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FOUNDATION, INC. [US/US]; Boyd Graduate Studies
Research Center, Athens, GA 30602-7411 (US).

(72) Inventors; and

(71) Applicants : DHAR, Shanta [IN/US]; 1500 Timothy
Road, Athens, GA 30606 (US). MARRACHE, Sean
[US/US]; 7280 SW Palmer Way, Beaverton, OR 97007
(US).(74) Agent: CAMPBELL, Keith, M.; Mueting, Raasch &
Gebhardt, P.A., PO Box 581336, Minneapolis, MN 55458-
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(54) Title: MITOCHONDRIAL DELIVERY OF 3-BROMOPYRUVATE

Fig. 1A

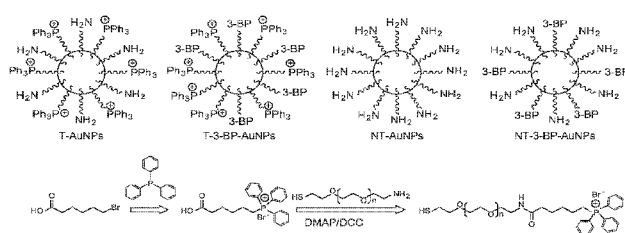
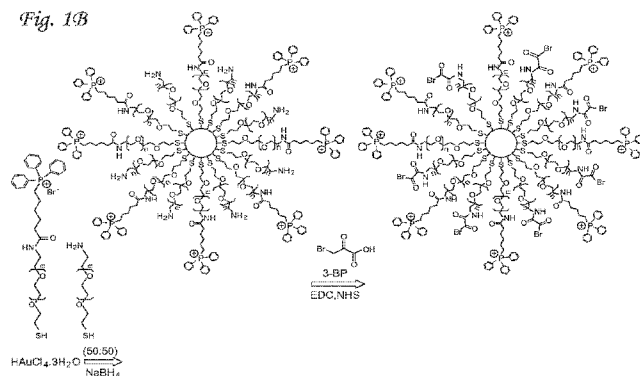


Fig. 1B



(57) Abstract: A mitochondria targeted gold nanoparticle (T-3-BP-AuNP) decorated with 3-bromopyruvate (3-BP) and delocalized lipophilic triphenylphosphonium (TPP) cations to target the mitochondrial membrane potential ($\Delta\psi_m$) was developed for delivery of 3-BP to cancer cell mitochondria by taking advantage of higher $\Delta\psi_m$ in cancer cell compared to normal cells. This construct showed remarkable anticancer activity in prostate cancer cells compared to non-targeted construct NT-3-BP-AuNP and free 3-BP. Anticancer activity of T-3-BP-AuNP was further enhanced upon laser irradiation by exciting the surface plasmon resonance band of AuNP and thereby utilizing a combination of 3-BP chemotherapeutic and AuNP photothermal effects. T-3-BP-AuNPs showed markedly enhanced ability to alter cancer cell metabolism by inhibiting glycolysis and demolishing mitochondrial oxidative phosphorylation in prostate cancer cells. Our findings demonstrated that mitochondria targeted and concerted chemo-photothermal treatment of glycolytic cancer cells with a single NP may have promise as a new anticancer therapy.

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MITOCHONDRIAL DELIVERY OF 3-BROMOPYRUVATE

RELATED APPLICATION

- [0001] This application claims the benefit of U.S. Provisional Application No. 61/953,159 filed on March 14, 2015, which application is hereby incorporated herein by reference in its entirety.

FIELD

- [0002] The present disclosure relates to nanoparticles configured to traffic agents that inhibit hexokinase, such as 3-bromopyruvate, to different compartments of mitochondria such as the outer mitochondrial membrane, the inner mitochondrial membrane, the inter membrane space and methods of use thereof.

INTRODUCTION AND SUMMARY

- [0003] The majority of cancers use aerobic glycolysis, which is accompanied by high rates of glucose consumption and lactate production, even when oxygen is available for oxidative phosphorylation (OXPHOS). Hexokinase (HK) is a key enzyme involved in the first step of glycolysis pathway in transferring a phosphate group from adenosine triphosphate (ATP) to glucose to produce glucose-6-phosphate. There are four different isoforms HK2-4 in mammalian cells which differ in their expression, subcellular localizations, and catalytic properties. HK 1 and 2 with abilities to bind to mitochondria through their interaction with voltage dependent anion channel (VDAC) play significant roles in the mitochondria homeostasis. The high glycolytic environment of aggressive cancers requires an up-regulation of HK2 protein level in cancer cells. Approximately 80% of HK2 are associated with mitochondria through their interaction with VDAC in cancer cells. Mitochondria-bound HK2 can acquire the required ATP for glucose phosphorylation to accelerate the glycolytic rate and

stimulate the tricarboxylic acid (TCA) cycle in the mitochondria. Interactions of VDAC and HK2 on the outer mitochondrial membrane (OMM) not only increase mitochondrial energy metabolism, but also down-regulate the apoptotic pathway by suppressing cytochrome c release. Increased association of HK2 with the mitochondria in cancer cells *via* VDAC, which has privileged access to mitochondrial ATP and regulates the opening of the mitochondrial permeability transition (MTP) pore causes resistance to apoptosis for cancer cells. This significant translocation of HK2 from cytoplasm to the OMM in cancer cells combined with its important roles in glycolytic pathway makes HK2 an attractive target for anticancer therapeutic modality development. A brominated derivative of pyruvic acid, 3-bromopyruvate (3-BP) is an anti-tumoral alkylating agent that selectively kills cancer cells by inhibiting HK2. 3-BP has the ability to suppress glycolytic capacity of tumor by abolishing the activity of mitochondrially-bound HK2. 3-BP also participates in the inhibition of mitochondrial succinate dehydrogenase, mitochondrial phosphate carrier (PIC), and adenine nucleotide carrier (ANC).

[0004] The characteristic alterations of mitochondrial function in cancer, association of HK2 to the OMM via VDAC, the ability of 3-BP in inhibition of HK2, and subsequent apoptosis of cancer cells led to many studies to evaluate the therapeutic potential of 3-BP. However, 3-BP showed limited clinical potential caused by non-specificity such as interaction with HK1 or other metabolic proteins and systemic toxicity. Once inside cells, 3-BP has several nonglycolytic targets, such as V-ATPases, sarcoplasmic reticulum Ca^{2+} -ATPases, carbonic anhydrases, and histone deacetylases. A family of proton-coupled monocarboxylate transporters (MCTs) are involved in the transport of monocarboxylic acids such as lactate, pyruvate, butyrate, and acetate across the plasma membrane.

[0005] Although these transporters are up-regulated in cancer cells to compensate the increased lactic acid production and consequent efflux by the cell, we hypothesized that 3-BP will face tremendous challenge for its uptake by the MCTs in cancer cells. To circumvent this, mitochondria-targeted local delivery strategies can be extremely important to enhance overall treatment efficacy and to reduce toxic effects of 3-BP.

- [0006] A variety of targeted-nanoparticles (NPs) have the ability to take the current medicine to the next level by precise delivery, improving pharmacokinetics (PK) and biodistribution (bioD) of small molecule based drugs. One of the most studied nanostructures in medicine is gold NPs (AuNPs) which can be constructed in different sizes, shapes, and have the ability to be decorated with drug molecules and targeting moieties. Furthermore, when AuNPs are illuminated with light of wavelengths of 650-900 nm, the photons will be strongly absorbed or scattered, the absorbed light can be transformed into heat, a process known as the photothermal effect, causing an increase in temperature. The heat generated from the photothermal effect can be used to destroy cancer cells directly.
- [0007] With these properties in mind, we hypothesized that the use of a mitochondria-targeted AuNP delivery system for 3-BP will allow the delivery of this HK2 inhibitor specifically to mitochondria and subsequent laser irradiation will further enhance the therapeutic efficacy of the engineered construct by photothermal effect. In this disclosure, we highlight the findings related to remarkable increase in anti-tumor activity of 3-BP by mitochondrial delivery using a triphenylphosphonium (TPP) cation modified AuNP (T-3-BPAuNP) (**FIG. 1**), efficient inhibition of glycolysis, and subsequent changes in the mitochondrial bioenergetics.
- [0008] In more general terms, nanoparticles, as described in embodiments herein, include a hexokinase-2 inhibitor and a mitochondrial targeting moiety. A gold nanoparticle may include any suitable hexokinase-2 inhibitor. Examples of suitable hexokinase-2 inhibitors include 3-bromopyruvate, 2-deoxy-D-glucose, lonidamine, or the like. The nanoparticles, in embodiments, include a radiation excitable agent that heats on exposure to radiation. Examples of such agents include gold nanoparticles, such as gold nanorods, quantum dots and silver nanoparticles. In embodiments, the nanoparticles comprise carbon nanotubes.
- [0009] Advantages of one or more of the various embodiments presented herein over prior nanoparticles, imaging methodologies, treatment modalities, or the like will be readily apparent to those of skill in the art based on the following detailed description when read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0010] FIG. 1:** Schematic drawing illustrating engineered AuNP and control NPs used in mitochondria-targeted delivery of 3-BP; including synthesis of mitochondria-targeted TPP-modified PEG ligand, T-AuNP, and conjugation of 3-BP.
- [0011] FIG. 2:** (A) Characterization of different AuNPs by DLS. (B) TEM of AuNPs. (C) Quantification of 3-BP by GADPH assay. (D) Non-immunogenic behavior of NT and T-AuNPs by ELISA.
- [0012] FIG. 3:** (A) Protease dependent release of 3-BP from T and NT-AuNPs. (B) HK2 binding profile of T-3-BP-AuNPs and NT-3-BP-AuNPs. (C) Mitochondrial and cytosolic distribution of T-AuNPs and NT-AuNPs and time-dependent distribution of T-AuNPs and T-3-BP-AuNPs in different mitochondrial.
- [0013] FIG. 4:** (A) Cells viability in PC3 and DU145 cells after treatment with T-AuNPs, NT-AuNPs, 3-BP, T-3-BP-AuNPs, NT-3-BP-AuNPs. Cell viability was assessed by the MTT assay after treatment with the indicated concentrations of the test articles for 72 h. The data are mean $\pm$ SD ($n = 3$ wells). (B) Cells viability in PC3 cells after treatment with T-AuNPs, NT-AuNPs, 3-BP, T-3-BP-AuNPs, NT-3-BP-AuNPs followed by 660 nm laser radiation for 1 min/well. The data are mean $\pm$ SD ($n = 3$ wells). (C) (Top) Apoptotic patterns induced in PC3 cells upon treatment with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 μ g/mL with respect to NP, 10 μ M with respect to 3-BP), NT-3-BP-AuNP (13.7 μ g/mL with respect to NP, 10 μ M with respect to 3-BP), free 3-BP (10 μ M) for 6 h at 37 °C in the dark. (Bottom) Apoptosis induced in PC3 cells upon treatment with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 μ g/mL with respect to NP, 10 μ M with respect to 3-BP), NT-3-BP-AuNP (13.7 μ g/mL with respect to NP, 10 μ M with respect to 3-BP), free 3-BP (10 μ M) for 4 h at 37 °C, followed by irradiation with 660 nm laser for 1 min, and further incubation for 12 h. Annexin V-PI assay results are presented in quadrants as analyzed by flow cytometry. The x-axis represents Alexa Fluor® 488 Annexin V and the y-axis represents PI. Live cells accumulate in Q4 (Annexin V and

PI negative), cells undergoing apoptosis accumulate in Q3 (Annexin V positive and PI negative), cells in end-stage apoptosis or necrotic stage accumulate in Q2 (Annexin V and PI positive).

[0014] **FIG. 5:** Intracellular (A) and extracellular (B) lactate levels in PC3 cells after treatment with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 µg/mL with respect to NP, 10 µM with respect to 3-BP), NT-3-BP-AuNP (13.7 µg/mL with respect to NP, 10 µM with respect to 3-BP), free 3-BP (10 µM) for 6 h at 37 °C. (C) Changes in intracellular ATP content in PC3 cells after treatment with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 µg/mL with respect to NP, 10 µM with respect to 3-BP), NT-3-BP-AuNP (13.7 µg/mL with respect to NP, 10 µM with respect to 3-BP), free 3-BP (10 µM) for 6 h at 37 °C in 5% CO₂ atmosphere. Results are represented as mean ± SD (n=3). Statistical analyses were performed by using one-way ANOVA with Tukey post hoc test.

[0015] **FIG. 6:** Bioenergetics analyses in PC3 cell line. (A) A representative graph of ECAR output from XF24 analyzer of control, T-3-BP-AuNP, NT-3-BP-AuNP, and free 3-BP treated PC3 cells and its response to glucose, oligomycin, and 2-DG and (B) comparison of glycolysis, glycolytic capacity, and glycolytic reserve in the treated cells. (C) A representative graph of OCR output from XF24 analyzer of control, T-3-BP-AuNP, NT-3-BP-AuNP, and free 3-BP treated PC3 cells and its response to oligomycin, FCCP, antimycin A/rotenone and comparison of (D) spare respiratory capacity, coupling efficiency, and (E) ETC accelerator response and basal respiration in the treated cells.

[0016] **FIG. 7:** Schematic drawing of various cellular targets and mechanism of action of 3-BP when it is delivered with a mitochondria-targeted delivery system.

[0017] The schematic drawings presented herein are not necessarily to scale.

DETAILED DESCRIPTION

- [0018] In the following detailed description, reference is made to the accompanying drawings that form a part hereof, and in which are shown by way of illustration several specific embodiments of devices, systems and methods. It is to be understood that other embodiments are contemplated and may be made without departing from the scope or spirit of the present disclosure. The following detailed description, therefore, is not to be taken in a limiting sense.
- [0019] Nanoparticles, as described in embodiments herein, include a hexokinase-2 inhibitor, a mitochondrial targeting moiety and a radiation excitable agent. In embodiments, the nanoparticles are gold nanoparticles.
- [0020] The nanoparticles may be formed in any suitable manner. For example, gold nanoparticles may be produced in a liquid by reducing chloroauric acid ($\text{H}[\text{AuCl}_4]$). After dissolving chloroauric acid, the solution can rapidly stirred while a reducing agent such as NaBH_4 is added, causing Au^{3+} ions to be reduced to neutral gold atoms. As more and more of these gold atoms form, the solution becomes supersaturated, and gold gradually starts to precipitate. Agents, such as mitochondrial targeting agents, hexokinase inhibitors and the like may be attached to the gold nanoparticles through an appropriate linker having a reducing group, such as a SH moiety. The agents may be attached to the linkers before or following attachment to the gold particles.
- [0021] In embodiments, the nanoparticles are silver nanoparticles or carbon nanotubes, which may be synthesized through any suitable technique. Several synthesis schemes for producing silver nanoparticles or carbon nanotubes having bound agents are well known to those of skill in the art. If the nanoparticles are not formed from a radiation excitable core (e.g., if the nanoparticles are not gold nanoparticle, silver nanoparticles, etc.), then a radiation excitable agent may be bond to the core.
- [0022] The nanoparticles described herein may optionally include a hydrophilic layer surrounding the core (e.g., surrounding a gold nanoparticle core). The hydrophilic layer may assist the nanoparticle in evading recognition by the immune system and may enhance circulation half-life of the nanoparticle.

