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(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, Oakland, California 94607-5200 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ZINK, Jeffrey I.** [US/US]; 3560 Valley Meadow Road, Sherman Oaks, California 91403 (US). **NEL, Andre E.** [US/US]; 4212 Murieta Avenue, Sherman Oaks, California 91423 (US). **XIA, Tian** [CN/US]; 11120 Queensland Street, Unit A5, Los Angeles, California 90034 (US). **Ji, Zhaoxia** [CN/US]; 3710 Los Feliz Boulevard, Apartment 42, Los Angeles, California 90027 (US). **MENG, Huan** [CN/US]; 3340 Sawtelle Boulevard, Apartment 204, Los Angeles, California 90066 (US). **LI, Zongxi** [CN/US]; 3245 South Sepulveda Boulevard, Apartment 303, Los Angeles, California 90034 (US). **LIONG, Monty** [US/US]; 103 9th Street, Apartment 126, Charlestown, Massachusetts 02129 (US).

Angeles, California 90066 (US). **LI, Zongxi** [CN/US]; 3245 South Sepulveda Boulevard, Apartment 303, Los Angeles, California 90034 (US). **LIONG, Monty** [US/US]; 103 9th Street, Apartment 126, Charlestown, Massachusetts 02129 (US).

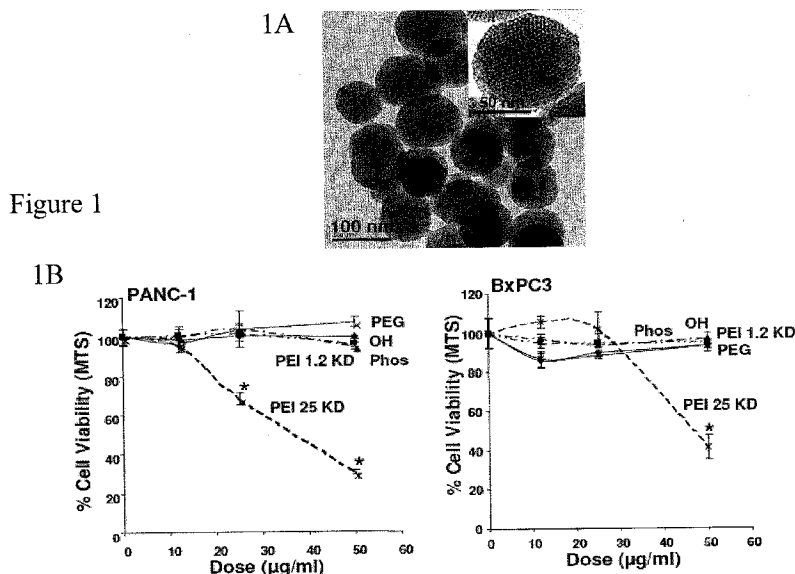
(74) Agents: **DALEY, Henry J.** et al.; VENABLE LLP, P.O. Box 34385, Washington, District of Columbia 20043-9998 (US).

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(54) Title: CATIONIC POLYMER COATED MESOPOROUS SILICA NANOPARTICLES AND USES THEREOF



(57) Abstract: A submicron structure having a silica body defining a plurality of pores and a cationic polymer on the surface of said silica body is described. The submicron structure may further include an oligonucleotide and be used to deliver the oligonucleotide to a cell. The submicron structure may further include a therapeutic agent and be used to deliver the therapeutic agent to a cell. An oligonucleotide and therapeutic agent may be used together. For example, when the oligonucleotide is an siRNA, the composition may be used to decrease cellular resistance to the therapeutic agent by decreasing translation of a resistance gene.

**Declarations under Rule 4.17:**

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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CATIONIC POLYMER COATED MESOPOROUS SILICA NANOPARTICLES AND USES THEREOF

CROSS-REFERENCE OF RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 61/363,945 filed July 13, 2010, the entire contents of which are hereby incorporated by reference.

[0002] This invention was made with Government support of Grant No. RC2 ES018766; R01 ES016746, and R01 CA133697, awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND

Field of Invention

[0003] The current invention relates to submicron structures having a silica body defining a plurality of pores and an outer surface between pore openings of said plurality of pores and a cationic polymer on the surface of said silica body. Such submicron structures may be combined with oligonucleotides and therapeutic compounds for drug delivery, transfection, and cancer therapy.

