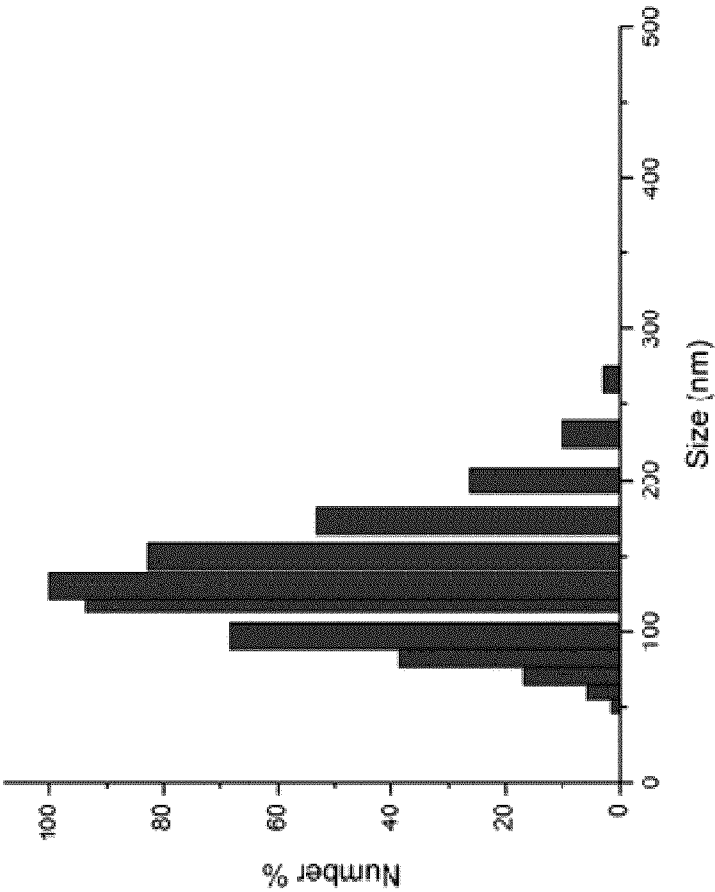


Fig. 14a

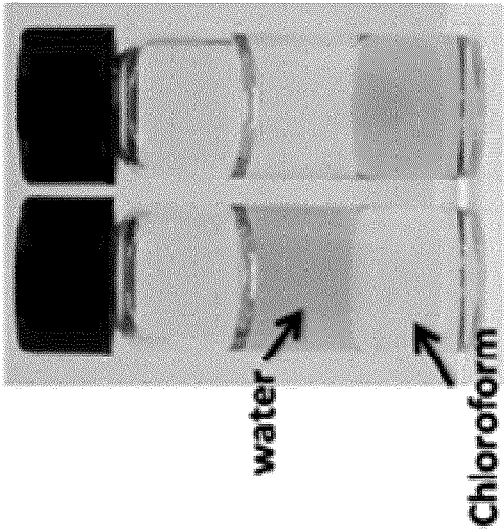


Fig. 15a

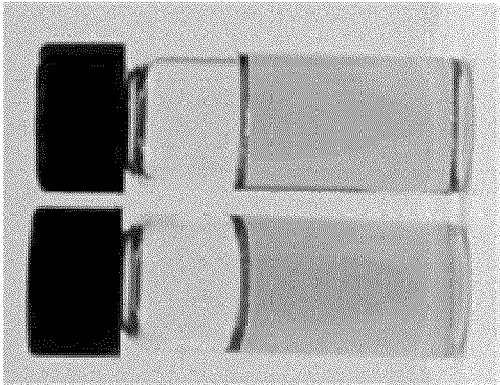


Fig. 15b

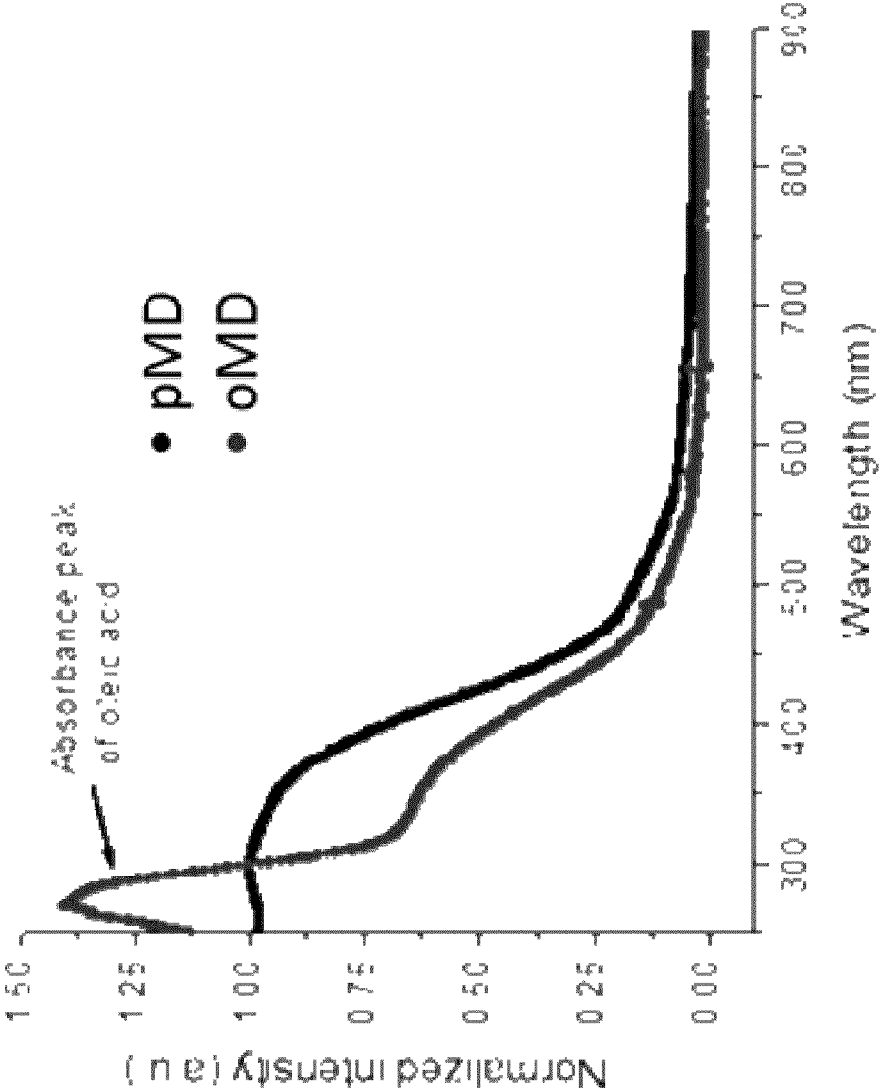