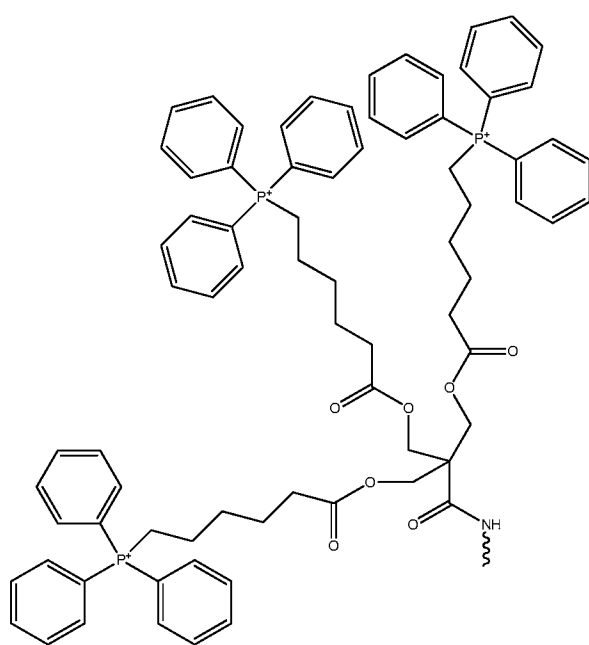
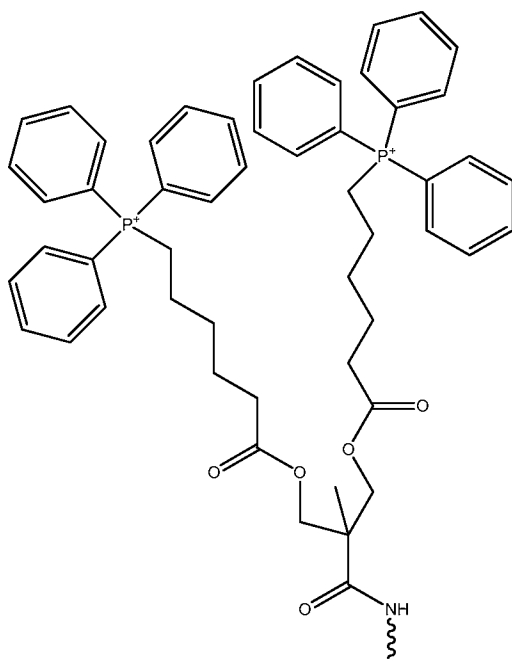
- [0023] The hydrophilic layer may be formed, in whole or in part, by a hydrophilic portion of an amphiphilic polymer, such as a block co-polymer having a hydrophobic block and a hydrophilic block; a polysaccharide or the like.
- [0024] Any suitable hydrophilic polymer or hydrophilic portion of an amphiphilic polymer may form the hydrophilic layer or portion thereof. The hydrophilic polymer or hydrophilic portion of a polymer may be a linear or branched or dendritic polymer. Examples of suitable hydrophilic polymers include polysaccharides, dextran, chitosan, hyaluronic acid, polyethylene glycol (PEG), polymethylene oxide, and the like.
- [0025] Nanoparticles having a hydrophilic layer may have any suitable diameter. In embodiments, nanoparticles have a diameter of about 250 nm or less; e.g. about 200 nm or less. In embodiments, a nanoparticle effective for mitochondrial targeting has a diameter of about 190 nm or less, about 180 nm or less, about 170 nm or less, about 160 nm or less, about 150 nm or less, about 140 nm or less, about 130 nm or less, about 120 nm or less, about 110 nm or less, about 100 nm or less, about 90 nm or less, about 80 nm or less, about 70 nm or less, about 60 nm or less, about 50 nm or less, about 40 nm or less, about 30 nm or less, about 20 nm or less, or about 10 nm or less. In embodiments, a nanoparticle has a diameter of from about 10 nm to about 250 nm, such as from about 20 nm to about 200 nm, from about 50 nm to about 160 nm, from about 60 nm to about 150 nm, from about 70 nm to about 130 nm, from about 80 nm to about 120 nm, from about 80 nm to about 100 nm, or the like.
- [0026] The nanoparticles described herein include one or more moieties that target the nanoparticles to mitochondria. As used herein, “targeting” a nanoparticle to mitochondria means that the nanoparticle accumulates in mitochondria relative to other organelles or cytoplasm at a greater concentration than substantially similar non-targeted nanoparticle. A substantially similar non-target nanoparticle includes the same components in substantially the same relative concentration (e.g., within about 5%) as the targeted nanoparticle, but lacks a targeting moiety.
- [0027] The mitochondrial targeting moieties may be tethered to the core in any suitable manner. In embodiments, a targeting moiety is bound to a gold nanoparticle core via a

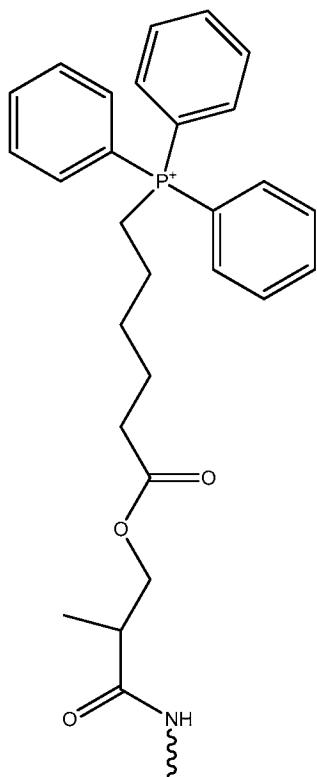
reduced sulfhydryl moiety of a linker. More than one targeting moiety may be bound to a given nanoparticle.

[0028] Examples of targeting moieties tethered to polymers presented throughout this disclosure for purpose of illustrating the types of reactions and tethering that may occur. However, one of skill in the art will understand that tethering of targeting moieties to nanoparticles may be carried out according to any of a number of known chemical reaction processes.

[0029] Targeting moieties may be present in the nanoparticles at any suitable concentration. In embodiments, the concentration may readily be varied based on initial *in vitro* analysis to optimize prior to *in vivo* study or use. In embodiments, the targeting moieties will have surface coverage of from about 5% to about 100%.

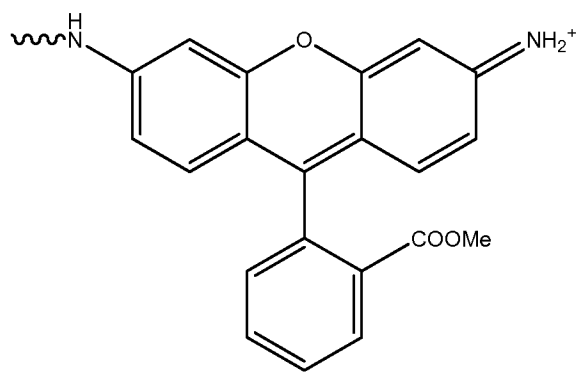
[0030] Any suitable moiety for facilitating accumulation of the nanoparticle within the mitochondria may be employed. Due to the substantial negative electrochemical potential maintained across the inner mitochondrial membrane, delocalized lipophilic cations are effective at crossing the hydrophobic membranes and accumulating in the mitochondria. Triphenyl phosphonium (TPP) containing compounds can accumulate greater than 10 fold within the mitochondrial matrix. Any suitable TPP-containing compound may be used as a mitochondrial matrix targeting moiety. Representative examples of TPP-based moieties may have structures indicated below in **Formula I**, **Formula II** or **Formula III**:





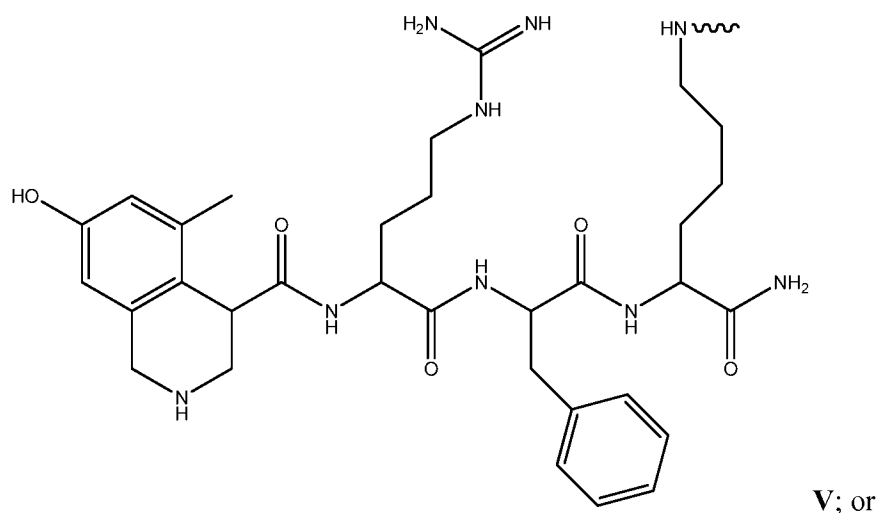
where the amine (as depicted) may be conjugated to a linker such as a polymer or other component for incorporation into the nanoparticle. Alternatively, the amine may be a sulfhydryl (not shown) or other suitable reducing moiety.

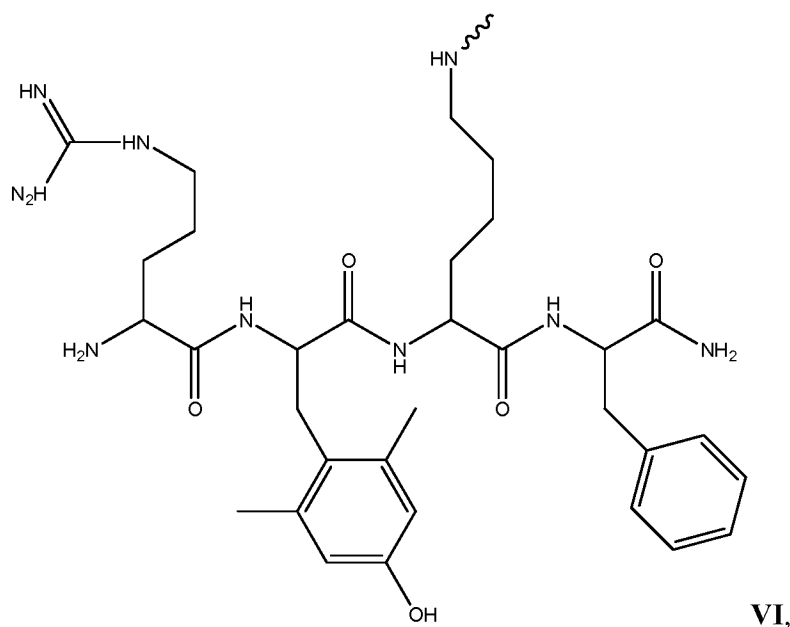
[0031] In embodiments, the delocalized lipophilic cation for targeting the mitochondrial matrix is a rhodamine cation, such as Rhodamine 123 having **Formula IV** as depicted below:



where the secondary amine (as depicted) may be conjugated to a linker such as a polymer, lipid, or the like for incorporation into the nanoparticle. Alternatively, the amine may be a sulfhydryl (not shown) or other suitable reducing moiety.

[0032] Of course, non-cationic compounds may serve to target and accumulate in the mitochondrial matrix. By way of example, Szeto-Shiller peptide may serve to target and accumulate a nanoparticle in the mitochondrial matrix. Any suitable Szeto-Shiller peptide may be employed as a mitochondrial matrix targeting moiety. Non-limiting examples of suitable Szeto-Shiller peptides include SS-02 and SS-31, having **Formula V** and **Formula VI**, respectively, as depicted below:





where the secondary amine (as depicted) may be conjugated to a linker such as a polymer, lipid, or the like for incorporation into the nanoparticle. Alternatively, the amine may be a sulfhydryl (not shown) or other suitable reducing moiety.

- [0033] A targeting moiety can be attached to a hydrophilic polymer or hydrophilic portion of a polymer so that the targeting moiety will extend from the core of the nanoparticle to facilitate the effect of the targeting moiety.
- [0034] A nanoparticle, as described herein, may include any one or more hexokinase inhibitors. The hexokinase inhibitor may be a hexokinase-2 inhibitor. Examples of suitable hexokinase inhibitors include 3-bromopyruvate, 2-deoxy-D-glucose, lonidamine, and the like.
- [0035] The one or more therapeutic agents may be attached to the core of the nanoparticle via a linker and may be released from the core at a desired rate.
- [0036] In embodiments, a therapeutic agent or precursor thereof is conjugated to a linker attached to the core in a manner similar to that described above with regard to targeting moieties. The therapeutic agent may be conjugated via a cleavable linker so that the agent may be released when the nanoparticle reaches the target location, such as mitochondria.

- [0037] The therapeutic agents may be present in the nanoparticle at any suitable concentration. For example, a therapeutic agent may be present in the nanoparticle at a concentration from about 0.01% to about 30% by weight of the nanoparticle.
- [0038] In embodiments, the nanoparticle is useful for treatment of cancer in a subject in need thereof. Any suitable cancer may be treated. In embodiments, the cancer is prostate cancer.
- [0039] The performance and characteristics of nanoparticles produced herein may be tested or studied in any suitable manner. By way of example, therapeutic efficacy can be evaluated using cell-based assays. Toxicity, bio-distribution, pharmacokinetics, and efficacy studies can be tested in cells or rodents or other mammals. Rodents, rabbits, pigs, or the like may be used to evaluate diagnostic or therapeutic potential of nanoparticles. Some additional details of studies that may be performed to evaluate the performance or characteristics of the nanoparticles, which may be used for purposes of optimizing the properties of the nanoparticles are described below. However, one of skill in the art will understand that other assays and procedures may be readily performed.
- [0040] Uptake and binding characteristics of nanoparticles may be evaluated in any suitable cell line, such as RAW 264.7, J774, jurkat, and HUVECs cells. The immunomodulatory role of nanoparticles may be assayed by determining the release of cytokines when these cells are exposed to varying concentrations of nanoparticles. Complement activation may be studied to identify which pathways are triggered using columns to isolate opsonized nanoparticles; e.g. as described in Salvador-Morales C, Zhang L, Langer R, Farokhzad OC, Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups, *Biomaterials* 30: 2231-2240, (2009). Because nanoparticle size is an important factor that determines biodistribution, Nanoparticles may be binned into various sizes (e.g., 20-40, 40-60, 60-80, 80-100, 100-150, and 150-300 nm) and tested according to size.
- [0041] Any cell type appropriate for a therapeutic agent employed in a nanoparticle may be used to evaluate therapeutic efficacy or proper targeting. Assays appropriate for the

therapeutic or pharmacologic outcome may be employed, as are generally understood or known in the art.

- [0042] Biodistribution (bioD) and pharmacokinetic (PK) studies may be carried out in rats or other suitable mammals. For PK and bioD analysis, Sprague Dawley rats may be dosed with targeted or similar nanoparticles without the targeting groups, through a lateral tail vein injection. Animals may be sacrificed; and brain, heart, intestine, liver, spleen, kidney, muscle, bone, lung, lymph nodes, gut, and skin may be excised, weighed, homogenized to evaluate distribution. Tissue concentration may be expressed as % of injected dose per gram of tissue (%ID/g).
- [0043] Therapeutic dosages of nanoparticles effective for human use can be estimated from animal studies according to well-known techniques, such as surface area or weight based scaling.
- [0044] All scientific and technical terms used herein have meanings commonly used in the art unless otherwise specified. The definitions provided herein are to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.
- [0045] As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” encompass embodiments having plural referents, unless the content clearly dictates otherwise. As used in this specification and the appended claims, the term “or” is generally employed in its sense including “and/or” unless the content clearly dictates otherwise.
- [0046] As used herein, “have”, “having”, “include”, “including”, “comprise”, “comprising” or the like are used in their open ended sense, and generally mean “including, but not limited to”. It will be understood that “consisting essentially of”, “consisting of”, and the like are subsumed in “comprising” and the like.
- [0047] As used herein, “disease” means a condition of a living being or one or more of its parts that impairs normal functioning. As used herein, the term disease encompasses terms such disease, disorder, condition, dysfunction and the like.