Discussion of Related Art

[0004] Based on properties such as large surface area and ordered porous channels that can be used to encapsulate molecules, mesoporous silica nanoparticles (MSNP) have emerged as an efficient drug delivery platform (Kim et al., *Angew. Chem., Int. Ed.*, vol. 47, pp. 8438-8441, 2008; Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008; Lu et al., *Small*, vol. 3, pp. 1341-1346, 2007; Slowing et al., *Adv. Drug Delivery Rev.*, vol. 60, pp. 1278-1288, 2008; Vallet-Regí et al., *Angew. Chem., Int. Ed.*, vol. 46, pp. 7548-7558, 2007). In addition to the well-developed surface chemistry, silica materials are known to be safe, biodegradable and potentially biocompatible (Borm et al., *Toxicol. Sci.*, vol. 90, pp. 23-32, 2006; Finnie et al., *J. Sol-Gel. Sci. Techn.*, vol. 49, pp. 12-18, 2009). This drug transport system is suitable for the delivery of anticancer drugs, including camptothecin, paclitaxel, and doxorubicin (Kim et al., *Angew. Chem., Int. Ed.*, vol. 47, pp. 8438-8441, 2008; Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008; Vivero-Escoto et al., *J. Am. Chem. Soc.*, vol. 131, pp. 3462-3463, 2009). The chemical stability of the particles contribute to their therapeutic utility by allowing the attachment of functional groups for

imaging and targeting applications along with the placement of a series of nanovalves for on-demand drug release (Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008; Nguyen et al., *Org. Lett.*, vol. 8, pp. 3363-3366, 2006; Rosenholm et al., *ACS Nano*, vol. 3, pp. 197-206, 2009).

[0005] RNA interference describes natural processes that lead to gene silencing by siRNA (Moazed et al., *Nature*, vol. 457, pp. 413-420, 2009). siRNA has been widely used as an experimental tool that is now also becoming the focus of the pharmaceutical industry (Blow et al., *Nature*, vol. 450, pp. 1117-1120, 2007). Currently there are a number of clinical trials underway that include the use of siRNAs to treat various disease processes (Davis et al., *Mol. Pharm.*, vol. 6, pp. 659-668, 2009; Judge et al., *Mol. Ther.*, vol. 13, pp. 494-505, 2006). As for most molecular therapies, *in vivo* delivery is a major hurdle in successful implementation and has sparked a number of strategies to increase siRNA circulatory half-life, facilitate transduction across biological membranes, and achieve cell-specific delivery (Davis et al., *Mol. Pharm.*, vol. 6, pp. 659-668, 2009; Judge et al., *Mol. Ther.*, vol. 13, pp. 494-505, 2006).

[0006] There are a number of circumstances where drug and siRNA delivery could achieve a synergistic therapeutic outcome. One example is the restoration of drug sensitivity in cancer cells by knockdown of genes that are involved in the resistance to one or more chemotherapeutic agents. An example is the inducible P-glycoprotein (Pgp) gene that encodes for a gene product known as the multiple drug resistance protein 1 (MDR-1) (Gottesman et al., *Annu. Rev. Med.*, vol. 53, pp. 614-627, 2002). Pgp is constitutively expressed in normal cells such as capillary endothelial cells in the blood brain barrier but also is selectively overexpressed in carcinomas of the stomach, breast, pancreas and cervix in response to a number of chemotherapeutics agents (Szakacs et al., *Nat. Rev. Drug Discov.*, vol. 5, pp. 219-234, 2006). If overexpressed, Pgp could lead to drug resistance because MDR-1 contributes to the formation of a drug efflux pump that prevents the intracellular buildup of chemotherapeutic agents (Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008).

SUMMARY

[0007] Embodiments of the invention include a submicron structure having a silica body defining a plurality of pores and an outer surface between pore openings of said plurality of pores, and a cationic polymer on the surface of said silica body. Said submicron structure has a maximum dimension less than one micron.