Fig. 15c

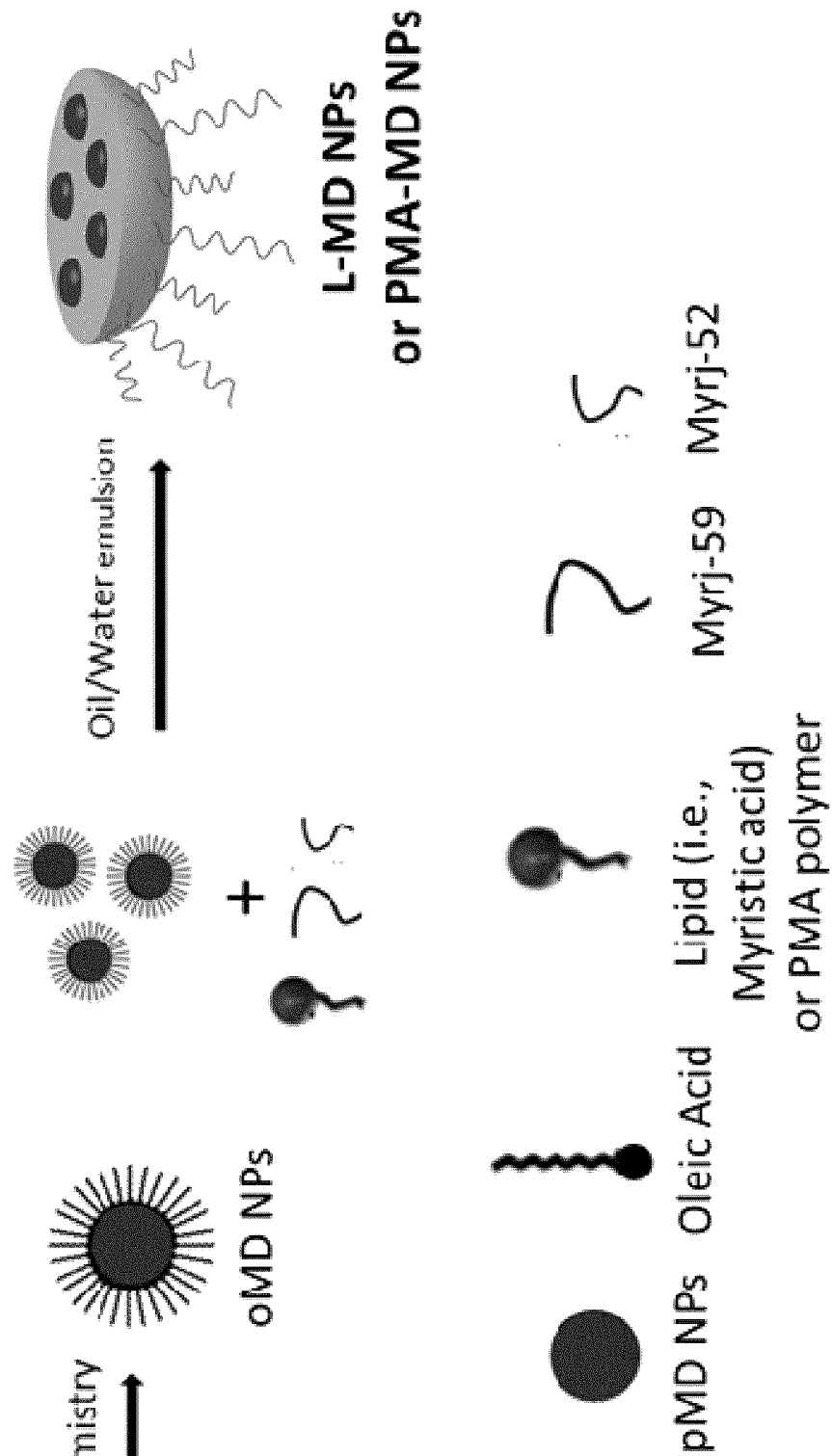


Fig. 16

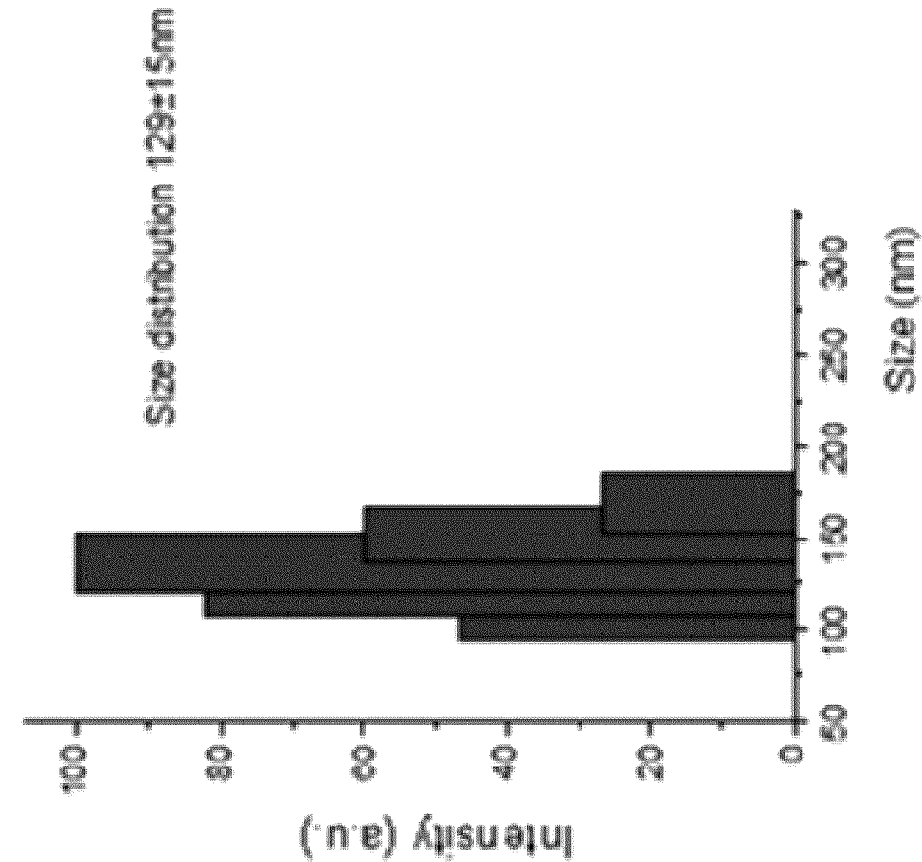


Fig. 17b

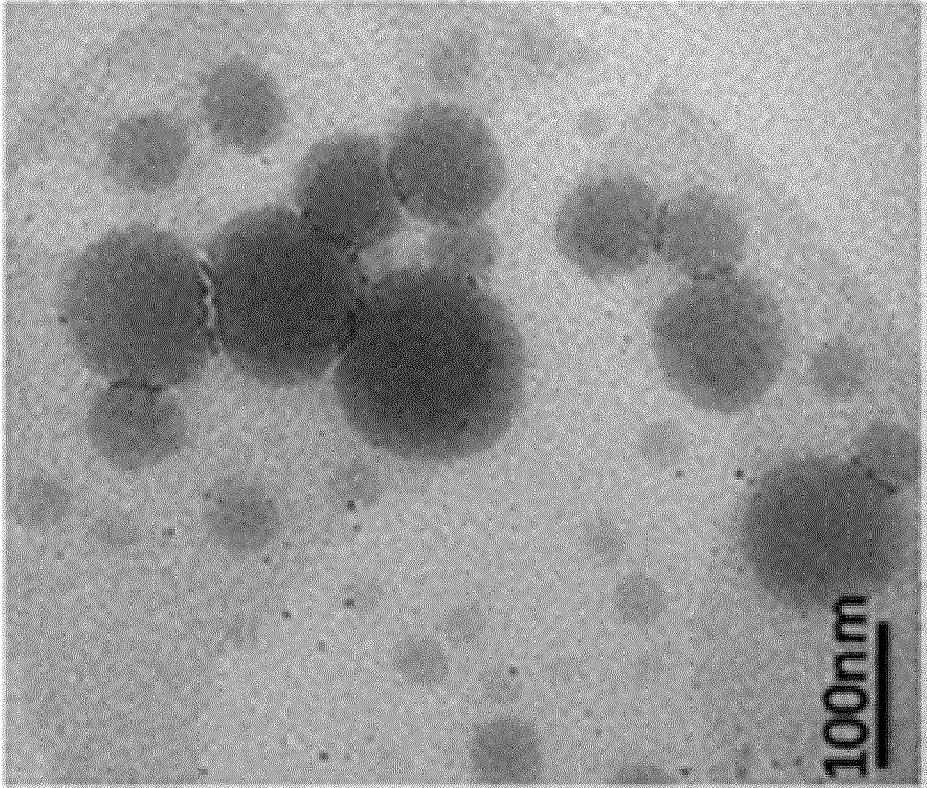