- [0048] As used herein, “treat” or the like means to cure, prevent, or ameliorate one or more symptom of a disease.
- [0049] As used herein, “bind,” “bound,” or the like means that chemical entities are joined by any suitable type of bond, such as a covalent bond, an ionic bond, a hydrogen bond, van der Waals forces, or the like. “Bind,” “bound,” and the like are used interchangeable herein with “attach,” “attached,” and the like.
- [0050] As used herein, a “derivative” of a compound is a compound structurally similar to the compound of which it is a derivative. Many derivatives are functional derivatives. That is, the derivatives generally provide a desired function similar to the compound to which it is a derivative. By way of example, triphenyl phosphonium (TPP) is described herein as a mitochondrial targeting moiety because it can accumulate, or cause a compound or complex (such as a nanoparticle) to which it is bound to accumulate, in the mitochondria. Accordingly, a functional derivative of TPP is a derivative of TPP that may accumulate, or cause a compound or complex to which it is bound to accumulate, in the mitochondria in a similar concentration as TPP (e.g., within about a 100 fold concentration range, such as within about a 10 fold concentration range).
- [0051] In the following, non-limiting examples are presented, which describe various embodiments of representative nanoparticles, methods for producing the nanoparticles, and methods for using the nanoparticles.

EXAMPLES

[0052] EXPERIMENTAL

- [0053] **Cell Line and Cell Culture.** Human prostate cancer PC3, DU145 cells and RAW 264.7 macrophages were procured from the American type culture collection (ATCC). DU145 cells were grown at 37 °C in 5% CO₂ in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin. PC3 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. RAW 264.7 macrophages were cultured in RPMI media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were passed every 3 to 4 days and restarted from frozen stocks upon reaching pass number 20.

- [0054] **Synthesis of TPP-(CH₂)₅-COOH.** 6-Bromohexanoic acid (0.5 g, 2.6 mmol) and triphenylphosphine (0.7 g, 2.7 mmol) were dissolved in acetonitrile (15 mL). The resulting mixture was heated to a reflux under nitrogen flow for 24 h. The solution was cooled to room temperature and concentrated under reduced pressure. The resulting viscous oil was washed with hexanes (2 x 15 mL) and diethyl ether (3 x 15 mL) to produce a white solid (0.85 g, 87% yield). ¹H NMR (CDCl₃): δ 7.8-7.6 [m, 15], 3.52 [t, 2H], 2.3 [t, 2H], 1.61 [m, 6H] ppm. ¹³C NMR (CDCl₃): δ 175, 135.2, 133.4, 130.2, 118.5, 34.3, 29.5, 23.9, 22.8, 22.4, 22.1 ppm. ³¹P NMR (CDCl₃): δ 24.39 ppm.
- [0055] **Synthesis of SH-PEG-TPP.** SH-PEG-NH₂ (0.2 g, 0.0571 mmol), TPP-(CH₂)₅-COOH (0.043 g, 0.1142 mmol), and 4-dimethylaminopyridine (DMAP) (0.014 g, 0.1142 mmol) were dissolved in 7 mL CH₂Cl₂, cooled to 0°C while stirring, and a solution of dicyclohexylcarbodiimide (DCC) (0.024 g, 0.1142 mmol) in 1 mL CH₂Cl₂ was added. The mixture was warmed to room temperature and stirred overnight. The reaction by-product, dicyclohexylurea precipitated was filtered off and the filtrate was precipitated in chilled diethyl ether. The resulting solid was centrifuged at 5000 revolutions per minute (rpm) for 15 min. The supernatant was decanted and the resulting wet solid was lyophilized to result white solid. Yield: 50 % (0.11 g). ¹H NMR (CDCl₃): δ 7.82-7.57 [m, 15], 3.81 [m, 2H], 3.71 [m, 2H], 3.64 [m, 4H], 2.88 [m, 2H], 1.73 [m, 2H] ppm. ¹³C NMR (CDCl₃): δ 207.15, 131.51, 131.48, 129.99, 129.9, 126.95, 126.83, 66.9 ppm. ³¹P NMR (CDCl₃): δ 24.63 ppm.
- [0056] **Synthesis of T and NT-AuNPs.** For T-AuNP synthesis, a solution of SH-PEG-TPP (0.1 g, 0.25 mmol) and SH-PEG-NH₂ (0.1 g, 0.25 mmol) were prepared in 15 mL H₂O and gold(III) chloride trihydrate (HAuCl₄·3H₂O) (0.05 g, 0.12 mmol) was added. This mixture was vigorously stirred for 10 min until all of the gold salt was dissolved. The reduction was carried out by adding drop wise a freshly prepared aqueous solution of

sodium borohydride (0.05 g, 1.2 mmol in 3 mL H₂O) with vigorous stirring and 15 mL of deionized (DI) water was then added to the mixture. After 24 h, the solution was dialyzed using a Spectra/Por® dialysis membrane of MW cutoff of 5,000 Da against H₂O for 12 h. The water was changed every hour. The NPs were further purified by differential centrifugation at 7,000 rpm for 30 min. After centrifugation the supernatant was kept and the pellet was discarded to obtain monodispersed T-Au-NPs. The non-targeted NPs, NT-Au-NPs were prepared following the same synthetic method as mentioned for T-AuNPs using SH-PEG-NH₂ (0.2 g, 0.5 mmol) and HAuCl₄·3H₂O (0.05 g, 0.12 mmol).

[0057] Synthesis of T-3-BP-AuNPs and NT-3-BP-AuNPs. 3-BP was conjugated to the surface of the T-AuNP and NT-AuNP surface by amide coupling. 3-BP (30 mg, 0.18 mmol), *N*-hydroxysuccinimide (NHS) (39 mg, 0.12 mmol), and ethyl(dimethylaminopropyl) carbodiimide (EDC) (30 mg, 0.18 mmol) were dissolved in H₂O (5 mL). 3-BP was allowed to activate for 6 h at 25 °C. To this, T-AuNPs or NT-AuNPs (10 mg/mL, 5 mL) was added and stirred overnight at 25 °C. NPs were purified by dialysis (MW cutoff of 2,000 Da) against water for 8 h changing the water every hour. NPs were lyophilized and freshly resuspended in nanopure water prior to use.

[0058] Determination of 3-BP Loading by GADPH Assay. The activity of 3-BP on GAPDH was used to determine 3-BP loading on AuNPs using a KDalert™ GAPDH assay kit. GAPDH was treated with predetermined concentration of 3-BP to generate a standard curve. AuNPs (100 µL, 10 mg/mL) were treated with 1.0 mM potassium iodide (KI) to dissolve the gold core and then 10 µL of the NPs were then added to GADPH enzyme (10 µL, 0.133 U/mL) and subjected to the activity assay. The NP/GADPH enzyme suspension (10 µL) was then added to the KDalert™ master mix (90 µL) in a 96 well plate. The mixture was then gently shaken to ensure full mixing and the fluorescence was measured (excitation: 560 nm, emission: 590 nm).

[0059] Immune Response from NPs in RAW 264.7 Macrophages by ELISA. RAW 264.7 macrophages were plated at a concentration of 50,000 cells/mL in 96 well plates and allowed to grow for 12 h. The cells were incubated with T-AuNP (10 mg/mL), NT-

AuNP (10 mg/mL) for 24 h at 37°C. Additionally, LPS alone (100 ng/mL) was added to the macrophage culture to serve as a control. ELISA was performed on the supernatants against the pro-inflammatory cytokines IL-6 and TNF- α . Antibody coated plates were prepared by treating ELISA compatible thermo scientific Nunc 96-well plates with the antibody for 12 h at 4°C and then blocked with 10% FBS in PBS for 1 h at room temperature followed by 5 washings with the washing buffer (1X PBS with 0.05% v/v Tween® 20). Macrophage supernatants (100 μ L) were added to the plates and incubated for 2 h at room temperature, followed by 5 washes with the washing buffer and sequential incubations with the cytokine-biotin conjugate and streptavidin working solution. The substrate reagent containing 3,3',5,5' tetramethylbenzidine (100 μ L) was then added to each well, incubated for 15 min in the dark, and the reaction was stopped by adding 50 μ L stop solution containing 0.1 M H₂SO₄. The absorbance was recorded at 450 nm using a BioTek Synergy HT well plate reader.

[0060] Release of 3-BP from T-3BP-AuNP and NT-3-BP-AuNP. The ability of 3-BP to be cleaved from the NP surface was tested by using proteases from *Streptomyces griseus* type XIV. T-3-BP-AuNP and NT-3-BP-AuNPs (10 mg/mL with respect to AuNP and 1.4 mM with respect to 3-BP) were treated with 0.5 mg/mL of protease in water for up to 6 h at 37°C with gentle shaking. At a predetermined time point, the NPs were isolated by repeated centrifugations and resuspensions in nanopure water while discarding the supernatant each time at 12,000 rpm for 30 min. The resulting pellet was resuspended in 100 μ L water and the amount of 3-BP was quantified by KDaAlert™ GADPH assay described above.

[0061] HK2 Binding Assay. HK2 binding ability of T and NT 3-BP loaded AuNPs was tested by using human HK2 recombinant of molecular mass 104.1 kDa expressed in *Escherichia coli*. T-AuNPs, NT-AuNPs, T-3-BP-AuNP, and NT-3-BP-AuNPs (10 mg/mL with respect to AuNP and 1.4 mM with respect to 3-BP) were treated with 1 μ L solution of 1.0 mg/mL of HK2 (in 20 mM Tris pH 8.0 and 10% glycerol) for 1 h at 37 °C. HK2 bound NPs were then centrifuged at 12000 rpm for 30 min and supernatants were discarded to remove unbound HK2. This process was repeated 3 times and finally the NP pellet obtained was resuspended in water. BCA assay directly on the HK2-bound to AuNP did not show any signal. Therefore, the Au core was

dissolved using 1 mM KI and BCA assay was performed to quantify the amount of HK2 bound to 3-BP. A standard curve was constructed using bovine serum albumin (BSA). For protease mediated cleavage of HK2-loaded AuNPs, these NPs were treated with proteases for 12 h and subsequently BCA assay was performed.

[0062] Quantification of AuNPs in the Mitochondrial Compartments. PC3 cells were grown at a density of 5×10^5 /mL cells in 30 mL RPMI media overnight in T-150 flask. The media was changed and T-AuNPs or NT-AuNPs were added (1 mg/mL) and incubated for 12 h. The media was changed and the cells were rinsed with 1x phosphate buffered saline (PBS) (3x). The cells were isolated by trypsinization and centrifuged for 3 min at 1,800 rpm. The final cell suspension was resuspended in reagent A from the mitochondria isolation kit for mammalian cells and incubated on ice for 2 min. After 2 min, reagent B (10 μ L) was added and incubated on ice for 5 min, vortexing each min. These cells were treated with reagent C and the cells were centrifuged at $700 \times g$ for 10 min. The resulting supernatant was collected containing the mitochondrial and cytosolic fractions. This was further centrifuged at $12,000 \times g$ for 15 min at 4°C to yield a mitochondrial pellet and the cytosolic supernatant. The resulting pellet was further purified with a washing and centrifugation at $12,000 \times g$ for 5 min to yield the mitochondrial fraction. These fractions were analyzed for the amount of gold by ICP-MS and the amount of protein recovered by BCA assay. To evaluate the exact location of the T-AuNPs and T-3-BP-AuNPs within the mitochondria, the mitochondria were further fractionated. PC3 cells were grown at a density of 5×10^5 /mL cells in 30 mL RPMI media overnight in T-150 flask. The media was changed and T-AuNPs or T-3-BP-AuNPs were added (1 mg/mL) and incubated for either 4 or 12 h. After the given time, the cells were trypsinized and repeatedly washed with PBS at 1,800 rpm for 3 min. The mitochondria were isolated as described above. This was further subfractionated into the OMM, the intermembrane space, the IMM, and the matrix. The freshly isolated PC3 mitochondria in PBS (1x) were incubated with protease inhibitor (0.125 mg/mL) and 0.6% digitonin for 10 min on ice. Immediately after incubation, the mitochondria were centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant (SN-I) contained the OMM fraction and the interstitial membrane space. The pellet was resuspended in 150 mmol/L KCl, protease inhibitor (0.125 mg/mL) and incubated on ice for 10 min. This was centrifuged at $10,000 \times g$ for

10 min at 4°C. The supernatant, which contained the mitochondrial matrix, was collected. To this, 50 µL of 1x cell lysis buffer (30 mM Tris-HCl, 0.1 mM EDTA, 20 % w/v sucrose) was added. This was subsequently sonicated and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant (SN-II) was collected, containing the purified IMM fraction and matrix. SN-I and SN-II were centrifuged at 105,000xg for 60 min. The pellet from SN-I contained the OMM fraction and the supernatant contained the interstitial membrane space. The pellet from SN-II was resuspended in PBS containing Lubrol WX (0.5 mg/mL), 37 % sucrose and incubated for 15 min on ice. This was once again centrifuged at 105,000xg for 60 min at 4°C. The pellet was collected containing the IMM fraction and the supernatant contained the matrix. The collected fractions were analyzed for Au concentration by ICP-MS. A BCA assay was performed on all the fractions in order to calculate the Cd (ng)/protein (pg).

[0063] MTT Assay and Data Analysis. Cytotoxicity profiles of T-AuNPs, NT-AuNPs, T-3-BPAuNPs, NT-3-BP-AuNPs, and 3-BP were studied using the MTT assay against prostate cancer PC3 and DU145 cell lines. Cells (2000 cells/well) were seeded on a 96-well plate in 100 µL of desired medium and incubated for 24 h. The cells were then treated with different constructs at varying concentrations and incubated for 12 h at 37°C and the media was changed. The cells were further incubated for additional 60 h at 37°C under 5% CO₂. For photothermal MTT studies, after 4 h treatment of cells with NPs, the cells were irradiated with 660 nm laser (power 20.0 mW) light with a fiber optics for 1 min per well and the irradiated cells were incubated for 12 h at 37°C, followed by a media change and further incubation for 60 h. The cells were then treated with 20 µL of MTT (5 mg/mL in PBS) for 5 h. The medium was removed, the cells were lysed with 100 µL of DMSO, and the absorbance of the purple formazan was recorded at 550 nm using a Bio-Tek Synergy HT microplate reader and background absorbance was measured at 800 nm. Each well was performed in triplicate and each experiment was repeated three times. Cytotoxicity was expressed as mean percentage increase relative to the unexposed control ± standard deviation (SD). Control values were set at 0% cytotoxicity or 100% cell viability. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a three parameters logistic model used to calculate the IC₅₀, which is the concentration of chemotherapeutics causing 50% inhibition in comparison to untreated controls. The mean IC₅₀ is the

concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent measurements that were reproducible and statistically significant. The IC₅₀ values were reported at $\pm 95\%$ confidence intervals. This analysis was performed with GraphPad Prism (San Diego, U.S.A).

[0064] Apoptosis Detection by Flow Cytometry. PC3 cells were plated at a density of 1×10^6 cells/mL and allowed to grow for 14 h. Media was changed and cells were treated with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), NT-3-BP-AuNP (13.7 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), free 3-BP (10 μM or 1 mM) for 6 h at 37°C. As positive controls, etoposide (100 μM , incubation time: 12 h) for apoptosis and H₂O₂ (1 mM, incubation time: 45 min) for necrosis were used. For apoptosis detection after laser treatment, cells were treated with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), NT-3-BP-AuNP (13.7 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), free 3-BP (10 μM) for 4 h at 37 °C, irradiated with laser of 660 nm for 1 min, and further incubated for 12 h. Cells were trypsinized, washed 3 times with cold 1x PBS, and centrifuged at 1800 rpm for 3 min, and the supernatants were discarded. Cells were counted and resuspended in 1X annexin binding buffer at a density of $\sim 1 \times 10^6$ cells/mL preparing a sufficient volume to have 100 μL per assay. To 100 μL of cell suspension, 5 μL Alexa Fluor® 488 annexin V and 1 μL 100 $\mu\text{g/mL}$ PI working solution were added, incubated for 15 min at room temperature, followed by addition of 400 μL 1X annexin-binding buffer, gently mixing in ice, and the samples were analyzed on the flow cytometer immediately.