[0008] In some embodiments, the submicron structure also includes a cationic therapeutic compound. The cationic therapeutic compound may be, for example, in the interior or in the pores of the submicron structure. In some embodiments, the submicron structure also includes an oligonucleotide electrostatically bound to the cationic polymer. Some embodiments include both a cationic therapeutic compound and an oligonucleotide. In some embodiments, the oligonucleotide is an siRNA that reduces translation of a protein that causes resistance in a cell. In some embodiments, the siRNA reduces translation of a protein that causes resistance to the therapeutic compound in the cell. In some embodiments, the siRNA reduces translation of p-glycoprotein. In some embodiments, the therapeutic compound is doxorubicin.

[0009] In some embodiments, the cationic polymer is electrostatically bound to the silica body. In some embodiments, the cationic polymer is selected from the group consisting of polyethyleneimine, polyamidoamine, polylysine, poly(allylamine), and poly(diallyldimethylammonium chloride). In some embodiments, the cationic polymer is polyethyleneimine.

[0010] Some embodiments include an oligonucleotide electrostatically bound to the cationic polymer. The oligonucleotide may be DNA or RNA. When the oligonucleotide is RNA, it may be a small inhibiting RNA (siRNA).

[0011] Some embodiments include a therapeutic compound within the silica body or pores of the silica body. The therapeutic compound may be hydrophobic, neutral (i.e. uncharged), cationic or anionic at physiologic pH.

[0012] In some embodiments, the silica body is mesoporous. In some embodiments, the pores are substantially cylindrical and have an ensemble average diameter between about 1 nm and about 10 nm.

[0013] In some embodiments, the silica body is substantially spherical and has a diameter between about 50 nm and about 1000 nm. In some embodiments, the substantially spherical silica body has a diameter between about 100 nm and about 500 nm.

[0014] In some embodiments, the silica body has a plurality of anionic molecules attached to an outer surface of the silica body. In some embodiments, the plurality of anionic molecules have a phosphonate moiety. In some embodiments, the plurality of anionic molecules are derived from reaction between the silica body surface and trihydroxysilylpropyl methylphosphonate.

[0015] In some embodiments, the submicron structure further includes a light-emitting compound, peptide, protein, oligonucleotide, sugar, oligosaccharide, or polysaccharide covalently bonded to the surface of the silica body. Some embodiments have a light emitting compound covalently bonded to the surface of the silica body.

[0016] In some embodiments, the submicron structure further includes a core structure within the silica body. In some embodiments, the core structure is a superparamagnetic nanocrystal, silver nanocrystal or gold nanocrystal. In some embodiments, the core structure is a superparamagnetic iron oxide nanocrystal

[0017] Embodiments of the invention include pharmaceutical compositions having a submicron structure according to the invention and a pharmaceutically acceptable carrier or excipient. Embodiments of the invention include use of the submicron structures according to the invention for the manufacture of a medicament or pharmaceutical composition for the treatment of a disease or disorder.

[0018] Embodiments of the invention include therapeutic methods have the step of administering an effective amount of a submicron structure according to the invention to a subject in need of treatment. Embodiments include use of the submicron structures according to the invention for the treatment of a disease or disorder by administering the submicron structure to a subject in need of treatment.

[0019] Embodiments of the invention include methods of treating drug resistant cancer where the submicron structure includes an siRNA that reduces translation of a protein causing resistance in the drug resistant cancer. Embodiments of the invention includes the use of a submicron structure according to the invention having an siRNA that reduces translation of a protein causing drug resistance to treat drug resistant cancer.

[0020] Embodiments of the invention include methods of transfecting a cell by administering a submicron structure according to the invention having an oligonucleotide. Embodiments of the invention include the use of a submicron structure according to the invention including an oligonucleotide to transfect a cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Further objectives and advantages will become apparent from a consideration of the description, drawings, and examples.