Fig. 17a

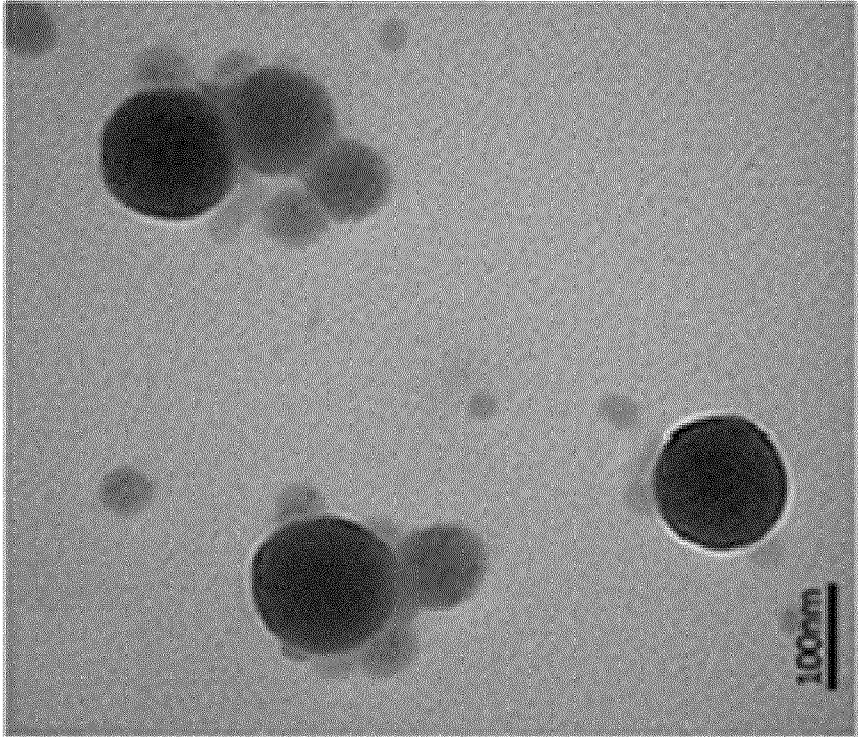


Fig. 18a

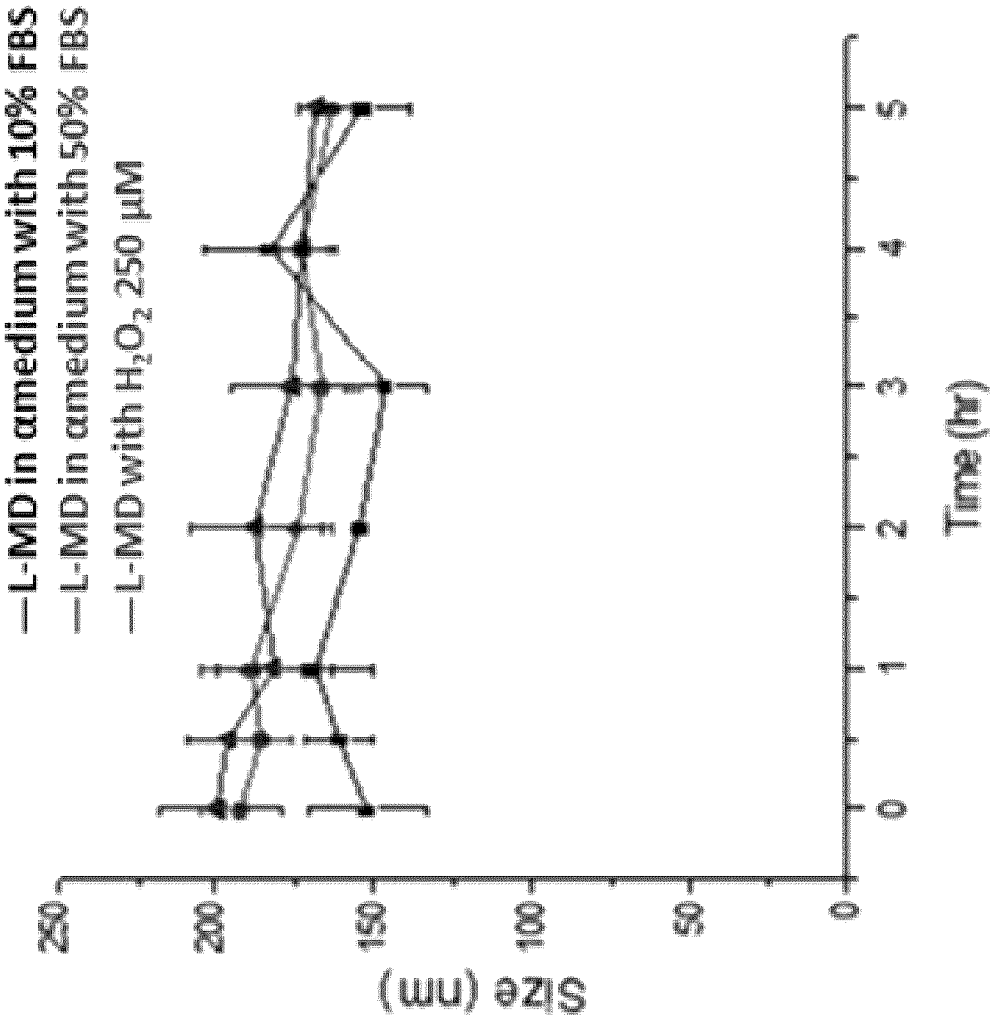


Fig. 17c

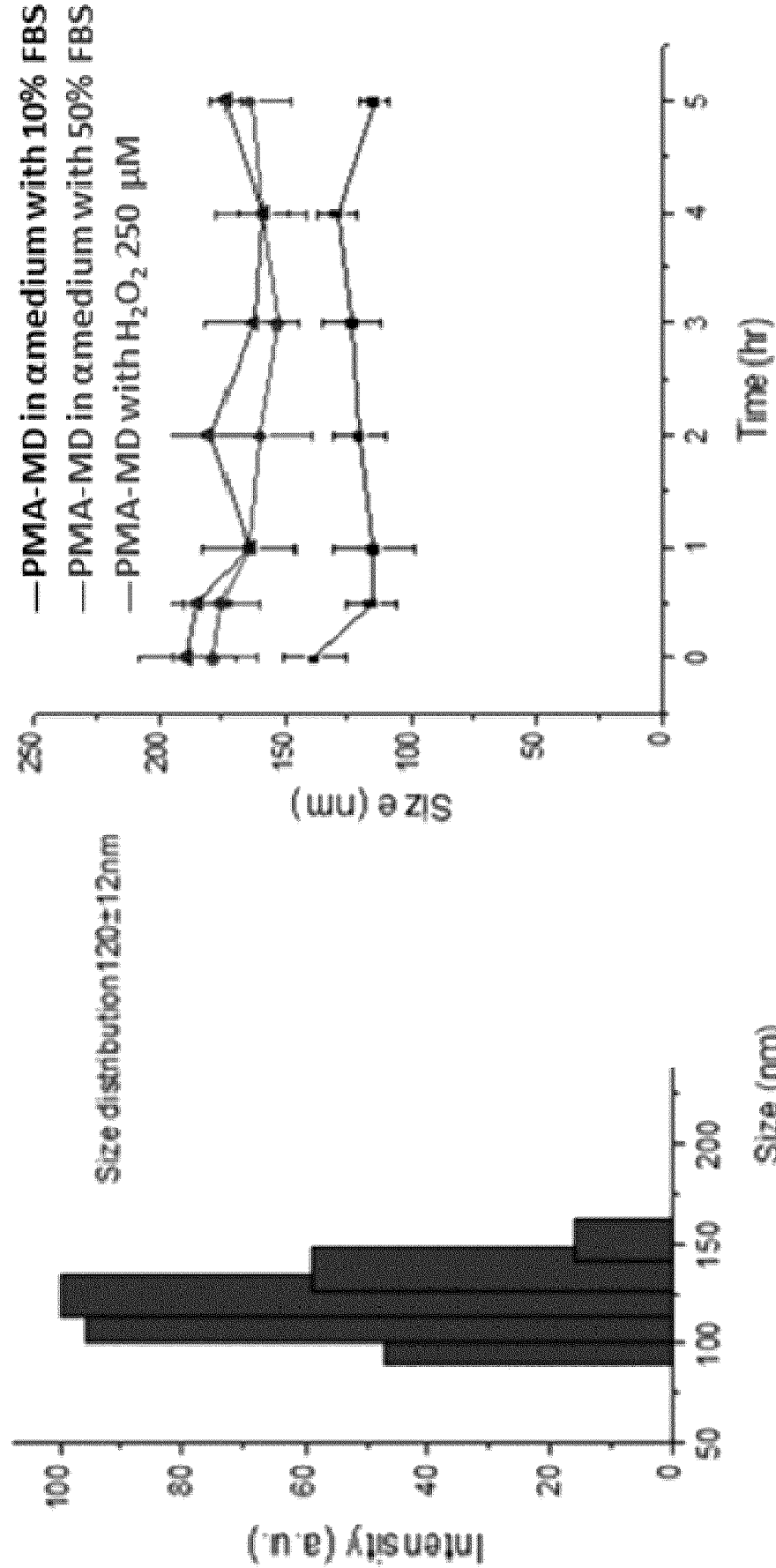


Fig. 18c

Fig. 18b