[0065] Lactate Determination. PC3 cells were seeded at a density of 1×10^6 cells/mL and allowed to grow for 12 h at 37 °C under 5% CO₂. Cells were treated with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), NT-3-BP-AuNP (13.7 $\mu\text{g/mL}$ with respect to NP, 10 μM with respect to 3-BP), free 3-BP (10 μM) for 6 h at 37 °C. After 6 h, the media was removed and the cells were homogenized. The lysate and the media supernatant were added to the enzyme and substrate working reagent mixture and incubated for 30 min.

Lactate concentration was measured using Bio-Tek Synergy HT microplate reader at 450 nm and comparing to a standard curve.

[0066] CellTiter-Glo® Luminescent ATP Quantification. PC3 cells were seeded at a density of 1×10^6 cells/mL in a 12 well plate and allowed to grow for 12 h at 37°C under 5% CO₂. Cells were treated with T-AuNP (1 mg/mL), NT-AuNP (1 mg/mL), T-3-BP-AuNP (14.1 µg/mL with respect to NP, 10 µM with respect to 3-BP), NT-3-BP-AuNP (13.7 µg/mL with respect to NP, 10 µM with respect to 3-BP), free 3-BP (10 µM) for 6 h at 37°C in 5% CO₂ atmosphere. Control wells containing medium without cells were used to obtain background luminescence value. Plates were then equilibrated at room temperature for ~30 min. A volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well was added, this mixtures were mixed for 2 min in a shaker to induce cell lysis. The plates were incubated at room temperature for additional 10 min to stabilize luminescent signal. Luminescence was recorded using a plate reader. ATP quantification was carried out from a standard curve using ATP disodium salt hydrate.

[0067] Bioenergetic Assay. Prior to the assay, XF sensor cartridges were hydrated. To each well of an XF utility plate, 1 mL of Seahorse Bioscience calibrant was added and the XF sensor cartridges were placed on top of the utility plate, and kept at 37°C incubator without CO₂ for a minimum of 12 h. PC3 cells were cultured in XF24-well cell culture microplates (Seahorse Bioscience) at a density of 5×10^4 cells/well (0.32 cm²) in 200 µL growth medium and then incubated for 24 h at 37°C in 5% CO₂ atmosphere. The three key parameters of glycolytic function: glycolysis, glycolytic capacity, and glycolytic reserve were assessed using a Seahorse XF glycolysis stress kit. After the cells were attached, an additional 100 µL growth medium was added and the cells were incubated for 24 h at 37°C in 5% CO₂ atmosphere. The cells were treated with T-3-BPAuNP (10 µM with respect to 3-BP), NT-3-BP-AuNP (10 µM with respect to 3-BP), and 3-BP (10 µM) for 6 h at 37 °C in 5% CO₂ atmosphere. After 6 h, all but 50 µL of the culture medium was removed from each well and the cells were rinsed two times with 600 µL of XF stress test glycolysis optimization medium pre-warmed to 37°C and finally 610 µL of glucose depleted optimization medium was added to each well and the plate was placed at 37 °C without CO₂ for 1 h prior to assay. The OCR

and the ECAR were measured simultaneously for 16 min to establish a baseline rate. Glycolysis, glycolytic capacity, and glycolytic reserve were calculated by subtracting the average rates before and after the addition of glucose (10 mM), ATP synthase inhibitor oligomycin (1.0 μ M), and 2-deoxy-D-glucose (2-DG) (100 mM). These three compounds were injected consecutively with specific time gap and ECAR values were measured after each injection. Different parameters of respiration: basal respiration, coupling efficiency, and spare respiratory capacity were investigated by using Seahorse XF-24 cell Mito Stress Test Kit. PC3 cells were plated and treated with different constructs as mentioned above. After 6 h incubation with the constructs, the media was changed as above with the optimization media supplemented with sodium pyruvate, L-glutamine, and D-glucose to a final volume of 500 μ L. Different parameters of respiration were calculated by subtracting the average respiration rates before and after the addition of the electron transport inhibitors oligomycin (1.0 μ M), FCCP (1.0 μ M), an ionophore that is a mobile ion carrier, and a mixture of antimycin-A (1.0 μ M) which is a complex III inhibitor and rotenone (1.0 μ M), a mitochondrial inhibitor that prevents the transfer of electrons from the Fe-S center in Complex I to ubiquinone. The parameters calculated included: basal respiration (baseline respiration minus antimycin-A post injection respiration), ATP turnover (baseline respiration minus oligomycin post injection respiration), maximal respiratory capacity (FCCP stimulated respiration minus antimycin-A post injection respiration) and reserve respiratory capacity (FCCP stimulated respiration minus baseline respiration). Test articles on each well had five replicates.

[0068] RESULTS AND DISCUSSION

[0069] **AuNPs for Targeted Delivery of 3-BP.** AuNPs are widely used in delivery of therapeutics due to their unique surface chemistry, tunable size, shape, and optical properties. However, one of the important issues in clinical translation of AuNPs is their toxicological effect. A recent study demonstrated that unmodified citrate coated AuNPs can cause inhibition of cell proliferation in a concentration and size dependent manner. Therefore, it may be important to design a biocompatible surface functionalized AuNP for delivery of 3-BP inside the mitochondria. Polyethylene glycol (PEG) is often used to protect surfaces from nonspecific interactions with

biomolecules. To deliver 3-BP inside the mitochondria, we constructed AuNPs covered with lipophilic cationic -TPP moieties attached to PEG. The lipophilic TPP cations easily permeate lipid bilayers driven by the plasma membrane potential (-30 to -60 mV) to enter cytoplasm and then concentrated several hundred-fold into mitochondria by the large mitochondrial membrane potential ($\Delta\psi_m$) (-150 to -180 mV) maintained across the inner mitochondrial membrane (IMM). To construct T-AuNPs, we synthesized TPP-PEG-SH by reacting NH_2 -PEG-SH with TPP-(CH_2)₅-COOH (**FIG. 1**). The -TPP group in TPP-PEG-SH allowed for mitochondria targeting and the -SH acted as anchoring group to AuNP surface. T-AuNPs were synthesized through the reduction of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and simultaneous stabilization with a mixture of NH_2 -PEG-SH and TPP-PEG-SH. The amine groups from T-AuNPs were conjugated with 3-BP using a standard amide coupling reaction to result T-3-BP-AuNPs (**FIG. 1**). To investigate the advantages of delivering 3-BP inside mitochondria, we constructed a non-targeted system, NT-AuNP, as a control. NT-AuNPs were constructed from $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ using NH_2 -PEG-SH and the amine groups were used to conjugate 3-BP to generate NT-3-BP-AuNPs. AuNPs were characterized by dynamic light scattering (DLS), zeta potential measurements, and by transmission electron microscopy (TEM) (**FIGS. 2A, 2B**). The intensity-weighted average or the Z average of these NPs were mostly between 20 to 30 nm and were narrowly distributed. In our previous studies, we demonstrated that positively charged NPs of suitable size have the ability to enter mitochondria of cells, whereas negatively charged particles or NPs which are not highly positively charged or larger in size mostly distribute in the cytosols. Zeta potential measurements indicated that T-AuNP surface is positively charged and conjugation of 3-BP on the surface lowered the potential by ~6 mV. Non-targeted NPs with surface $-\text{NH}_2$ groups showed positive zeta potential and conjugation of 3-BP diminished the positive charge by ~10 mV. The small sizes and high positively charged surface coated with highly lipophilic cationic TPP moieties in T-3-BP-AuNPs played significant roles in their navigation to the mitochondria. The morphology of these NPs was mostly spherical as demonstrated by TEM (**FIG. 2B**). To demonstrate the versatility in biomedical applications, the dispersion stability of these AuNPs was evaluated by freeze drying the NPs and assessing the formation of aggregates and change in color upon making aqueous

suspension at a concentration of 10 mg/mL. No visible aggregation or changes in the color was observed and the resuspended NPs demonstrated similar sizes and zeta potential. The T and NT-AuNPs showed surface plasmon band at 540 and 523 nm, respectively. Upon conjugation of 3-BP on the surface of both T and NT-AuNPs, no changes in the diameter, no broadening or red shift of the surface plasmon band was observed indicating that no aggregation of AuNPs occurred during surface modification.

- [0070] It is challenging to quantify 3-BP in AuNPs using conventional analytical methods. We therefore devised an enzyme inhibition assay to quantify coupling efficiency of 3-BP on NP surface based on the fact that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an intracellular target of 3-BP (**FIG. 2C**). Pyruvylation of GAPDH by 3-BP causes inhibition of the enzymatic function. The enzyme activity of GAPDH can be determined using a KDAlert™ GAPDH assay kit. Generation of a standard curve by treating GAPDH with known concentrations of 3-BP, dissolution of Au core of T and NT-3-BP-AuNPs with potassium iodide (KI), and subsequent treatment with GAPDH allowed us to quantify amount of 3-BP present on the NP surface (**FIG. 2B**). Similar coupling efficiencies of 3-BP on the surface of T and NTAuNPs were noted (~8%, Table S1).
- [0071] Macrophages are one of the principal immune effector cells, hence the immunological response of RAW264.7 macrophage cells when exposed to T and NT-AuNPs was studied by following the production of pro-inflammatory cytokines, tumor necrosis factor alfa (TNF- α) and interleukin-6 (IL-6), using enzyme-linked immunosorbent assay (ELISA) (**FIG. 2D**). Neither the control cells nor the cells treated with T and NT-AuNPs showed any secretion of either TNF- α or IL-6. Both the cytokines were detected in the cells treated with bacterial lipopolysaccharide (LPS) as control (**FIG. 2D**). These results indicated that AuNPs do not elicit immunological response and do not induce production of pro-inflammatory cytokines even at high concentrations of 10 mg/mL.
- [0072] **Protease Dependent Release of 3-BP from AuNPs.** 3-BP can be a multitasking agent if it can be delivered to cancer cell, its mitochondria, and released at the target sites.

For inhibition of cytosolic glycolysis, 3-BP directly alkylates HK2 by reacting with the –SH groups from Cys 158 and Cys 606 which are present in the enzyme active sites. The terminal step of OXPHOS in making ATP in the mitochondria requires an ATP synthase (F(0)F(1)) comprised of two motors, PIC and ANC which carry phosphate and adenine nucleotide for ATP synthesis, respectively. 3-BP alkylates PIC and ANC that results in halting the final step of OXPHOS. Conformational changes associated with VDAC bound HK2 leads to its direct dissociation from the mitochondria that promotes mitochondrial injury provoked by pro-apoptotic proteins. Specific release of apoptosis-inducing factor (AIF), cytochrome c from the mitochondria to cytosol leads to eventual cell death. For successful execution of these processes, 3-BP needs to be released from the AuNPs at the target sites. In T-3-BP-AuNPs and NT-3-BP-AuNPs, 3-BP was conjugated to the AuNP surface via amide bonds in order to take advantage of the cellular proteases to catalyze the hydrolysis of these linkages to release 3-BP. Moreover, various proteases within mitochondria regulate mitochondrial biogenesis to ensure complete degradation of excess or damaged proteins. To test 3-BP release profile from the AuNPs, T-3-BP-AuNP and NT-3-BP-AuNP were treated with proteases from *Streptomyces griseus* type XIV at 37 °C and the concentration of 3-BP released was followed using the GADPH assay (**FIG. 3A**). In the absence of protease, no release of 3-BP from T-AuNP or NT-AuNP was observed under these conditions. In the presence of 0.5 mg/mL protease, ~50% of 3-BP was released from both T and NT-AuNPs in 4 h and >80% release of 3-BP was observed after 6 h. Thus, based on these data, we believe that some 3-BP will be released in the cytosol by the cytosolic proteases and T-3-BPNPs that will enter mitochondria will take advantage of high concentration of proteases in the mitochondrial matrix for 3-BP release (**FIG. 3A**).

[0073] HK2 Binding Affinity of Released 3-BP from T- and NT-AuNPs. The ability of 3-BPs from T-3-BP-AuNPs and NT-3-BP-AuNPs to covalently modify HK2 was tested using human HK2 recombinant of molecular mass 104.1 kDa expressed in *Escherichia coli*. Incubation of HK2 with T-3-BP-AuNPs and NT-3-BP-AuNPs, quantification of 3-BP-bound HK2 using the bicinchoninic acid (BCA) assay after dissolving the Au core with KI indicated that 3-BP from both T and NT-AuNPs has the ability to covalently bind to HK2 (**FIG. 3B**). Incubation of HK2 bound AuNPs with protease and subsequent protein quantification by BCA assay showed less protein

concentration, which further confirmed that, 3-BP covalently modified HK2 (**FIG. 3B**). T and NT-AuNPs without 3-BP did not show presence of any protein under these conditions. These data rule out the possibility that HK2 are simply adsorbed on the surface without interaction with 3-BP.

[0074] Intracellular Location of T-3-BP-AuNPs. 3-BP has several intracellular targets, thus the distribution of the T-AuNPs is the key factor in determining the efficacy of 3-BP. Where T-AuNPs or NT-AuNPs are located is essential information for understanding intracellular compartmentation of 3-BP. We first investigated distribution of NT and T-AuNPs in the mitochondria and cytosol in prostate cancer (PCa) PC3 cells. Treatment of these cells with the NPs for 12 h, isolation of mitochondrial (mito) and cytosolic (cyto) fractions, and quantification of gold by inductively coupled plasma mass spectrometry (ICP-MS) indicated that the overall uptake of T-AuNPs is higher than that of NT-AuNPs (**FIG. 3C**). Most of T-AuNPs are associated with the mitochondria. Only a small fraction of NT-AuNPs were associated with the mitochondria which might be attributed from the positively charged amine groups on the surface of these NPs. Highly lipophilic cationic -TPP groups are responsible for greater association of T-AuNPs with the mitochondria. We next assessed the exact location of T-AuNPs and T-3-BPAuNPs inside the mitochondria and investigated the time dependency of NP accumulation in different mitochondrial compartments. PC3 cells were treated with T-AuNPs and T-3-BP-AuNPs for two time points, 4 h and 12 h, and mitochondrial sub-fractions, OMM, IMM, intermembrane space, and matrix were isolated from the treated cells at both the time points, and gold was quantified in these fractions by ICP-MS (**FIG. 3C**). T-AuNPs and T-3-BP-AuNPs show very similar patterns in their mitochondrial distribution; however, the overall concentrations of T-3-BP-AuNPs were higher in the mitochondrial compartments compared to the T-AuNPs without any 3-BP. At early stage after 4 h incubation, both T-AuNPs and T-3-BP-AuNPs were found in the OMM and at 12 h, almost all T-AuNPs and T-3-BP-AuNPs were located inside the matrix. These observations indicated that the T-3-BP-AuNPs distributed in the OMM will be able to modify HK2 bound to VDAC. Complete localization of T-3-BP-AuNPs at 12 h might also be due to opening of the VDACs upon deactivation of HK2 by 3-BPs. Intra-mitochondrial distribution patterns of T-3-BP-AuNPs in a time dependent manner indicated that 3-

BP delivered by T-AuNPs will be able to attack all of its intracellular targets for maximum efficacy.