[0022] **Figure 1** shows transmission electron microscopy (TEM) of the MSNP and cell viability detection by the MTS assay. **Figure 1A** shows TEM image shows the particle size and the ordered pore structure. **Figure 1B** shows cell viability after addition of appropriately dispersed MSNP exhibiting a range of surface modifications to pancreatic cancer cell lines at doses ranging from 12.5-50 $\mu\text{g/ml}$ for 16 hrs, cells were incubated with the MTS reagent for 30 min and the absorbance was measured at 490 nm. All the MTS values were normalized according to the value of the control (no particle exposure) – this was regarded as 100% cell viability. The IC_{50} values of MSNP-PEI-25 KD in PANC-1 and BxPC3 cells were 37 $\mu\text{g/ml}$ and 46 $\mu\text{g/ml}$, respectively. The results were reproduced 3 times.

[0023] **Figure 2** shows cellular uptake of FITC-labeled MSNP in PANC-1 cells. MSNP were labeled with FITC as described in Example 1. **Figure 2A** shows a representative histogram showing the shift in fluorescence intensity in PANC-1 cells treated with 25 $\mu\text{g/ml}$ FITC-MSNP that contain different surface modifications (left panel). The fold-increase in MFI after 3 hr was calculated and used to generate the graph. FITC-labeled MSNP-Phos served as a control particle to show that coating with PEI leads to enhanced uptake in the same particle type in the same cell (right panel). **Figure 2B** shows confocal microscopy used to study the cellular uptake of FITC-MSNP in PANC-1 cells. Cells were exposed to 25 $\mu\text{g/ml}$ FITC-labeled particles for 3 hr. After cell membrane staining with 5 $\mu\text{g/ml}$ red fluorescent wheat germ agglutinin (WGA), cells were visualized using a Confocal 1P/FCS Inverted microscope. Data are representative of 3 separate experiments. $*p<0.01$ compared with control.

[0024] **Figure 3** shows cellular uptake of FITC-labeled MSNP in BxPC3 cells. BxPC3 cells were exposed to FITC-labeled MSNP and flow cytometry and confocal microscopy were conducted as in **Figure 2**. **Figure 3A** shows a representative histogram showing the shift in fluorescence intensity (left panel). The fold-increase in MFI after 3 hr was calculated and used to generate the graph. FITC-labeled MSNP-Phos served as a control particle to show that coating with PEI leads to enhanced uptake in the same particle type in the same cell (right panel). **Figure 3B** shows confocal microscopy used to study the cellular uptake of FITC-MSNP. Cells were exposed to 25 $\mu\text{g/ml}$ FITC-labeled particles for 3 hr. After cell membrane staining with 5 $\mu\text{g/ml}$ red fluorescent wheat germ agglutinin (WGA), cells were visualized using a Confocal 1P/FCS Inverted microscope. Data are representative of 3 separate experiments. $*p<0.01$ compared with control.

[0025] **Figure 4** shows Cell viability detection by the MTS assay. After incubation with particles coated with polymers of MW 0.6-25 KD at doses of 6-100 $\mu\text{g/ml}$ for 16 hrs, PANC-1 (**Figure 4A**), BxPC3 (**Figure 4B**), and HEPA-1 (**Figure 4C**) cells were incubated with the MTS reagent for 30 min and the absorbance was measured at 490 nm. All the MTS values were normalized as described in **Figure 1**. The experiment was reproduced 3 times.

[0026] **Figure 5** shows Gel retardation and DNase I protection assays. Agarose gel electrophoresis of PEI-MSNP/plasmid DNA (pEGFP) (**Figure 5A**) and PEI-MSNP/siRNA (**Figure 5B**) complexes at various nanoparticle to nucleic acid (N/P) ratios. Anionic phosphonate-coated MSNP was used as a control. M=MW marker. **Figure 5C** shows DNase I protection assay. M: DNA marker. Φ : naked plasmid DNA (pEGFP), as negative control. Lane 1, pDNA/PEI-1.2 KD complex. Lane 2, pDNA/PEI 25 KD complex. Lane 3, naked pDNA treated with DNase I, positive control. Lane 4, pDNA/PEI 1.2 KD complex treated with DNase I before pDNA was released by 1% SDS. Lane 5, pDNA/PEI 25 KD complex treated with DNase I before pDNA was released by 1% SDS. Lane 6, pDNA/PEI 1.2 KD complex treated with 1% SDS. Lane 7, pDNA/PEI 25 KD complex treated with 1% SDS.