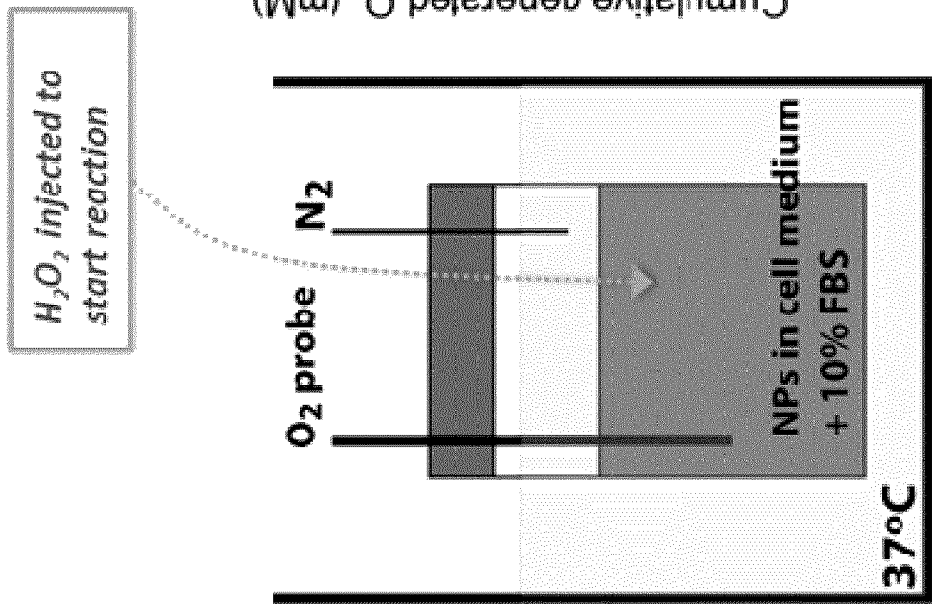


Fig. 19

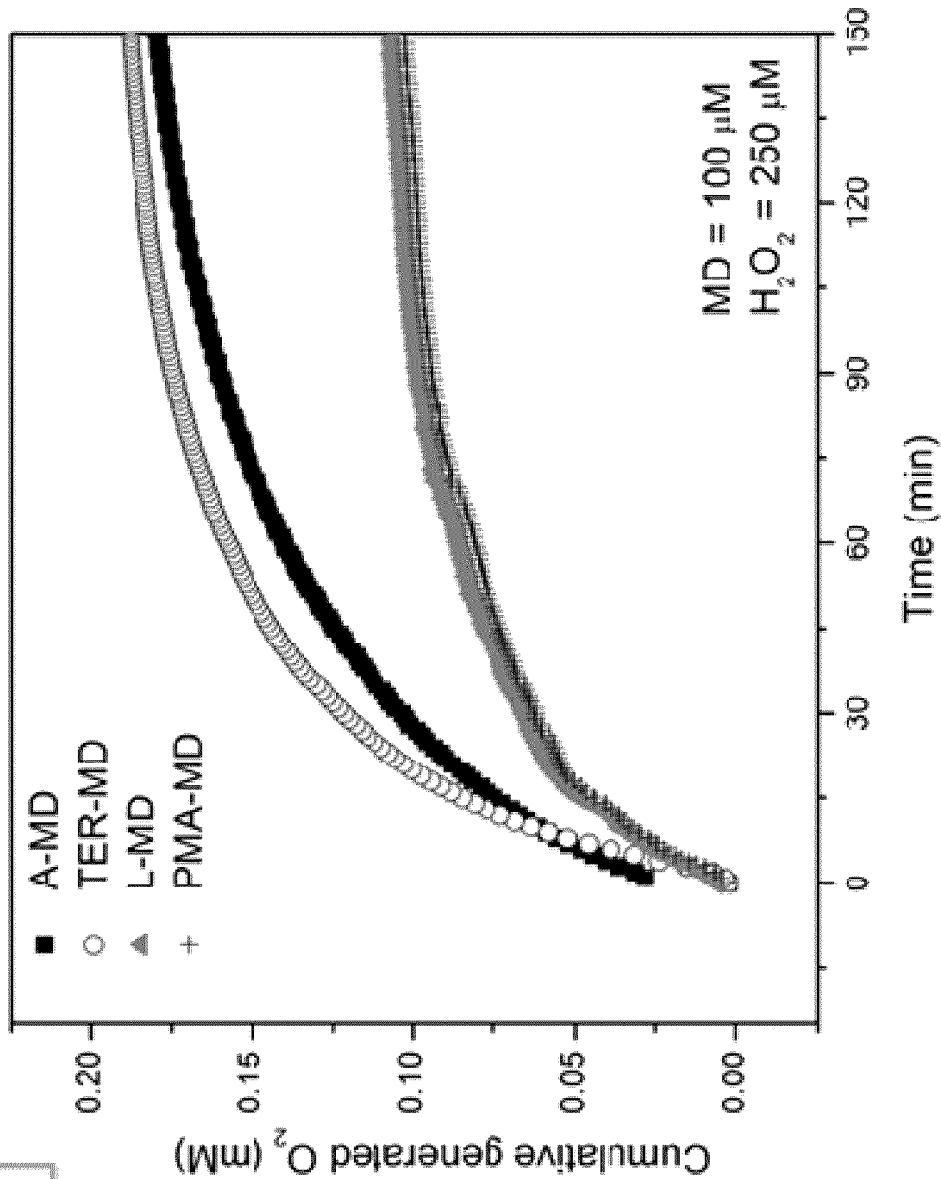


Fig. 20

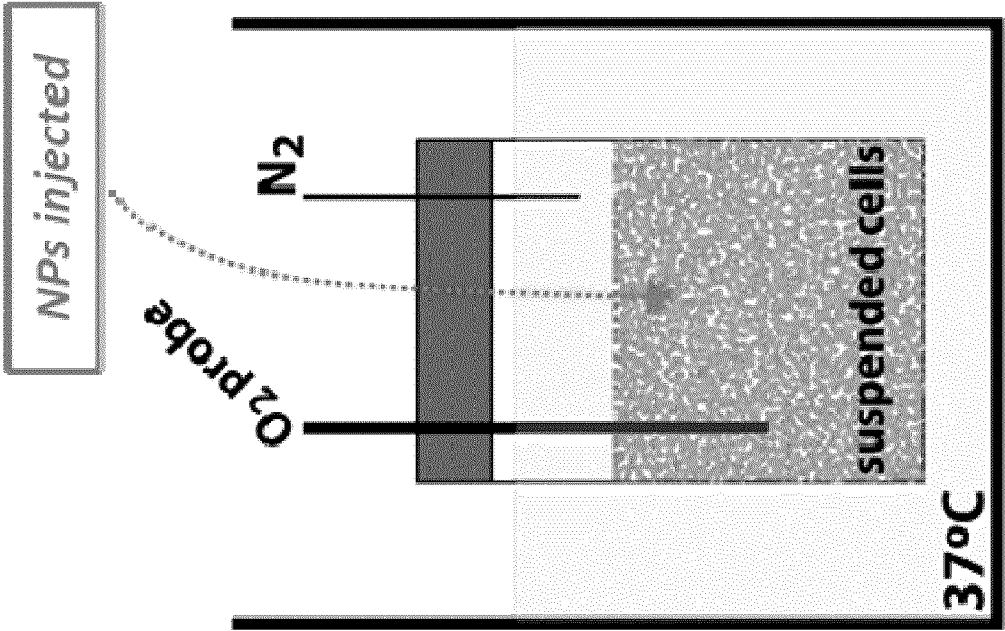


Fig. 21

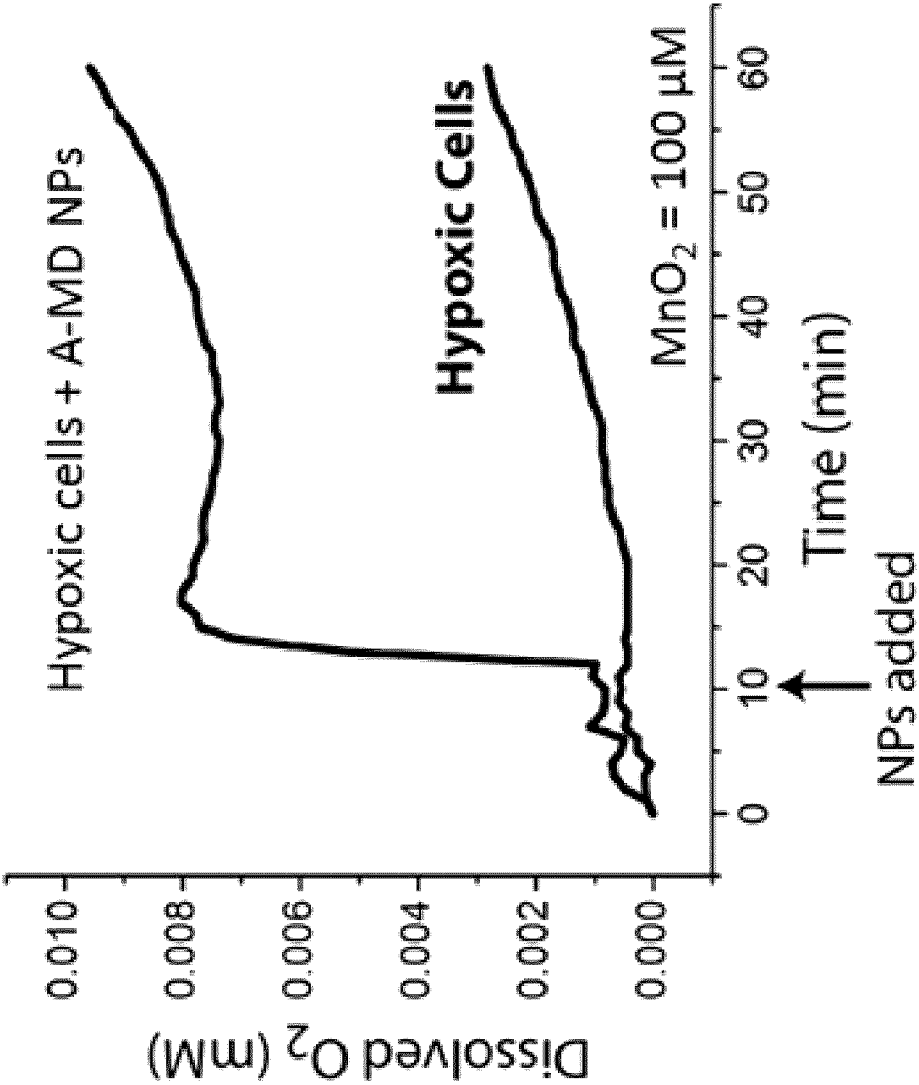