[0075] Anti-proliferative Effects of T-3-BP-AuNPs. We determined the effect of AuNPs on proliferation of two different PCa cell lines-PC3 and DU145 cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The widely used PCa cell lines PC3 and DU145 show differences in their metabolic phenotype. Both DU145 and PC3 lines have higher rates of lactate production, however these cell lines differed in glycolytic rate. PC3 cells show higher mRNA levels for glycolytic enzymes. The glycolytic reliance of PC3 and DU145 cells is believed to be due to OXPHOS insufficiency and the reduced reliance on OXPHOS is due to a mitochondrial dysfunction. T-3-BP-AuNPs exhibited highest efficacy in inhibiting proliferation of both PC3 and DU145 cells, NT-3-BP-AuNPs demonstrated significantly reduced inhibition compared to the T-3-BP-AuNPs, and free 3-BP showed only a modest inhibition (**FIG. 4A, Table 1**). No inhibition in cell growth was observed with T-AuNPs and NT-AuNPs without 3-BP (**FIG. 4A, Table 1**). These findings indicated preclinical proof of the concept that delivery of 3-BP to mitochondria using NP system results in enhanced uptake and cytotoxicity. Moreover, T-3-BP-AuNPs demonstrated an enhanced cytotoxic activity when combined with a 660 nm laser irradiation than NT-3-BP-AuNPs or free 3-BP plus laser treatment. It was interesting to note that T-AuNPs without any 3-BP showed an enhanced cytotoxicity under laser irradiation, whereas NT-AuNPs did not show any significant toxicity in presence of photo-irradiation (**FIG. 4B, Table 1**). These findings suggested that the higher accumulation of T-AuNPs inside mitochondria might be responsible for its toxicity under photo-irradiation by inducing local mitochondrial damage. These results together support that T-AuNP is highly effective for selective delivery of 3-BP and T-3-BP-AuNPs demonstrated the effects of targeted chemotherapy and photothermal effect.

Table 1. IC₅₀ with standard deviation calculated from three independent experiments

	PC3 ^a (μ M)	DU145 ^a (μ M)	PC3 + Laser ^b (mg/mL)
T-AuNPs	N/A	N/A	0.55 $\pm$ 0.09
NT-AuNPs	N/A	N/A	N/A
3-BP	271 $\pm$ 9	541 $\pm$ 37	N/A
T-3-BP-AuNPs	13 $\pm$ 3	28 $\pm$ 8	0.010 $\pm$ 0.003
NT-3-BP-AuNPs	45 $\pm$ 6	109 $\pm$ 7	0.060 $\pm$ 0.003

^aWith respect to 3-BP; ^bWith respect to AuNP

[0076] Induction of apoptosis is an important mechanism whereby 3-BP can suppress tumorigenesis. The pro-apoptotic effect of T-3-BP-AuNPs, NT-3-BP-AuNPs, and free 3-BP was studied in PC3 cells using Alexa Fluor® 488 Annexin V-propidium iodide (PI) assay by flow cytometry both in the dark and in presence of 660 nm laser irradiation for 1 min (**FIG. 4C**). Treatment of PC3 cells with T- and NT-3-BP-AuNPs showed increased percentage of apoptotic cells from 0% in control group to 99%. No necrotic cell population was noted in T and NT-3-BP-AuNP treated cells. Free 3-BP under same conditions with a concentration of 10 μ M was unable to show any apoptotic activity, however at a much higher concentration of 1 mM, significant apoptosis was observed in 3-BP treated PC3 cells. Under photo-irradiation of PC3 cells for 1 min with a 660 nm laser, T-AuNPs, with the ability to accumulate in the mitochondria, showed apoptotic activity. The apoptosis inducing properties of T-3-BP-AuNPs and NT-3-BP-AuNPs were found to be very similar (**FIG. 4C**). Our data thus suggested that 3-BP can promote apoptosis at a much lower concentration when delivered with a mitochondria-targeted delivery system and use of AuNPs as delivery system further enhances its activity by utilizing photothermal activity of gold nanocrystals.

[0077] Lactate Reduction and ATP Depletion. The final product of glycolysis is lactate. Hence, we quantified intracellular and extracellular lactate levels in PC3 cell lines treated with 3-BP, T-3-BP-AuNPs, NT-3-BP-AuNPs, T-AuNP, and NT-AuNPs.

Extracellular lactate levels were found to be much higher than those observed in the cells (**FIGS. 5A and 5B**). Our results showed that the levels of both intracellular and extracellular lactate declined with treatment with free 3-BP, T-3-BP-AuNPs, and NT-3-BP-AuNPs. This decrease was more significant in cells which were treated with T-3-BP-AuNPs compared to the cells treated with either 3-BP or 3-BP conjugated to NT-AuNPs (**FIGS. 5A and 5B**). Treatment with T-3-BP-AuNPs, NT-3-BPAuNPs, and 3-BP showed a decrease in the level of ATP in PC3 cells (**FIG. 5C**). The PC3 cells were found to be much more sensitive to T-3-BP-AuNPs than free 3-BP or NT-3-BP-AuNPs as inferred from the intracellular ATP levels at 6 h (**FIG. 5C**). The present data showed T-3-BPAuNPs has the ability to show depletion of ATP and reduction in lactate levels at lower 3-BP concentrations compared to free 3-BP or NT-3-BP-AuNPs. These observations further supported the importance of delivering 3-BP to the mitochondria in modulation of its efficacy.

[0078] Perturbation of Bioenergetic Functions of PC3 Cells by T-3-BP-AuNPs. We performed real-time measurements of extracellular acidification rate (ECAR), an indicator of glycolysis and oxygen consumption rate (OCR) which is a marker of OXPHOS in highly glycolytic PC3 cells after treatment with T-3-BP-AuNPs, NT-3-BP-AuNPs, and free 3-BP using a Seahorse XF24 extracellular flux analyzer (**FIG. 6**). Glycolytic parameters were calculated by monitoring changes in ECAR in response to sequential addition of D-glucose to assess glycolysis, oligomycin to measure maximal glycolytic capacity, and 2-deoxy-D-glucose (2-DG) as a measure of glycolytic reserve capacity (**FIGS. 6A and 6B**). Administration of excess of glucose to T-3-BP-AuNP treated PC3 cells in glucose-depleted medium showed only modest increase in the ECAR levels indicating remarkable activity of T-3-BP-AuNPs in glycolysis inhibition. Under similar conditions, 3-BP delivered by NT-AuNPs showed less efficiency in glycolysis inhibition and free 3-BP at this low concentration of 10 μ M did not show any glycolysis inhibitory effect (**FIG. 6A**). Significant inhibition of glycolysis by T-3-BP-AuNPs in PC3 cells was further supported by sequential administration oligomycin and 2-DG, the cells treated with T-3-BPAuNPs did not show any significant changes in the ECAR levels. These data demonstrated remarkable ability of 3-BP at a low concentration of 10 μ M in inhibition of glycolysis only when delivered

with a mitochondria-targeted delivery system. We next assessed the effects of T-3-BPAuNPs on the mitochondrial OXPHOS pathway and compared the results when 3-BP was either delivered with a NT-AuNP or in the free form (**FIGS. 6C-E**). By following the changes in the OCR values (**FIG. 6C**) in response to oligomycin to give maximal glycolytic capacity, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), an uncoupling agent that allows maximum electron transport and therefore a measure of maximum OXPHOS respiration capacity, and a combination of mitochondrial complex III inhibitor antimycin A and mitochondrial complex I inhibitor rotenone that allow precise measurement of mitochondrial uncoupling, spare respiratory capacity, coupling efficiency, ETC accelerator response, and basal respiration (**FIGS. 6D and 6E**) were measured. Significant decrease in the OCR in the PC3 cells that were treated with T-3-BP-AuNPs was noted.

[0079] We believe that the origin of this reduction was due to the ability of T-3-BP-AuNPs to target mitochondria-bound HK2 leading to loss of the mitochondrial integrity. Free 3-BP however has no preference for mitochondrial and cytosolic HK2. NT-3-BP-AuNPs showed preference for cytosolic HK2 as shown by their ability to reduce glycolysis and induce apoptosis, however, NT-3-BP-AuNPs did not affect the overall mitochondrial integrity. T-3-BP-AuNPs suppressed basal levels of OXPHOS and affected the ability of cancer cells to upregulate OXPHOS in response to agents such as FCCP that uncouple the mitochondrial proton gradient from ATP production. The effect of T-3-BP-AuNPs on OXPHOS, at a low 3-BP concentration of 100 μ M is an important finding because other non-mitochondrial effects of free 3-BP may account for its cytotoxic effect at least at high doses. We believe that T-3-BP-AuNPs do not exert its effects via a single molecular target within the mitochondrial respiratory chain, but through several collective disruptions that leads to impairment of OXPHOS.

[0080] **Potential Intracellular Targets of 3-BP Using Mitochondria-Targeted Delivery System.** Altogether, our data showed that T-3-BP-AuNPs have enormous potential in enhancing the efficacy of 3-BP. The overall mechanism of action of 3-BP when delivered with T-AuNP system is represented in **FIG. 7**. MCT is implicated as a 3-BP transporter and free 3-BP might not enter cells in the absence of MCT. When 3-BP is conjugated to a mitochondria-targeted NP system, as shown in T-3-BP-AuNPs, even

in the absence of MCT or when MCT are engaged in cellular efflux of excess lactate in highly proliferative glycolytic cancers, T-3-BP-AuNPs with $\Delta\psi$ -targeted -TPP moieties will be taken up by cancer cells by utilizing the natural uptake mechanisms of AuNPs and the fact that cancer cells frequently have more negatively charged $\Delta\psi$. Once inside the cells, a small portion of 3-BP released in the cytosol by the cytosolic proteases will inhibit cytosolic HK2. T-3-BP-AuNPs localized in the OMM at the early stage of trafficking will release 3-BP for potential dissociation of HK2 from VDAC thereby promoting cellular apoptosis. T-3-BP-NPs localized in the mitochondrial matrix will play significant roles in shutting down of mitochondrial OXPHOS by inhibiting succinate dehydrogenase and other enzymes such as PIC and ANC.

[0081] CONCLUSIONS

[0082] In conclusion, this study showed that 3-BP delivered by a mitochondria-targeted NP system has enormous potential in increasing the therapeutic window of free 3-BP. To the best of our knowledge, this is the first demonstration of engineering of such a NP system for mitochondrial delivery of 3-BP with superior efficacy. Furthermore, we demonstrated that mitochondrial compartmentalization of 3-BP when delivered with a T-AuNP system is time-dependent which utilizes different targets of 3-BP in the mitochondrial compartments further enhancing 3-BP activity. 3-BP released from T-AuNP by a protease mediated hydrolysis showed HK2 inhibition, anti-proliferative effects on highly glycolytic PC3 and DU145 cell lines, inhibited lactate production, complete inhibition of glycolysis, and blocked energy metabolism in these cells, finally triggered cell death and apoptosis. The efficiencies of all these activities were much higher compared to 3-BPs when delivered using a non-targeted NP system or the free formulation. Treatment-derived toxicity, cell-insensitivity to metabolic drugs such as free 3-BP, and lack of therapeutic selectivity are still the major issues in developing strategies leading to cancer cure. Given its ability to selectively deliver 3-BP inside mitochondria, T-3-BP-AuNPs could constitute a leading construct in the development of chemotherapeutics that target the unique neoplastic alterations of the cell glucose metabolism and up regulation of HK2. This study showed the potential that delivery of 3-BP using a mitochondria-targeted delivery system is an effective means of limiting

systemic toxicity while enhancing efficacy thus providing new strategies for 3-BP therapy.

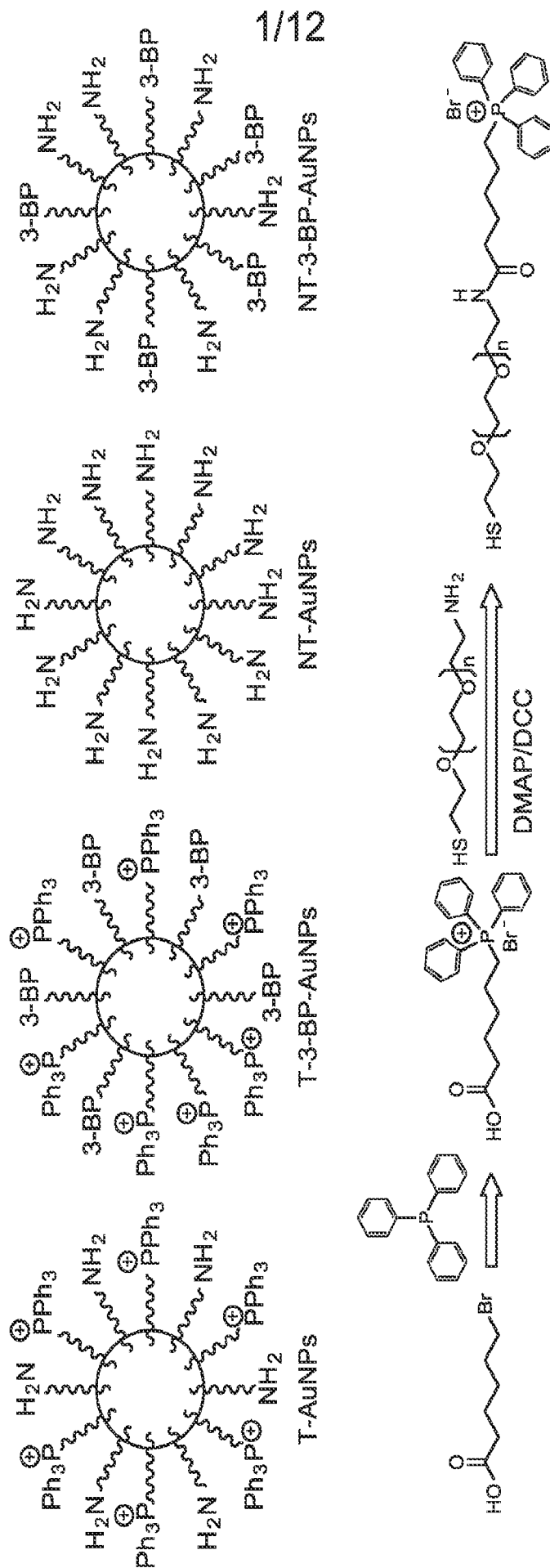
[0083] Thus, embodiments of MITOCHONDIRAL DELIVERY OF 3-BROMOPYRUVATE are disclosed. One skilled in the art will appreciate that the nanoparticles and methods described herein can be practiced with embodiments other than those disclosed. The disclosed embodiments are presented for purposes of illustration and not limitation.