[0027] **Figure 6** shows GFP knockdown by siRNA in stable transfected GFP-HEPA cells. HEPA-1 cells with stable GFP expression were used for siRNA knockdown assays. MSNP coated with different size PEI polymers were used to transfect GFP-specific or scrambled siRNA and the results compared with Lipofectamine 2000 as transfection agent. **Figure 6A** shows GFP knockdown assessed by flow cytometry in which GFP MFI was normalized to the value of control untransduced cells (100%). **Figure 6B** shows confocal pictures showing GFP knockdown in GFP-HEPA cells. TEX 615-labeled siRNA was used to show the cellular localization of the nucleic acid bound particles (red dots). "X" represents scrambled siRNA. The experiment was reproduced 3 times.

[0028] **Figure 7** shows GFP plasmid DNA transfection into HEPA-1 cells. HEPA-1 cells were used for GFP plasmid DNA transfection. MSNP coated with different size PEI polymers were used to transfect GFP plasmid DNA and the results were compared with Lipofectamine 2000 as transfection agent. **Figure 7A** shows a representative histogram showing the shift in green fluorescence intensity in HEPA-1 cells after transfection with Lipofectamine 2000 or MSNP-PEI-10 KD. **Figure 7B** shows confocal pictures showing GFP expression in transfected HEPA-1 cells. This demonstrates differences in the transfection efficiency as judged

by fluorescent intensity and proportion of cells in the population showing GFP expression. The experiment was reproduced 3 times.

[0029] **Figure 8** shows Drug delivery to PANC-1 and BxPC3 cells using PEI-MSNPs. A MTS assay was conducted for the paclitaxel-loaded MSNP delivered to these cells at doses of 3-50 $\mu\text{g/ml}$ over a 48 hrs period in PANC-1 (**Figure 8A**) and BxPC3 (**Figure 8B**) cells. The controls were cells treated with particles only and cells treated with paclitaxel suspended in culture medium with and without the addition of DMSO carrier. The experiment was reproduced 2 times.

[0030] **Figure 9** shows MSNP size distribution in aqueous solutions. Dynamic light scattering (DLS) for MSNP exhibiting different surface modifications was performed in water, DMEM plus 10% FCS, BEGM or BEGM plus 2 mg/ml BSA. The presence of serum and BSA in the cell culture media improves MSNP dispersity.

[0031] **Figure 10** shows *assessment of cell viability and mitochondrial membrane potential (MMP) in RAW 264.7 and BEAS-2B cells*. **Figure 10A** shows cell viability following treatment with MSNP displaying different surface modifications, as determined by the MTS assay as described in **Figure 1**. Cells were exposed to MSNP at doses of 12.5-50 $\mu\text{g/ml}$ for 16 hrs. All the MTS values were normalized as outlined in **Figure 1**. The IC_{50} values for image NP-PEI-25 KD in RAW 264.7 and BEAS-2B cells are 40.6 $\mu\text{g/ml}$ and 9.7 $\mu\text{g/ml}$, respectively. **Figure 10B** shows cell death and mitochondrial depolarization after treatment with MSNP-phosphonate and MSNP-PEI backspace-25kD was determined using PI and JC-1, respectively.

[0032] **Figure 11** shows effect on cell viability after conversion of primary amines to COOH. Succinic anhydride was used to convert the primary NH_2 - to COOH- groups on MSNP-PEI-25 KD. **Figure 11A** shows cell viability comparing non-modified with succinic anhydride treated particles in RAW 264.7 cells using MTS assay. **Figure 11B** shows the conversion confirmed using fluorescamine, which yields green fluorescence when complexed to the primary NH_2 groups. The decline in fluorescence intensity was followed in a fluorometer.

[0033] **Figure 12** shows determination of the stability of PEI coating on the MSNP surface. Rhodamine-B labeled PEI was used to coat the surface of FITC-labeled MSNP and the dual-labeled particles were added to RAW 264.7 cells prior to the performance of confocal microscopy. The composite overlay confirms that the polymer and the particle co-localize at the same intracellular site at 3 and 6 hrs.