Fig. 22

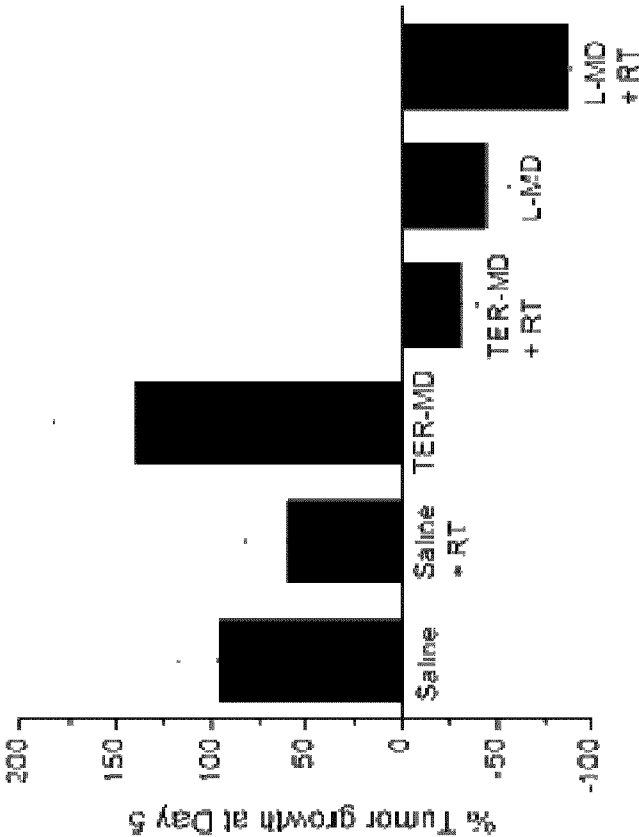


Fig. 23b

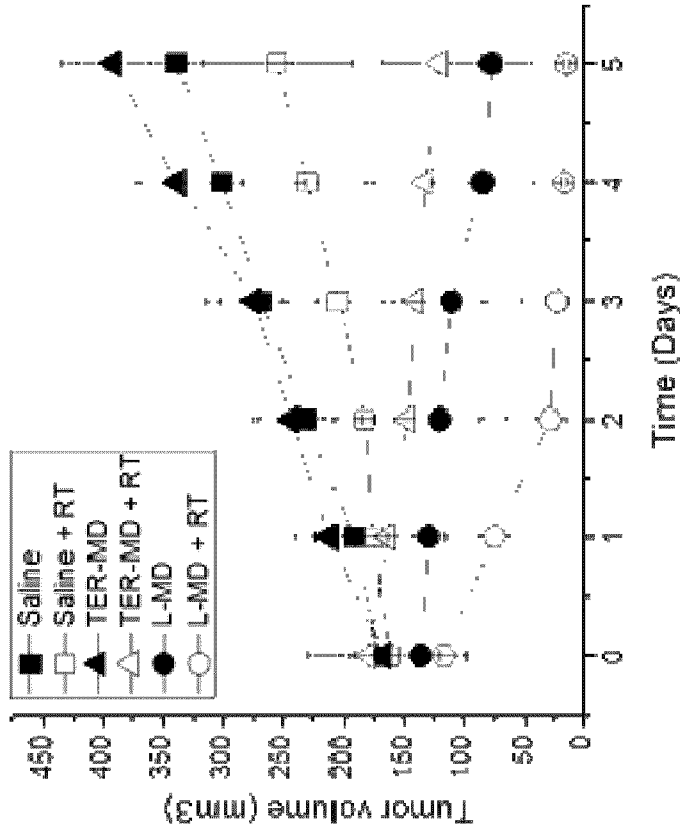


Fig. 23a

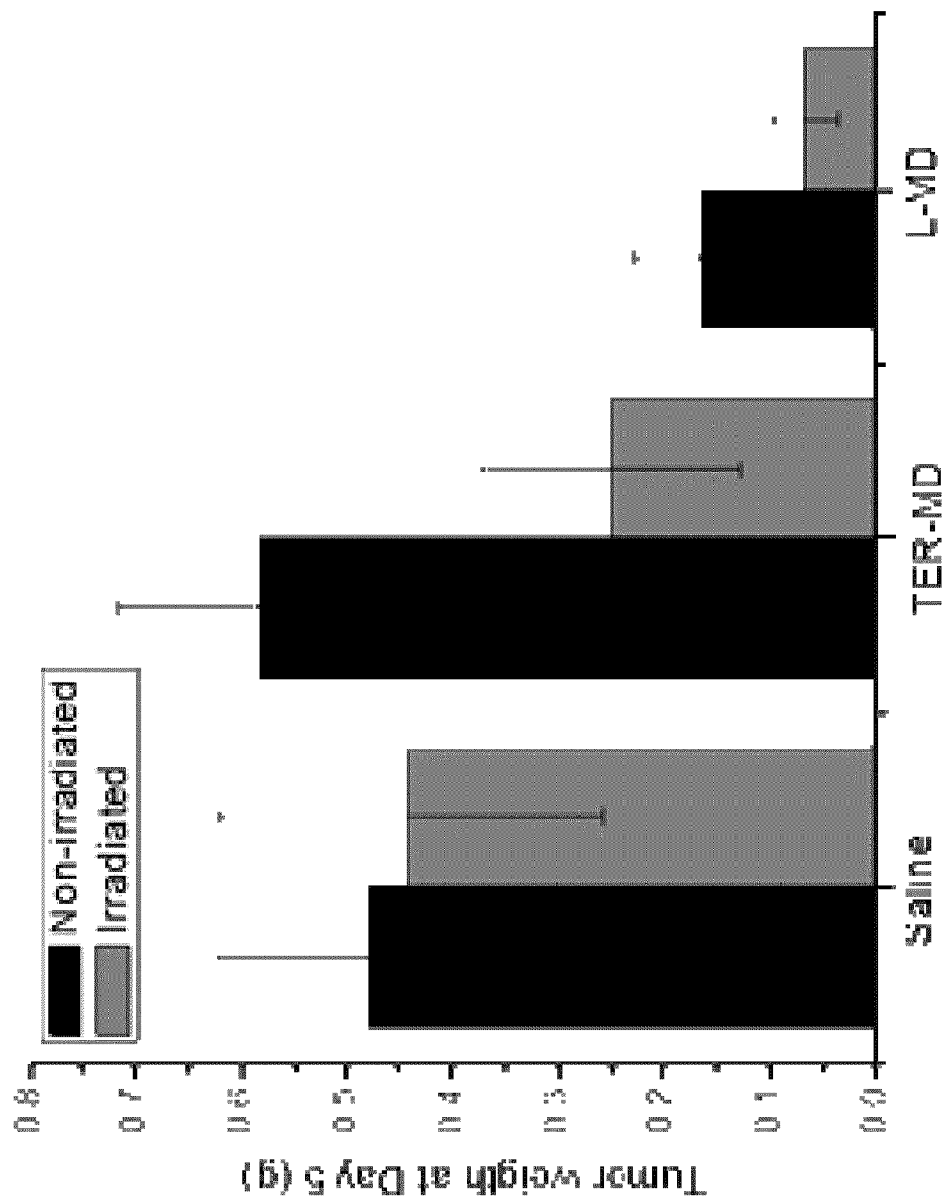


Fig. 24a