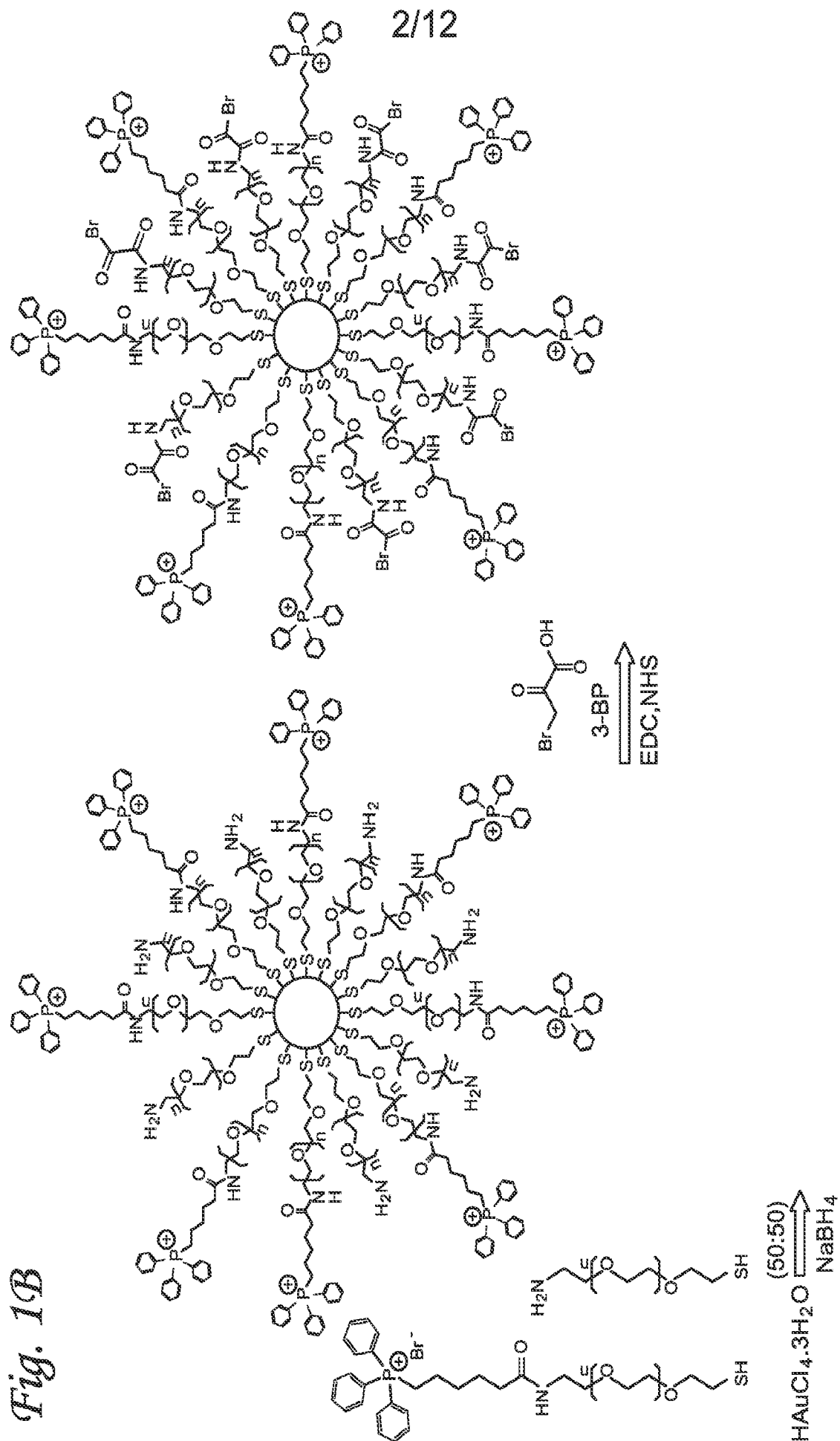
What is claimed is:

1. A nanoparticle, comprising:
a core;
hexokinase-2 inhibitor attached to the core; and
a mitochondrial targeting moiety attached to the core;
wherein the core comprises a radiation excitable component or wherein the nanoparticle further comprises a radiation excitable component attached to the core.
2. A nanoparticle according to claim 1, wherein the core is a radiation excitable gold nanoparticle core.
3. A nanoparticle according to claim 1 or claim 2, wherein the mitochondrial targeting moiety comprises a moiety selected from the group consisting of a triphenyl phosphonium (TPP) moiety, a Szeto-Shiller peptide, and a rhodamine cation.
4. A nanoparticle according to claim 1 or claim 2, wherein the mitochondrial targeting moiety comprises a triphenyl phosphonium (TPP) moiety or a derivative thereof.
5. A nanoparticle according to any one of the preceding claims, wherein the hexokinase-2 inhibitor is selected from the group consisting of 3-bromopyruvate, 2-deoxy-D-glucose, lonidamine, and combinations thereof.
6. Use of a nanoparticle according any one of the preceding claims for treating a patient in need thereof.

7. Use of a nanoparticle according any one of claims 1-5 in the manufacture of a medicament for treating a patient in need thereof.
8. A method comprising administering a nanoparticle according to any of claims 1-5 to a subject.
9. A method for treating a patient at risk or suffering from cancer, comprising administering an effective amount of a nanoparticle according any of claims 1-5 to the patient.
10. A method according to claim 9, wherein the cancer is prostate cancer.
11. A method according to any one of claims 8-10, further comprising laser irradiating cells containing the nanoparticles after administering the nanoparticles to the subject to cause the radiation excitable component to heat.

Fig. 1A





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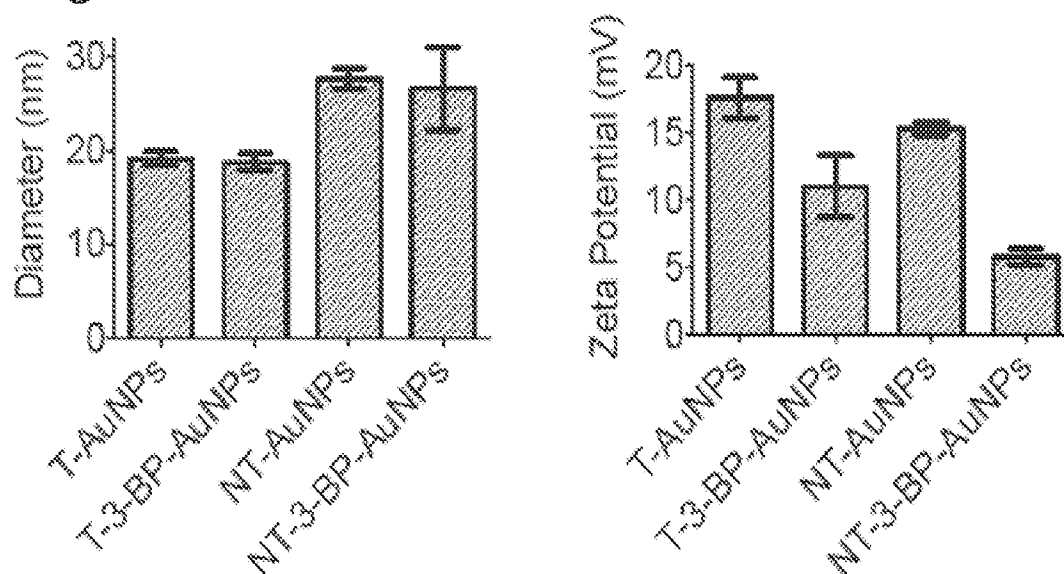
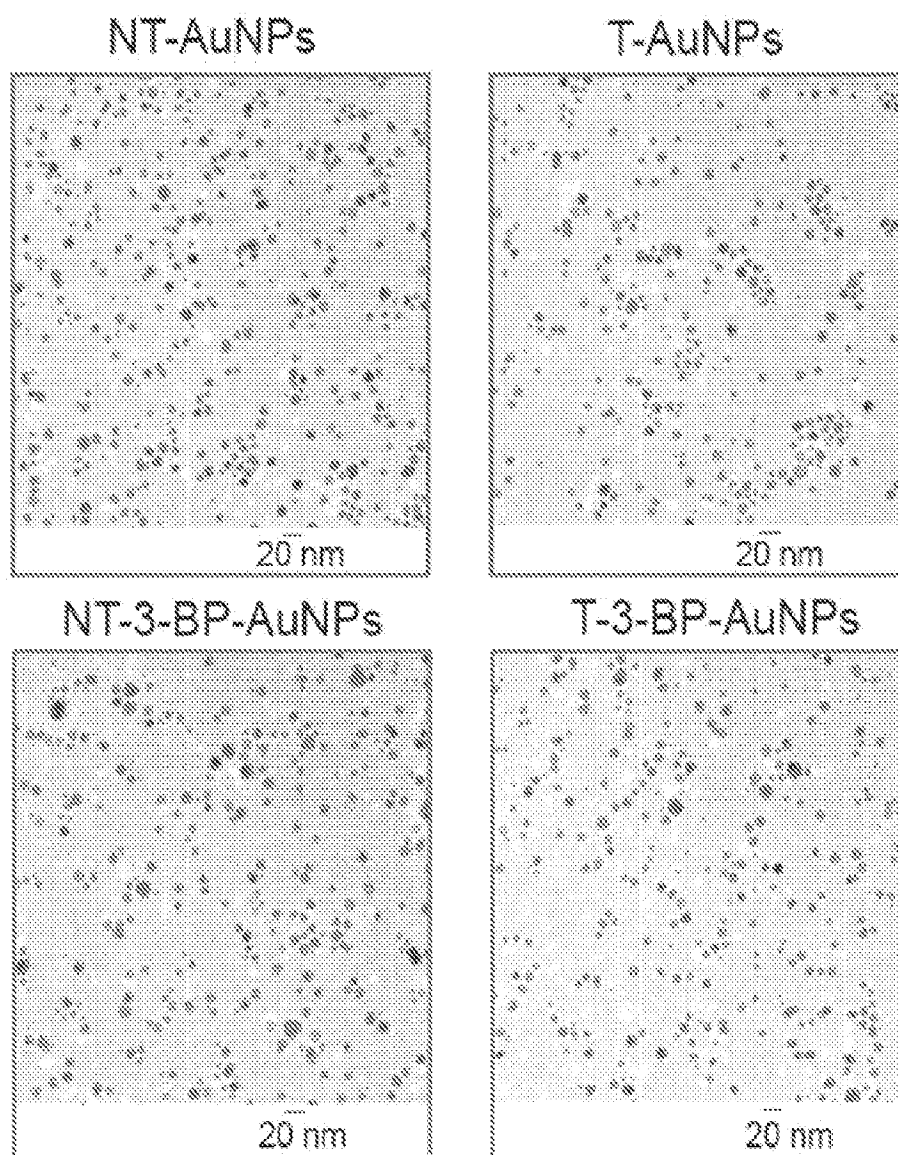
Fig. 2A*Fig. 2B*

Fig. 2C

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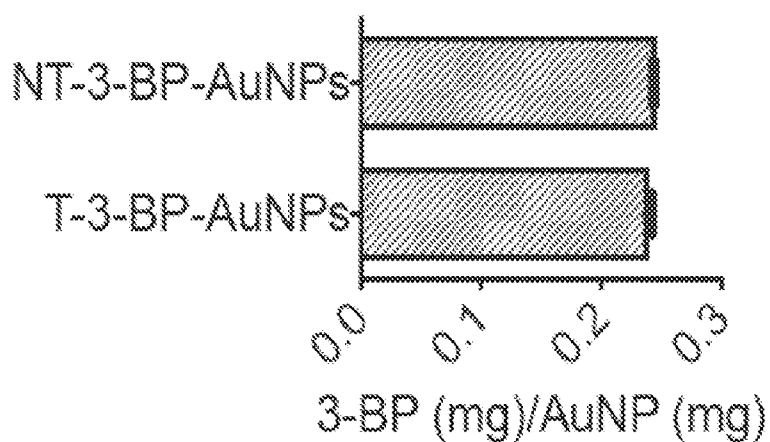
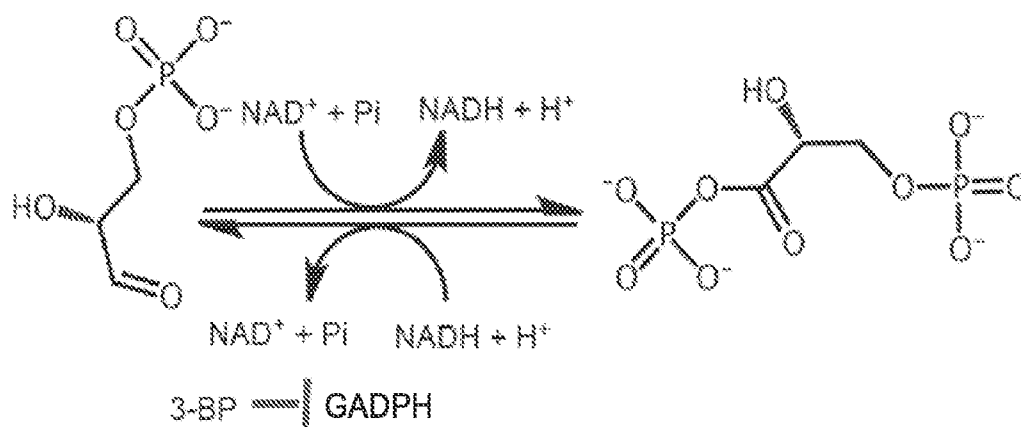
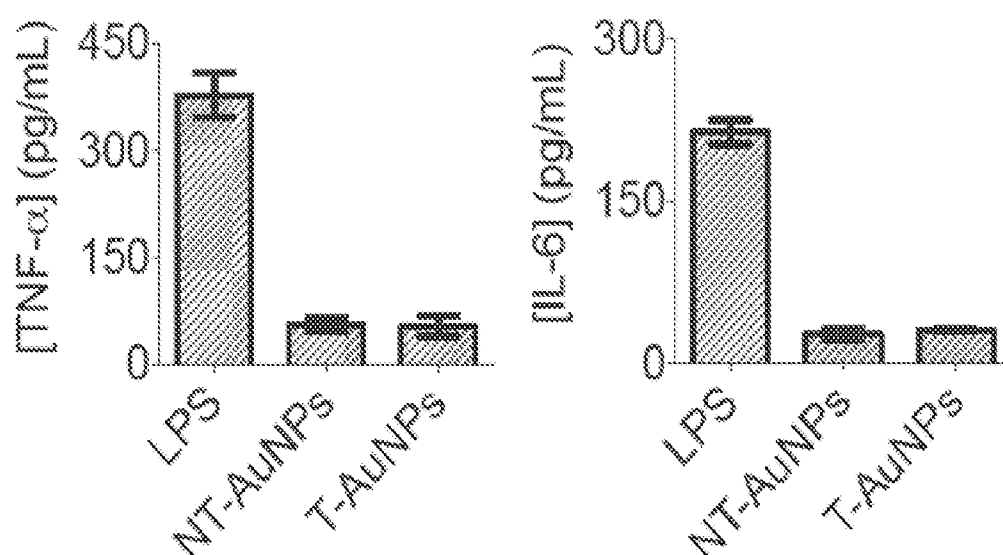