[0034] **Figure 13** shows cellular uptake of FITC-labeled MSNP in RAW 264.7 cells. MSNP were FITC labeled as described in the Examples. **Figure 13A** shows a representative histogram showing the shift in fluorescence intensity in RAW 264.7 cells treated with 25 µg/ml FITC-MSNP exhibiting different surface modifications for 3 hrs (left panel). The fold-increase in MFI was calculated and used to generate the graph. RITC-labeled MSNP was used as a control as discussed in **Figure 2** (right panel). **Figure 13B** shows confocal microscopy to study the cellular uptake of FITC-MSNP in RAW 264.7 cells. Cells were exposed to 25 µg/ml FITC-labeled particles for 3 hr. After the cell membrane was stained with 5 µg/ml red fluorescent wheat germ agglutinin (WGA), cells were visualized in a Confocal 1P/FCS Inverted microscope. Data are representative of 3 separate experiments. * $p < 0.01$ compared with control.

[0035] **Figure 14** shows Cell viability detection by the MTS assay in RAW 264.7 (**Figure 14A**) and BEAS-2B (**Figure 14B**) cells. After incubation with particles coated with different length PEI polymers at doses from 6.25-100 µg/ml for 16 hrs, the MTS assay and data calculation were performed as described in **Figure 1**.

[0036] **Figure 15** shows detection of GFP knockdown by siRNA using western blotting in HEPA-1 cells. **Figure 15A** shows HEPA-1 cells with stable GFP expression was used for siRNA knockdown as described in **Figure 6**. The procedure, including use of particles coated with different polymer lengths and the control agent, Lipofectamine 2000, was carried out as described in **Figure 6**. However, instead of using confocal microscopy, cells were lysed and the lysates used to conduct anti-GFP immunoblotting. The blotting membrane was also overlaid with an antibody recognizing β -actin to correct for protein loading. **Figure 15B** shows MFI of siRNA was calculated to show relative quantity of siRNA uptake into cells transfected by PEI-MSNP in HEPA-1 cells (arbitrary units).

[0037] **Figure 16** shows quantification of paclitaxel (Pac) loading capacity in MSNP. MSNP with different surface modifications were loaded with paclitaxel. Methanol was used for complete release of the drug from washed particles and the amount of paclitaxel in the supernatant was determined by UV absorbance at 230 nm.

[0038] **Figure 17** shows animal weight and histology of major organs. **Figure 17A** shows animal weight was monitored after particle injections. **Figure 17B** shows histology of liver, kidney, spleen was performed by UCLA Division of Laboratory Animal Medicine (DLAM) diagnostic laboratory services. The sections were stained with hematoxylin-eosin and examined by light microscopy.

[0039] **Figure 18** shows physicochemical characterization of PEI coated MSNP. **Figure 18A** shows TEM images of phosphonate-MSNP before and after coating with the 10 kD PEI polymer. The arrows indicate that the polymer decorates the MSNP surface but leaves the porous interior accessible to drug loading. **Figure 18B** shows particle size and zeta potential in pure water, after stabilization with 1 mg/mL BSA in water, or in DMEM cell culture medium were measured. All of the size and zeta potential data do not significantly change when the MSNP were loaded with Dox and siRNA (**Table 3**).

[0040] **Figure 19** shows effective Pgp siRNA delivery and gene knockdown in KB-V1 cells. **Figure 19A** shows agarose gel electrophoresis of PEI-coated MSNP to which Pgp siRNA was complexed at various nanoparticle to nucleic acid (N/P) ratios. M is molecular weight marker. The Φ lane contains Pgp siRNA only. Dox loading did not change the N/P ratio or the electrophoretic mobility. The results indicate that all siRNA was bound when the N/P ratio ratio >16 (PEI 1.8kD), >16 (PEI 10 kD), and >8 (PEI 25 kD). **Figure 19B** shows confocal microscopy to demonstrate Texas red-labeled siRNA uptake in association with FITC-labeled PEI coated MSNP. The cell membrane and nucleus were stained by WGA 633 and Hoechst 33342, respectively. The panels on the right show merging of the images to show Pgp siRNA co-localization with FITC-MSNP. **Figure 19C** shows quantitative comparison of labeled Pgp siRNA uptake by measuring fluorescent intensity of Texas red in various PEI groups, using Imaging J software. * $p < 0.05$. **Figure 19D** shows detection of Pgp knockdown by siRNA-PEI-MSNP using western blotting. Lipofectamine 2000 was used as positive control. The relevant Pgp expression was calculated by the signal intensity of the protein bands. "X" stands for cells treated by scrambled siRNA-PEI-MSNP.