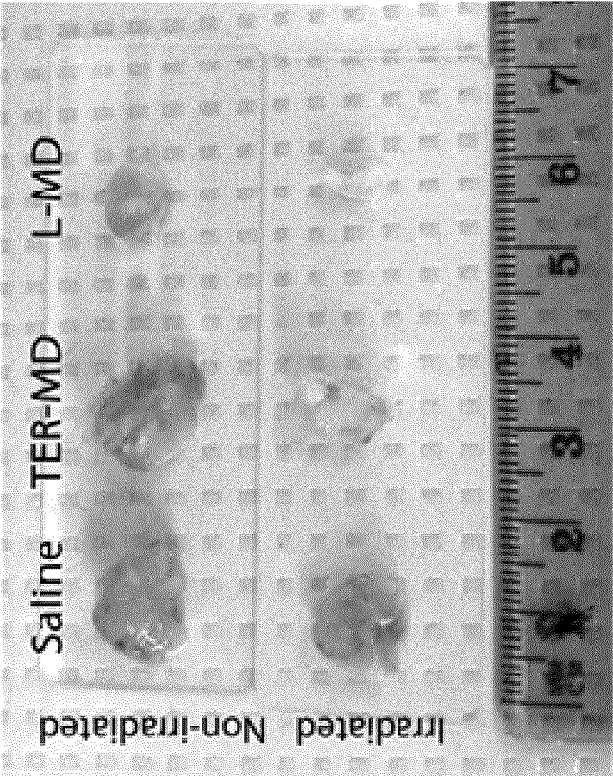


Fig. 24b

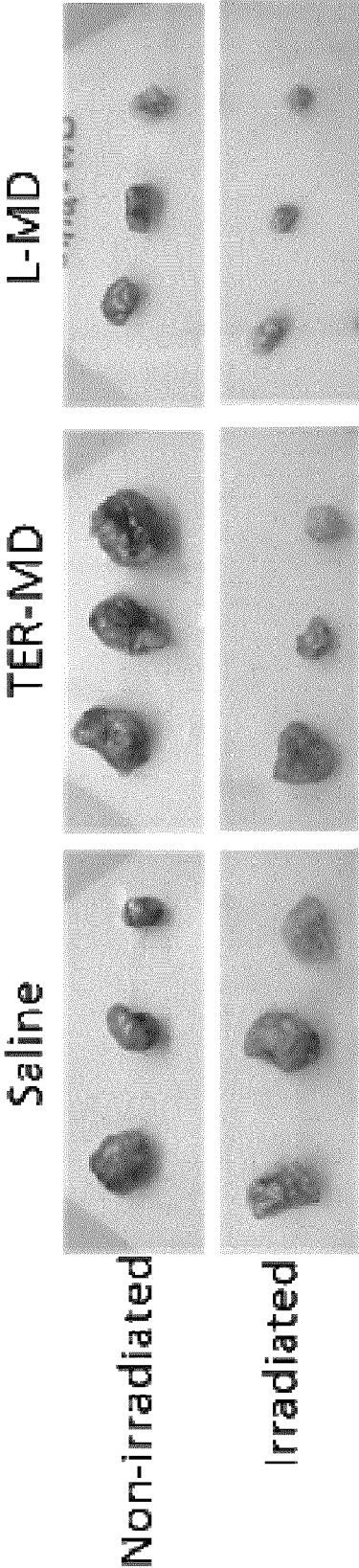


Fig. 24c

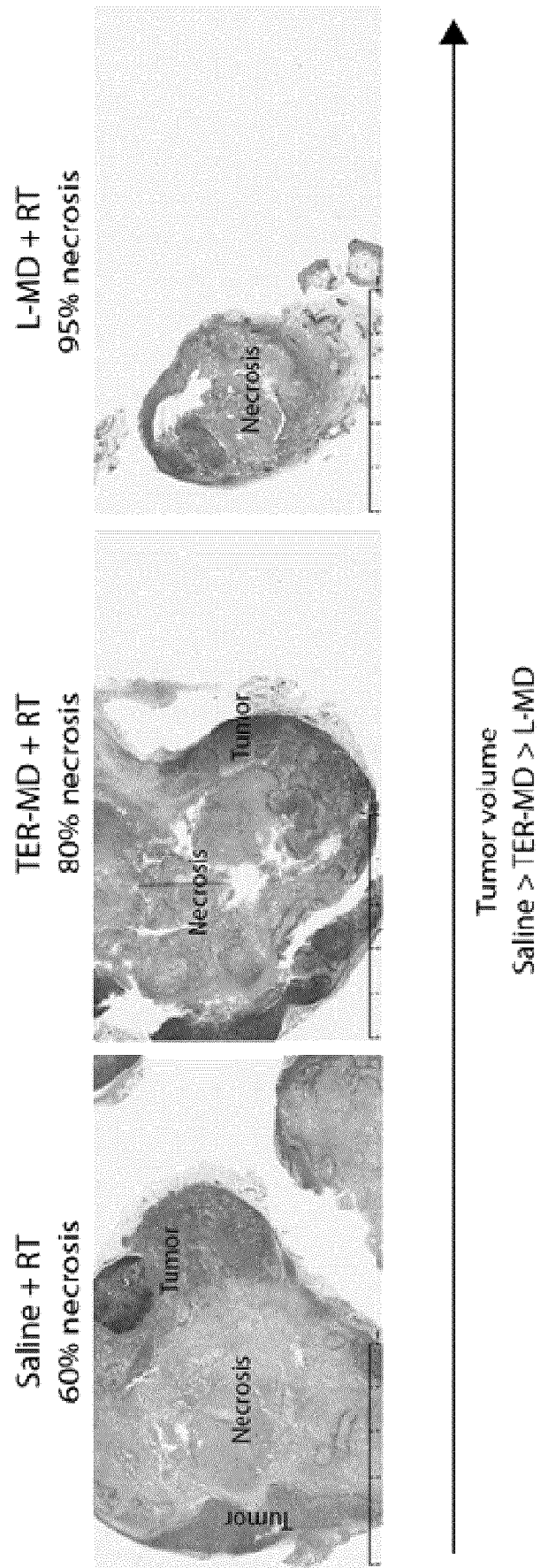


Fig. 25