Fig. 2D



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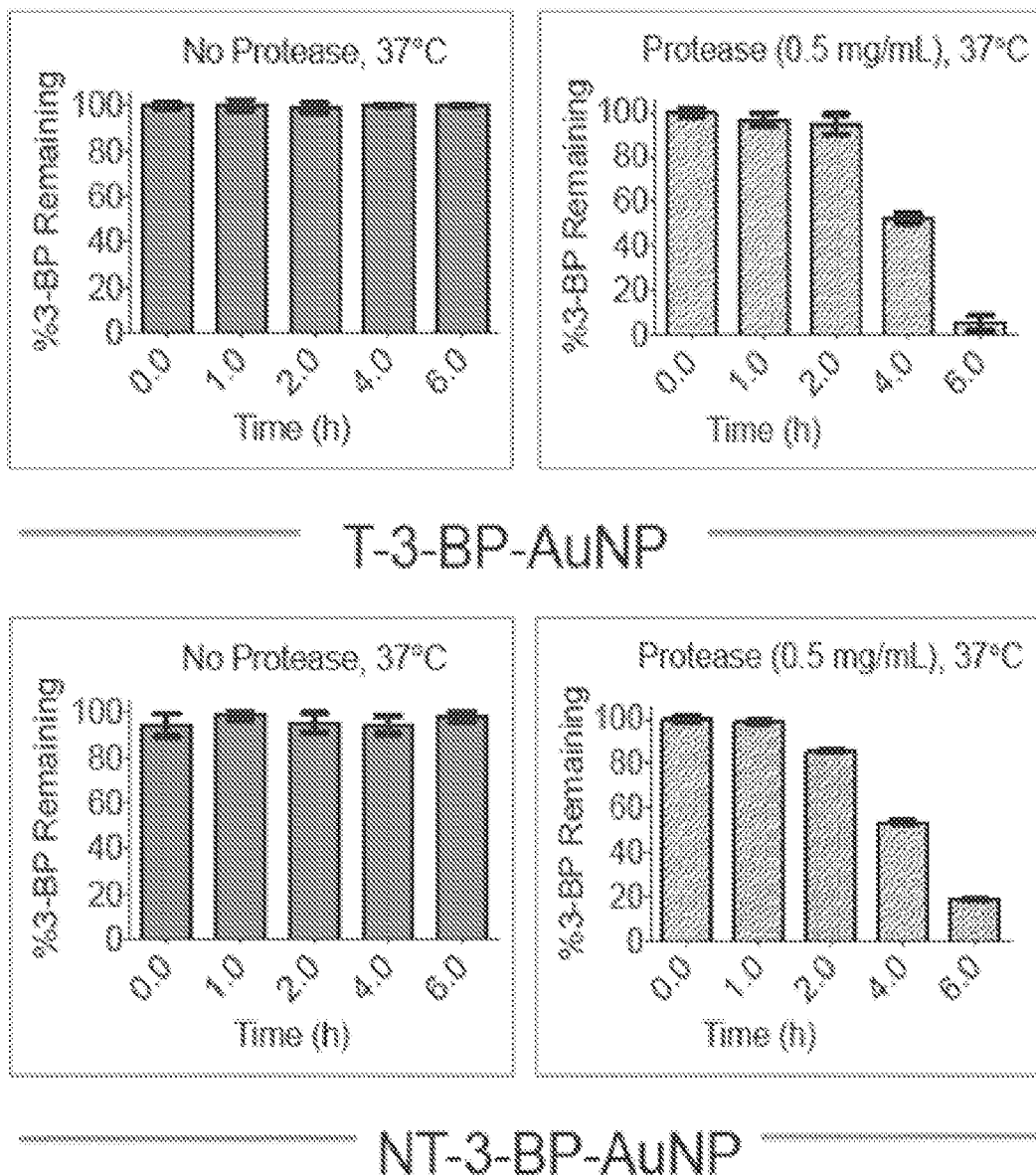
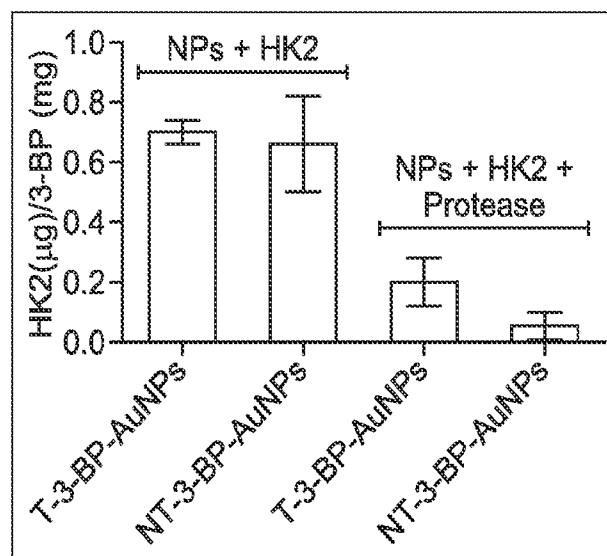
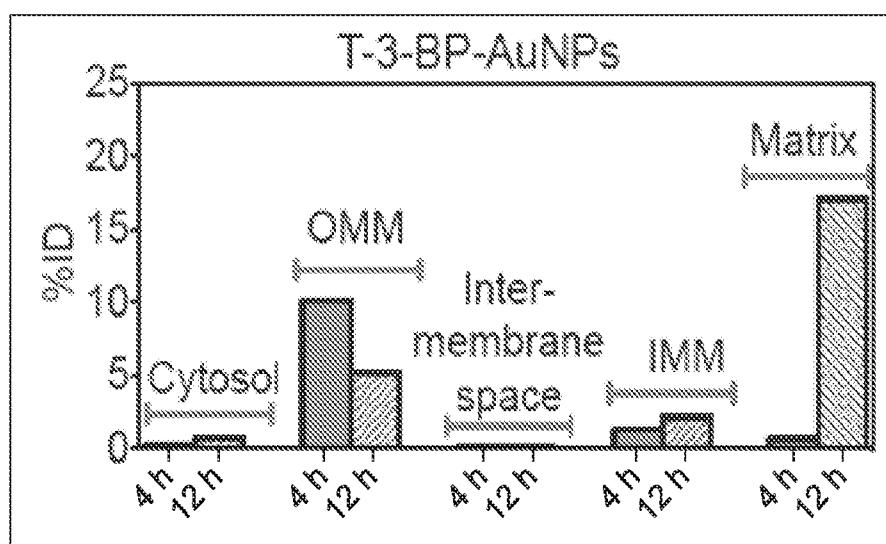
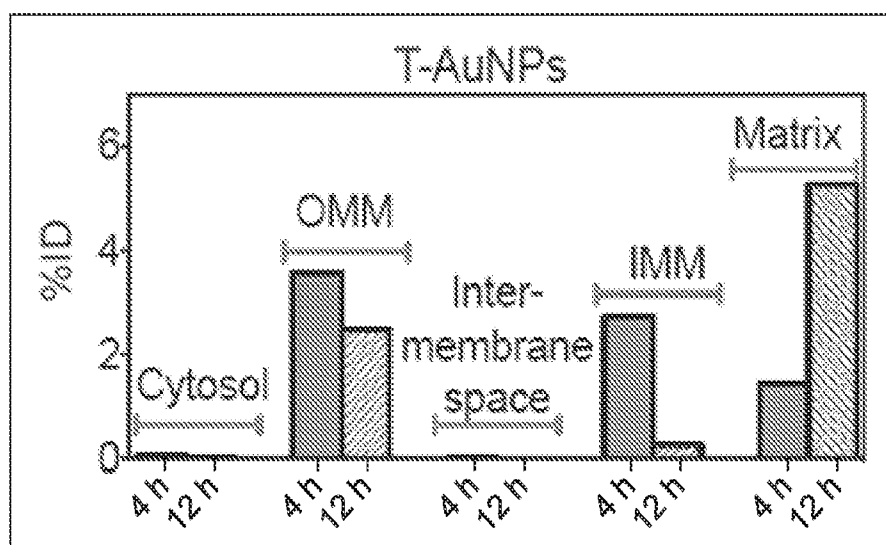
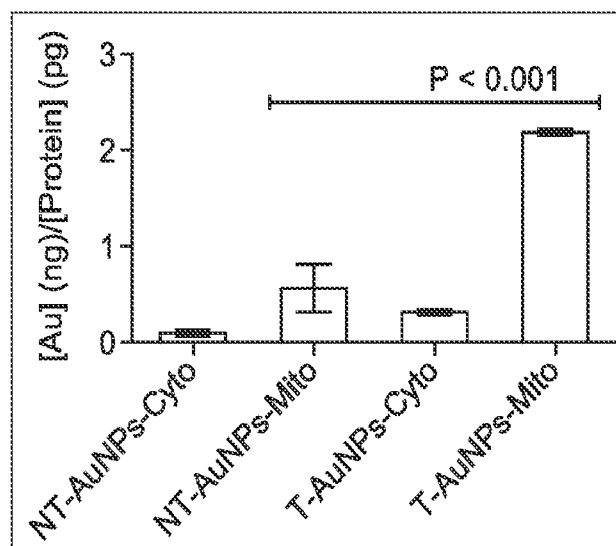
Fig. 3A*Fig. 3B*

Fig. 3C

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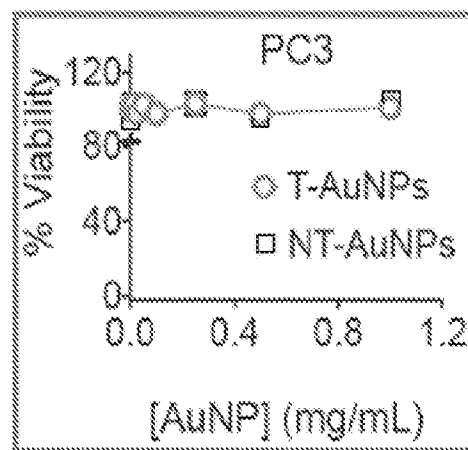
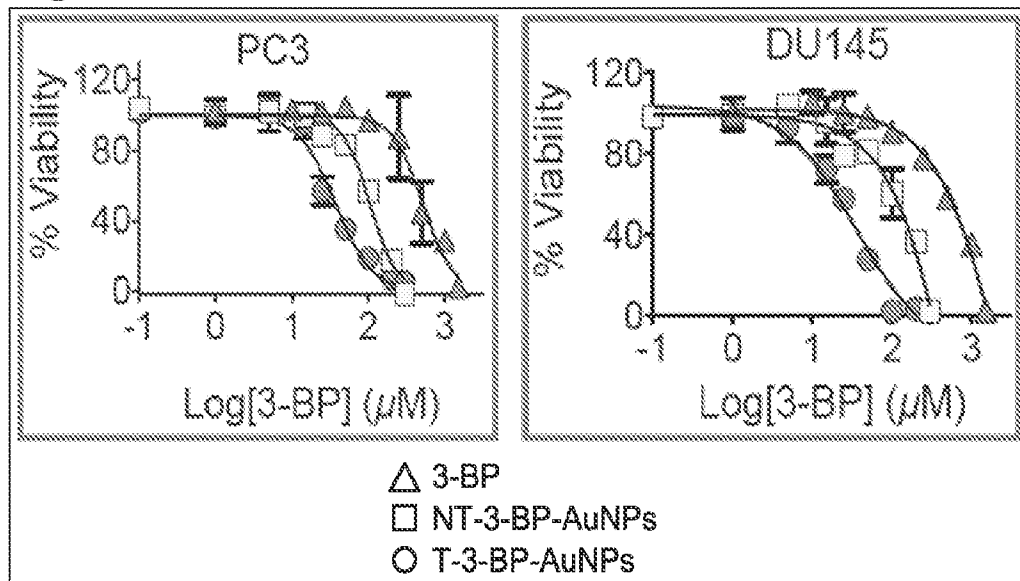
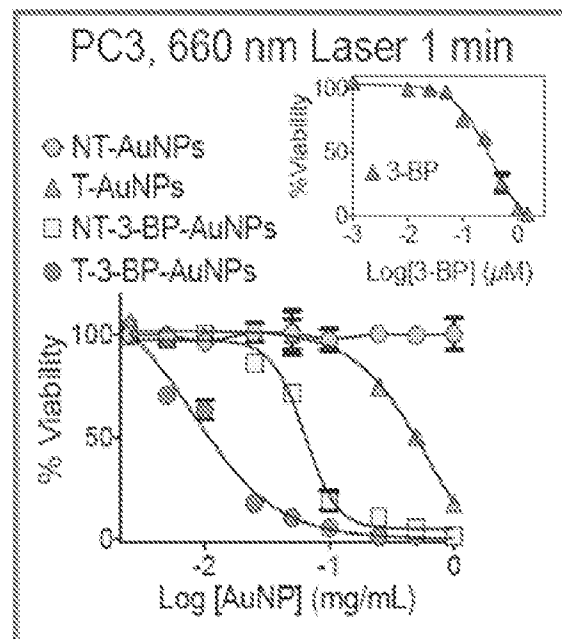
Fig. 4A*Fig. 4B*