[0041] **Figure 20** shows that phosphonate-MSNP effectively binds Dox *via* a proton-sensitive mechanism. **Figure 20A** shows modeling studies using positively and negatively charged MSNP under abiotic conditions. Loading yield of Dox in MSNP with various surface modifications. A photograph of the Dox-loaded MSNP (20 mg/ml) containing various surface modifications were taken. Consistent with loading yield, the phosphonate-MSNP was more intensively stained (red) than other particle types. **Figure 20B** shows loading yield measurements for PEI-coated phosphonate MSNP. The yields were similar to that of non-coated particles in (A). **Figure 20C** shows time-dependent release profile of Dox from drug loaded phosphonate MSNP in phenol red free DMEM acidified to pH 5.0. The effect of PBS or treatment with PBS containing 10% ethanol is shown for comparison. * $P < 0.05$. I_t is the

fluorescent intensity of released Dox at certain time point; I_0 as the total Dox fluorescence signal intensity that can be recovered by repeated acid washing (considered as 100% release). The release percentage equals $(I_t/I_0) \times 100\%$. **Figure 20D** shows Dox release from phosphonate-MSNPs coated with the 10 kD PEI polymer under similar acidification conditions; this demonstrates that the polymer does not interfere in drug release. **Figure 20E** shows Confocal microscopy showing FITC-labeled MSNP uptake into the LAMP-1⁺ compartment in KB-V1 cells. The yellow spots in the merged image show the co-localization. Calculation of co-localization ratio by Imaging J software indicates >55% co-localization of the green-labeled particles with the red-labeled lysosomes. **Figure 20F** shows confocal microscopy showing Dox release from the MSNP to the nucleus in KB-V1 cells 72 hrs after the introduction of the particles. The bottom panel shows that the lysosomal pH neutralizer, NH_4Cl , interferes in drug release.

[0042] **Figure 21** shows simultaneous delivery of Dox and Pgp siRNA to the nucleus leads to a synergistic increase in cellular and nuclear Dox levels in KB-V1 cells. **Figure 21A** shows quantitative comparison of Dox levels using a fluorescent readout of cellular drug levels 72 hrs after introduction of treatment, using 2 $\mu\text{g}/\text{ml}$ free Dox or the equivalent amount of drug loaded into MSNP before or after PEI coating or PEI coating followed by the attachment of Pgp siRNA. **Figure 21B** shows confocal images showing drug uptake in KB-V1 cells that treated by 5 $\mu\text{g}/\text{ml}$ free Dox or the equivalent amount of drug loaded into various MSNPs for 72 hrs. Please note that while free Dox could not be maintained intracellularly, Dox delivered by MSNP (Dox-MSNPs) were retained in the particles that localized in the peri-nuclear region. PEI-Dox-MSNPs significantly enhanced particle uptake compared to the unmodified MSNP. However, while much of the drug remained confined to the particles, nuclear staining could be observed when Pgp siRNA was added to this platform. Thus, Pgp knockdown is likely effective at maintaining the Dox that is released from the particles long enough to allow the drug to find its way to the nucleus. The cell membrane was stained by Alexa 633-conjugated WGA (cyan color). Dox staining is in red. **Figure 21C** shows quantitative analysis of the nuclear fluorescence signal in KB-V1 nuclei was performed by the use of Image J software.

[0043] **Figure 22** shows comparison of the cytotoxic effects of different delivery modalities of Dox in KB-V1 cells. **Figure 22A** shows MTS cell viability assay showing that MSNP delivery of Dox concomitant with Pgp siRNA is capable of improving the induction of cytotoxicity by free Dox or Dox delivered by PEI-coated MSNP not attached to siRNA. The broken line is the cell killing curve of free Dox in parental cell line (KB-31, Dox sensitive).