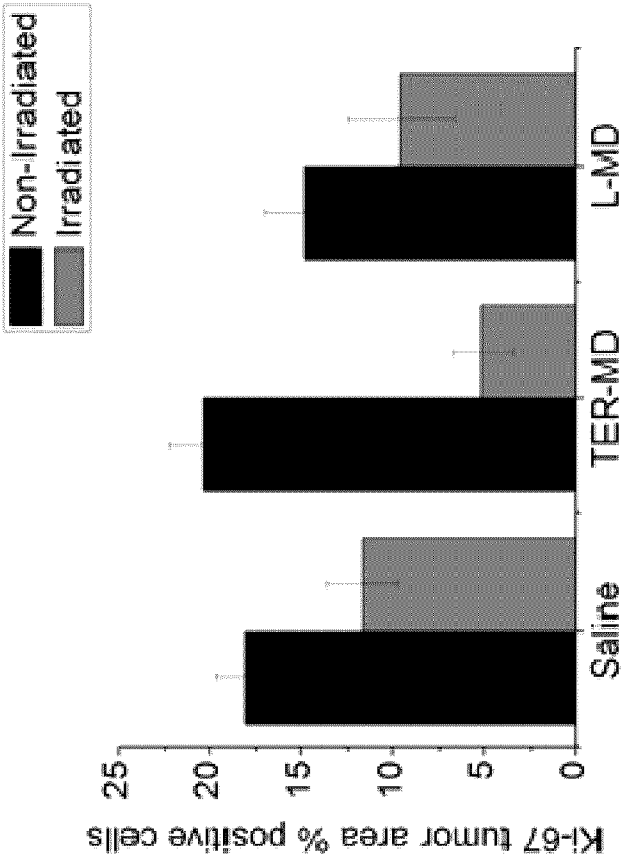


Fig. 26b

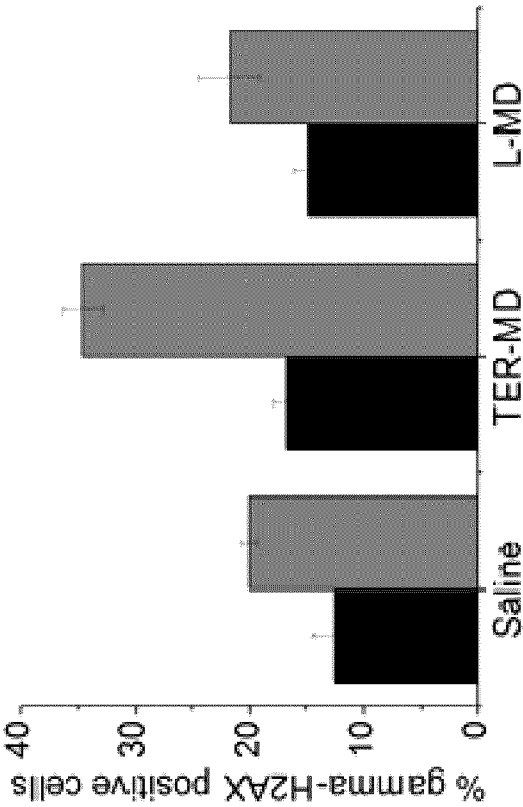


Fig. 26a

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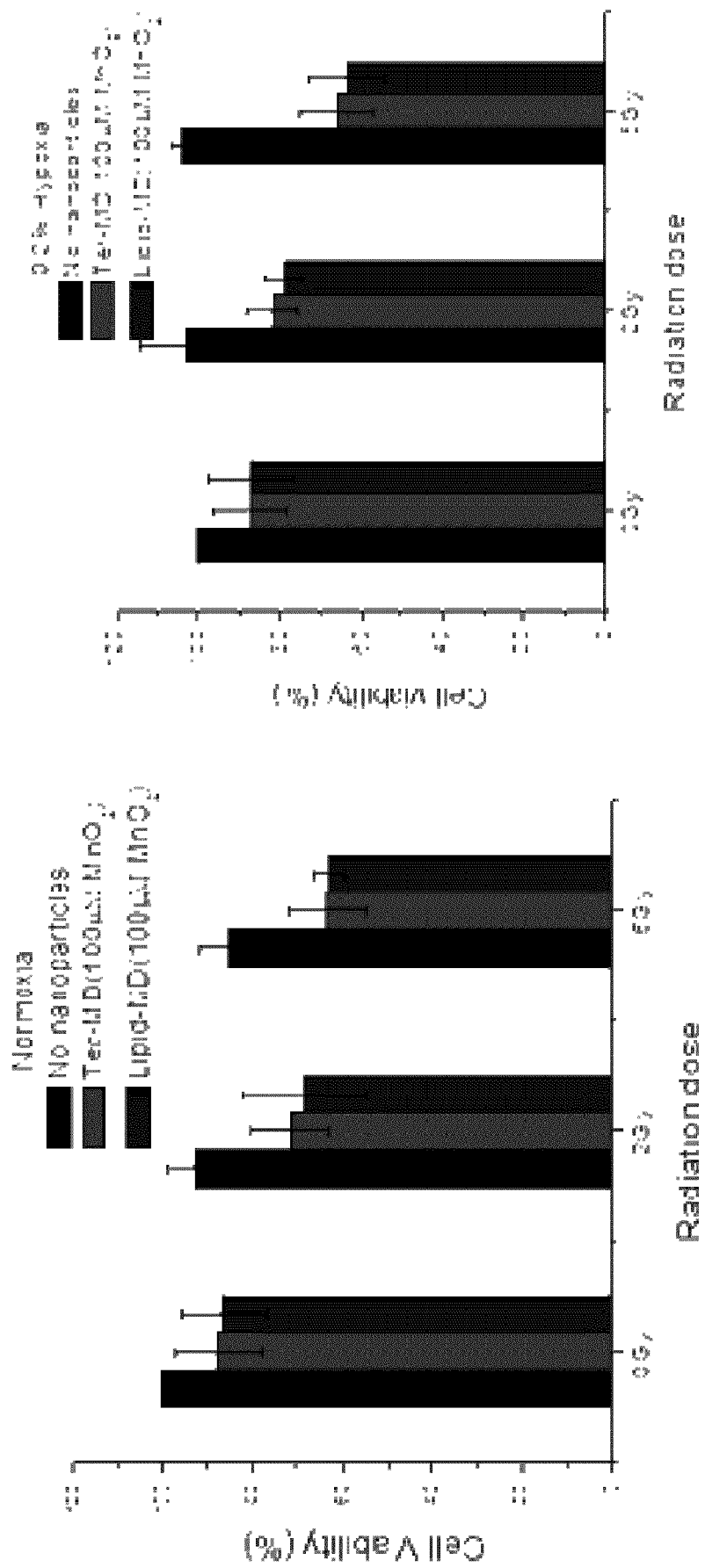