Fig. 4C

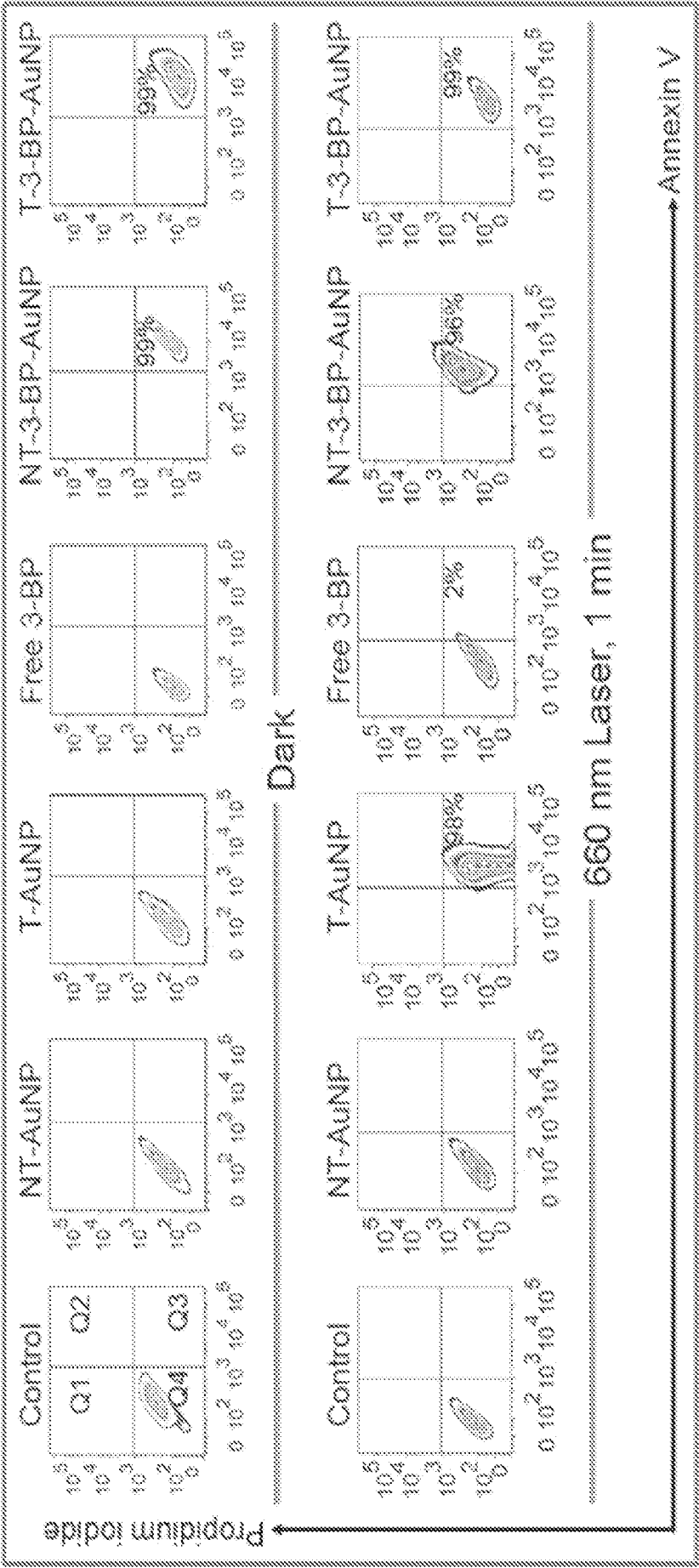
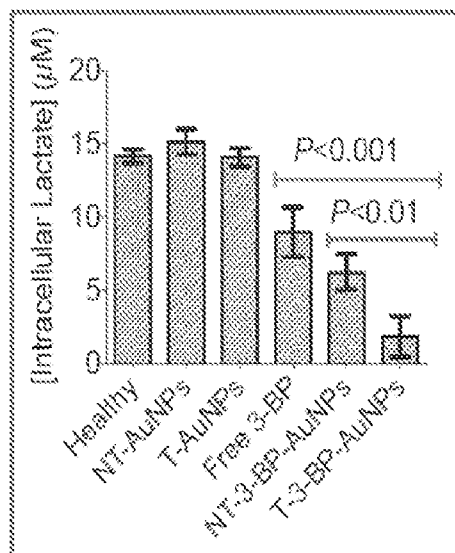
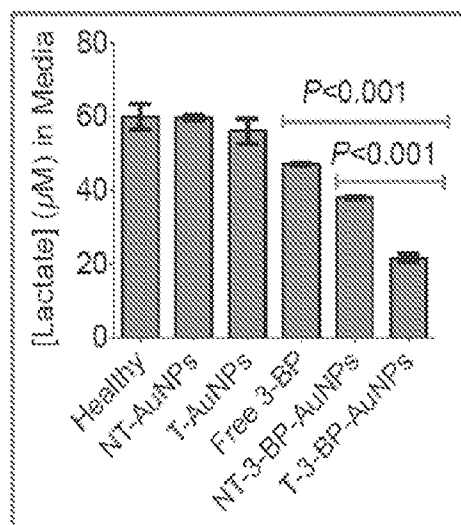
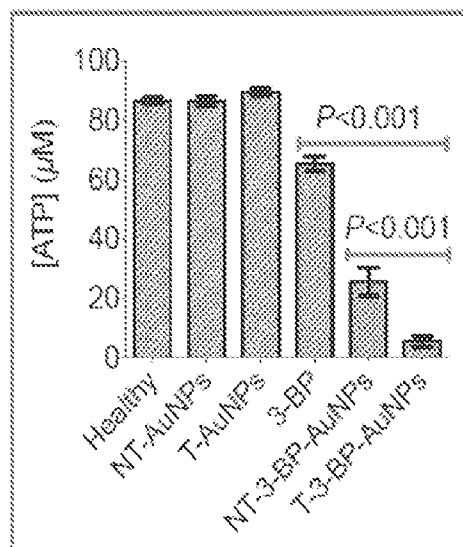
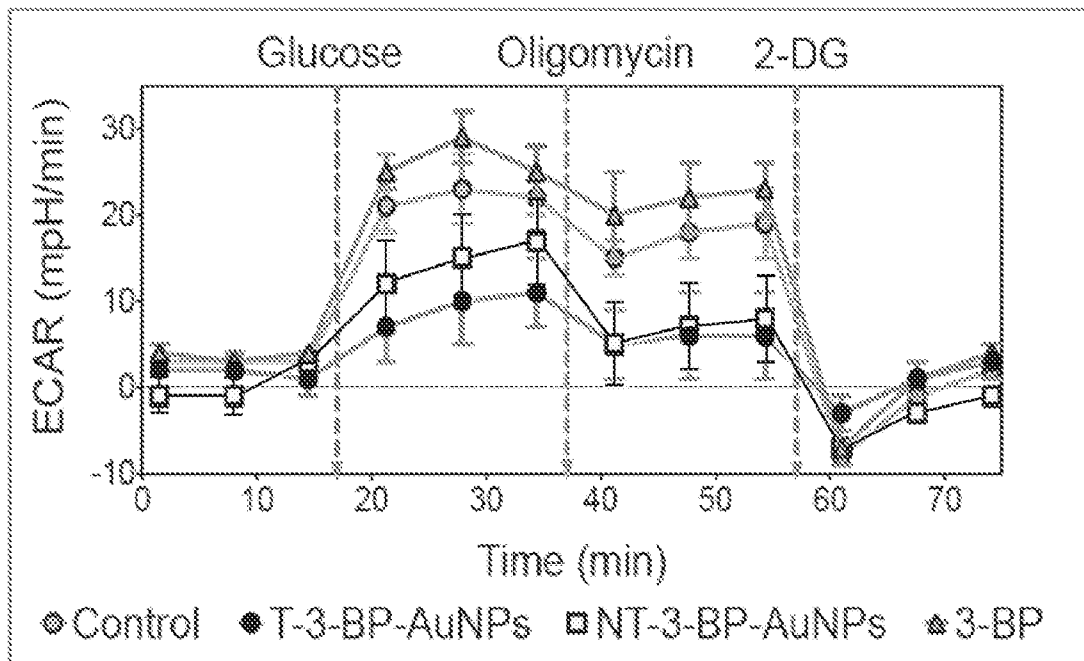
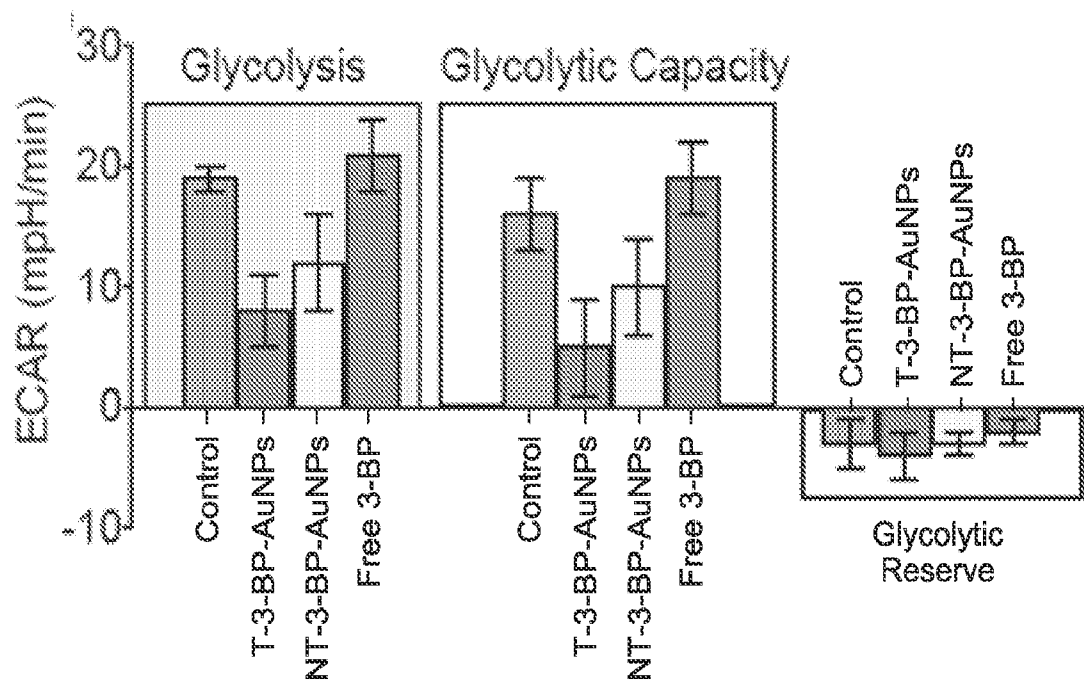


Fig. 5A

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*Fig. 5B**Fig. 5C*

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Fig. 6A*Fig. 6B*

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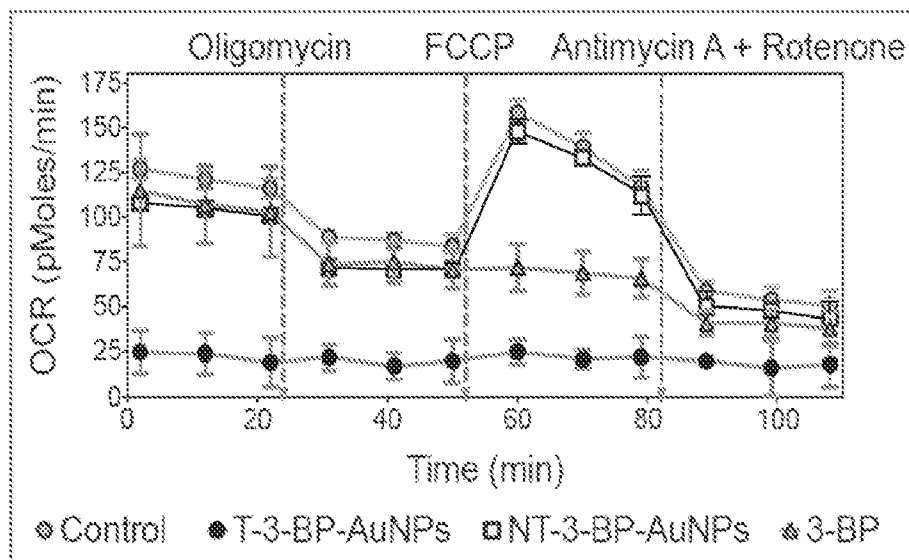
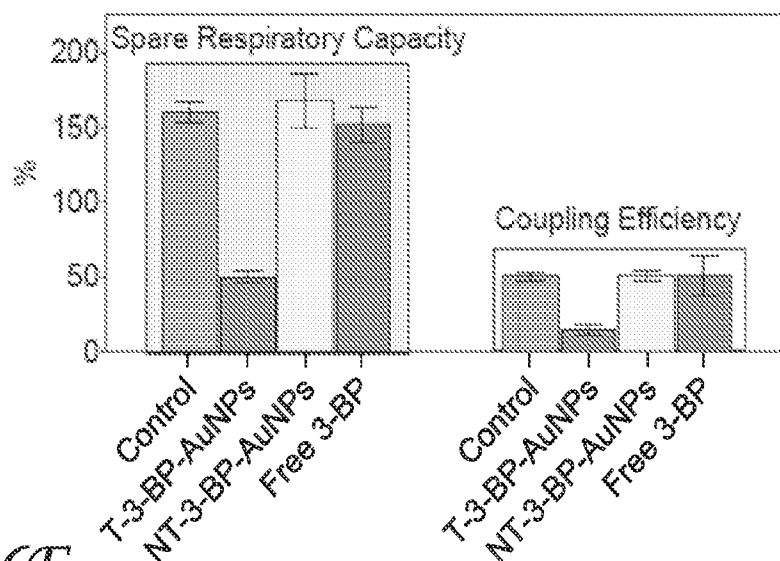
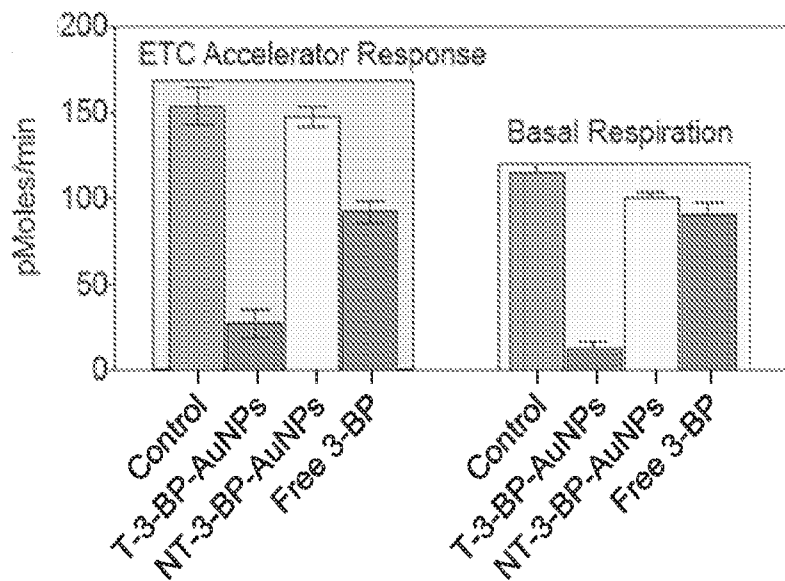
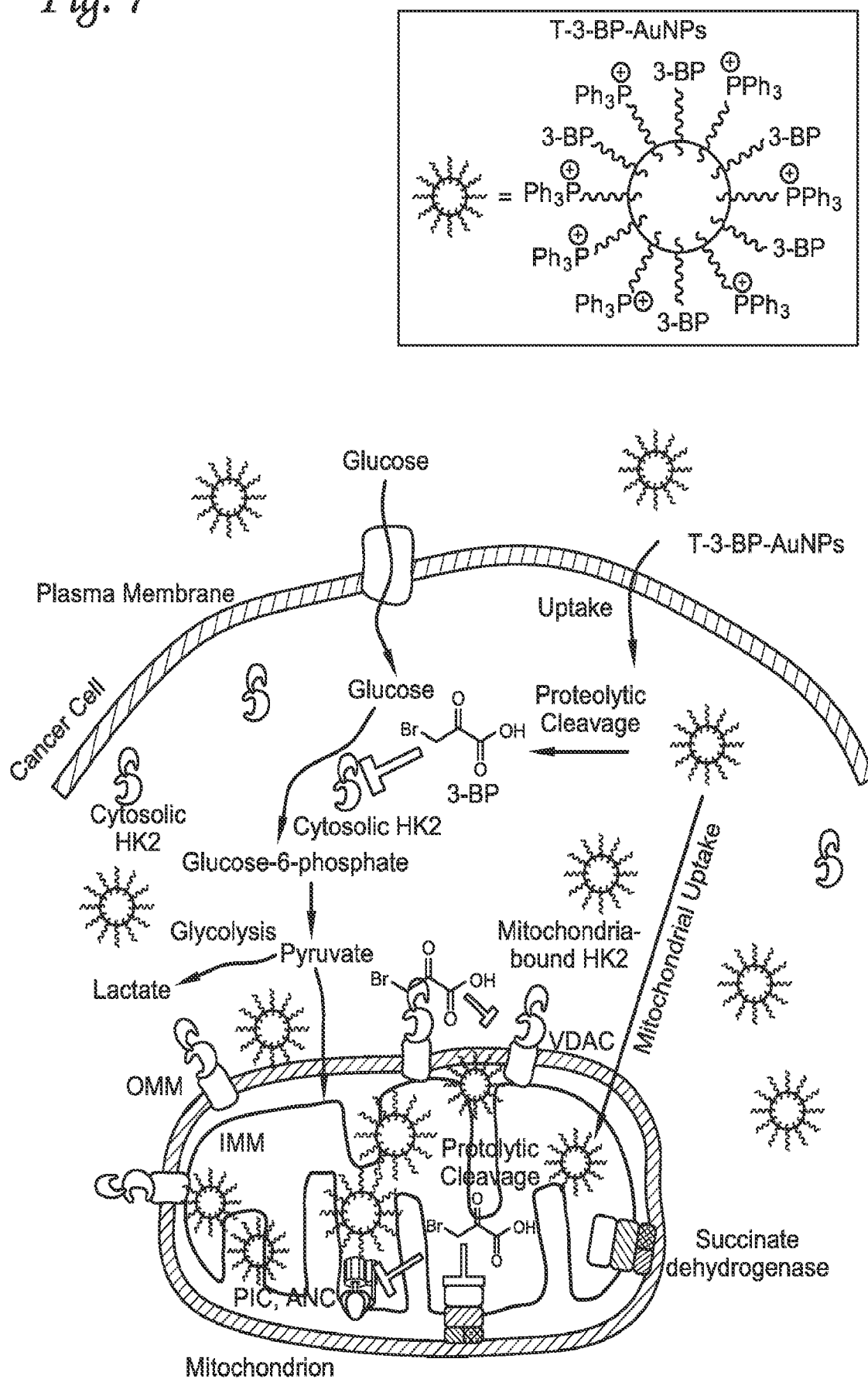
Fig. 6C*Fig. 6D**Fig. 6E*

Fig. 7

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/020591

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/66 (2015.01)

CPC - A61K 31/66 (2015.04)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 9/51, 31/66; B82Y 5/00 (2015.01)

CPC - A61K 9/51, 31/66, 47/48884 49/0067; B82Y 5/00 (2015.04) (keyword delimited)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/375; 556/021; 977/773, 905 (keyword delimited)

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

Orbit, Google Patents, Google, Google Scholar

Search terms used: Hexokinase, mitochondria, nanoparticle, triphenyl phosphonium.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0300532 A1 (JAHNEN-DECHENT et al) 08 December 2011 (08.12.2011) entire document	1-4
Y	US 2011/0008418 A1 (KO) 13 January 2011 (13.01.2011) entire document	1-4
A	WO 2013/123298 A1 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION INC) 22 August 2013 (22.08.2013) entire document	1-4
X, P	MARRACHE et al.: The energy blocker inside the power house: mitochondria targeted delivery of 3-bromopyruvate. Chemical Science 6(3); 1832-1845, 2015. [retrieved on 08.05.2015]. Retrieved from the Internet. <URL: http://pubs.rsc.org/en/content/articlepdf/2015/SC/C4SC01963F >. entire document	1-4

☐ Further documents are listed in the continuation of Box C.

* 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

08 May 2015

Date of mailing of the international search report

10 JUN 2015

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Blaine R. Copenheaver

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/020591

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims 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. ☒ Claims Nos.: 5-11
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 claims 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 claims 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.