Fig. 27a

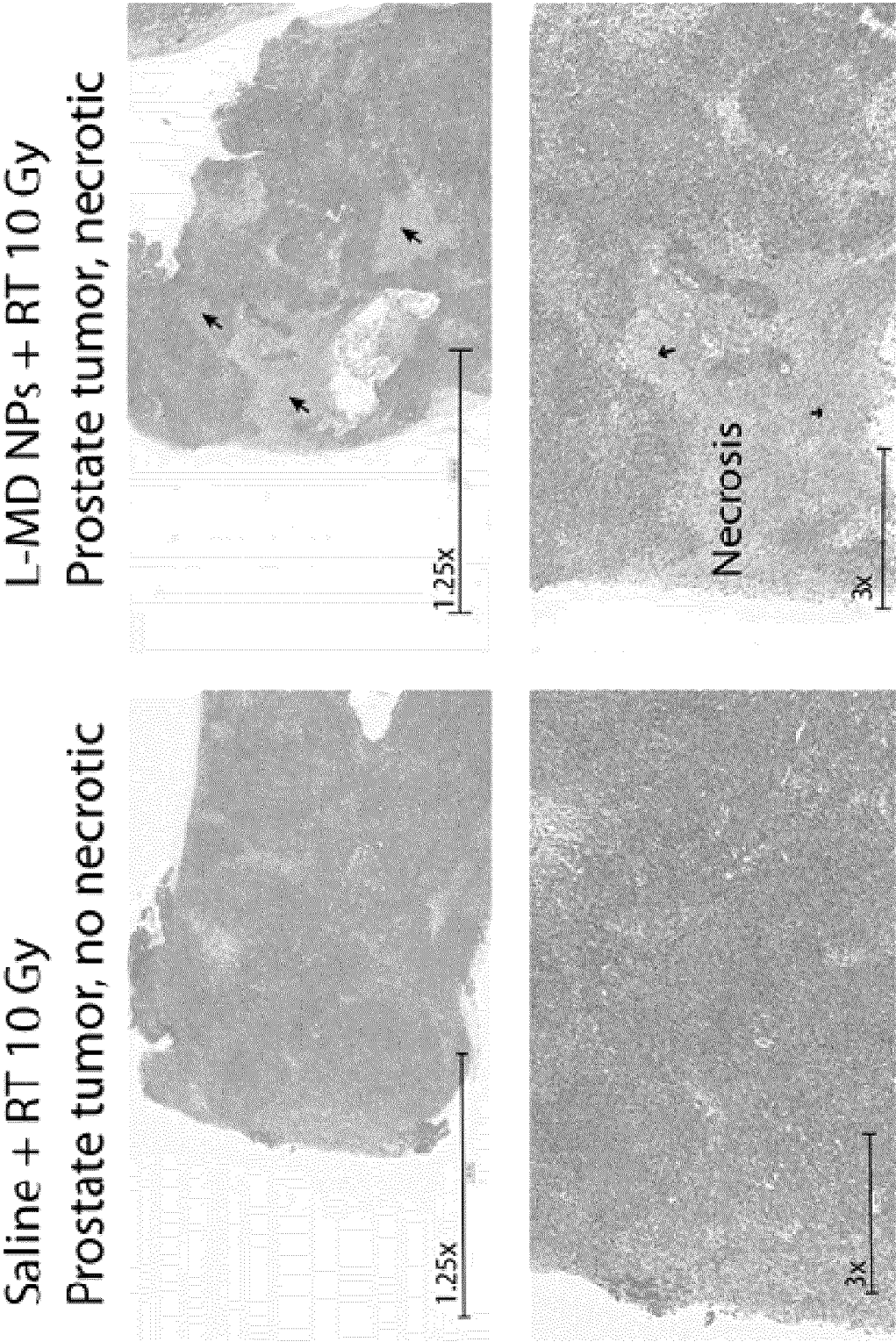


Fig. 27b

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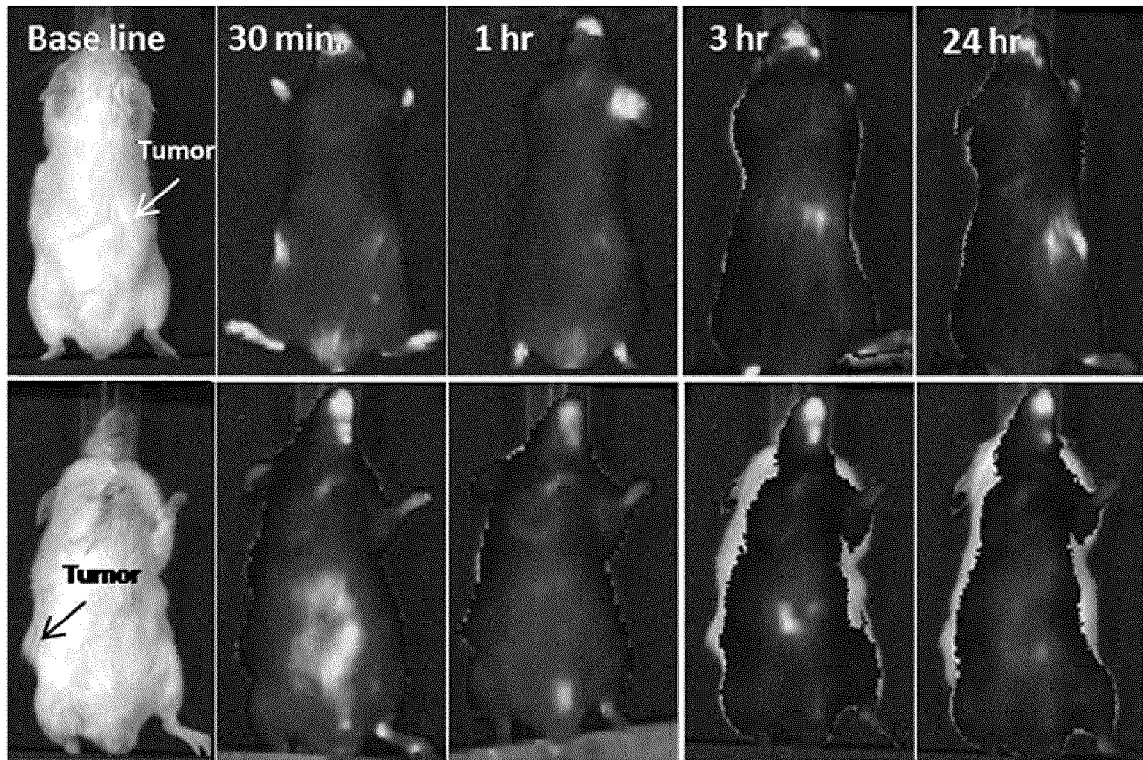


Fig. 27c

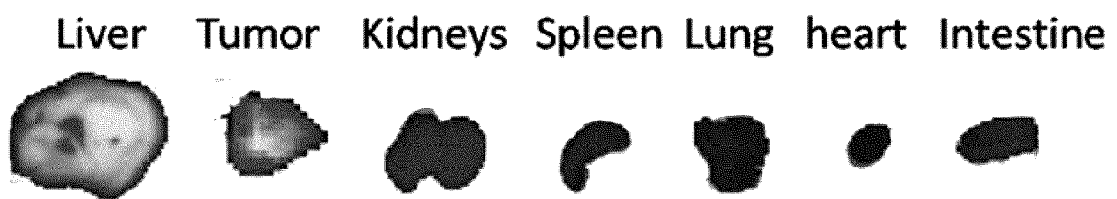


Fig. 27d

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2015/050277

A. CLASSIFICATION OF SUBJECT MATTER IPC: <i>A61K 9/14</i> (2006.01), <i>A61K 47/02</i> (2006.01), <i>A61K 47/30</i> (2006.01), <i>A61K 47/42</i> (2006.01), <i>A61L 31/12</i> (2006.01), <i>A61N 5/00</i> (2006.01) (more IPCs on the last page)		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K 17/00 A61K 9/14 A61K 47/02 A61K 47/30 A61K 47/42 A61L 31/12 A61N 5/00 A61P 35/00 C01B 13/00 C01G 1/02 C07H 21/00 C07K 17/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Questel Orbit database, Google Scholar, SCOPUS, Canadian Patent Database. Search terms used: manganese dioxide, matrix, coat, nanoparticle, metal oxide, allylamine, allylamine hydrochloride, bovine serum albumin, PAH,		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	D1 WO 2004/85998 (SHASTRI, V., CHEN, I., ZNIDARSIC, W.) 07-10-2004 (07 October 2004). Page 8, claims.	1-5, 7-10, 19, 23, 24, 33-41, 42, 46, and 48 --- 1-64 and 76-77
X	D2 US 2012/0114564 (HYEON, T., An, K., NA, H., LEE, J.) 10-05-2012 (10 May 2012). Claims.	1-8, and 76-77
Y	D3 <i>Poly(allylamine) stabilized iron oxide magnetic nanoparticles</i> . (EL KHOURY, J.M, CARUNTU, D, O'CONNOR, C.J, JEONG, K.-U, CHENG, S.Z.D., Hu, J.). Journal of Nanoparticle Research. Vol. 9. No. 5. October 2007. Pp. 959-964.	11-18, 20-22, 25-32, 43-45, 49-64
A	D4 <i>Multifunctional uniform core-shell Fe₃O₄@mSiO₂ mesoporous nanoparticles for bimodal imaging and photothermal therapy</i> (LI, J. JIANG, H., YU, Z., XIA, H., ZOU, G., ZHANG, Q., YU, Y.) February 2013. Vol. 8. No. 2. Pp. 385-391.	1-64 and 76-77
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 13 July 2015		Date of mailing of the international search report 22 July 2015 (22-07-2015)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer ADAM MACKENZIE (819) 994-6514

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2015/050277

A61P 35/00 (2006.01), *C01B 13/00* (2006.01), *C01G 1/02* (2006.01), *C07H 21/00* (2006.01),
C07K 17/00 (2006.01)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2015/050277

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 65-75
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 65-75 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to examine under Rule 67.1(iv) of the PCT. Moreover, this Authority has established a written opinion based on the alleged use of the claimed nanoparticles in the claimed treatment.
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2015/050277

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2004085998A2	07 October 2004 (07-10-2004)	WO2004085998A2 WO2004085998A3 US2007026069A1	07 October 2004 (07-10-2004) 29 December 2005 (29-12-2005) 01 February 2007 (01-02-2007)
US2012114564A1	10 May 2012 (10-05-2012)	US2012114564A1 AU2008211871A1 EP2117606A1 JP2010516760A KR20080071463A KR20080071472A KR20090119867A WO2008093999A1	10 May 2012 (10-05-2012) 07 August 2008 (07-08-2008) 18 November 2009 (18-11-2009) 20 May 2010 (20-05-2010) 04 August 2008 (04-08-2008) 04 August 2008 (04-08-2008) 20 November 2009 (20-11-2009) 07 August 2008 (07-08-2008)