Figure 22B shows annexin V- SYTOX staining showing enhanced apoptosis and cell death by siRNA-PEI-Dox compared to the other Dox modalities mentioned in **Figure 22A**. The flow cytometry data was further confirmed by TUNEL staining assay (**Figure 29**).

[0044] **Figure 23** shows identification of Dox sensitivity in KB-31 and KB-V1 cells. **Figure 23A** shows cytotoxicity profiles of Dox in KB-31 (parental line) and KB-V1 cells (resistant cell line). **Figure 23B** shows immunoblotting showing Pgp expression in KB-31 and KB-V1 cell.

[0045] **Figure 24** shows agarose gel electrophoresis of PEI 10 kD-coated MSNP to which Pgp siRNA was complexed at various nanoparticle to nucleic acid (N/P) ratios. The Φ lane contains Pgp siRNA only. The threshold N/P ratio (N/P=16) remains the same with or without Dox loading (**Figure 19A**, middle panel).

[0046] **Figure 25** shows Pgp expression was significantly knocked down (~80%) by siRNA-PEI 10 kD-Dox-MSNP treatment at the dose of 10 μ g/ml for 72 hrs. Knockdown of Pgp expression by siRNA is not influenced by Dox loading.

[0047] **Figure 26** shows assessment of PEI-MSNP safety in KB-V1 cells. **Figure 26A** shows MTS assay assessment of the viability of the cells incubated with the polymer-coated MSNP. **Figure 26B** shows MTS assay assessment of cell viability in response to 100 μ g/ml MSNP coated with the 10 kD polymer. The cell viability began to decrease at 36 hrs time-point (* p <0.05)

[0048] **Figure 27** shows loading and release profile of Hoechst 33342 loaded MSNP with various surface modifications. **Figure 27A** shows loading yield of Hoechst 33342 in MSNP with different surface modifications. Positively charged Hoechst 33342 dye (pKa=11.9), has a strong interaction with the particles with a negatively charged porous surface, showing higher loading yield. **Figure 27B** shows Hoechst 33342-loaded MSNP was incubated in H^+ (pH=5.0) and 10% (v/v) ethanol containing DMEM for 3 hrs. The supernatant was collected for released dye measurement. H^+ induced Hoechst 33342 release is significantly higher than ethanol induced release (* p <0.05).

[0049] **Figure 28** shows that the Dox release profile remains same with or without siRNA binding on PEI 10 kD-MSNP. The experiment was performed in acidifying phenol red free DMEM medium (pH=5) at the 12 hr time point. PBS was used as control. The data show that Dox release will not be influenced by siRNA that binds to PEI polymer where locates at the exterior of MSNP.

[0050] **Figure 29** shows a TUNEL detection kit used according to the manufacturer's instructions to study Dox-induced apoptosis. Percentage of TUNEL positive cell showing enhanced apoptosis by siRNA-PEI-Dox compared to the other Dox modalities. The result shows the same trend of flow cytometry data (**Figure 22B**)

[0051] **Figure 30** shows cell viability analyzed after longer exposure periods (4 days) because Pgp knockdown by siRNA may need a certain amount of time, but did not observe a significant improvement in cytotoxicity.

[0052] **Figure 31** shows that co-administration of free Dox and Tariquidar (50 nM) partially restores drug sensitivity in KB-V1 cells for 72 hrs. This combination significantly improved cell killing capability in KB-V1 cells, but was not able to completely restore Dox sensitivity to the level seen in KB-31 cells.

[0053] **Figure 32** shows loading and release profile of CPT loaded MSNP with various surface modifications. **Figure 32A** shows loading yield of CPT in modified MSNP. As shown in **Figure 32A**, MSNP with different surface modifications were compared for their loading capacity to CPT. The loading yield of MSNP varied from 3.8% (w/w) to 6.2% (w/w) when different surface modifications were used. The amounts of CPT stored within the surface functionalized particles were similar, although storing lesser amounts than the silanol surface. **Figure 32B** shows the release profile of CPT loaded into phosphonate-MSNP in response to acidification or induction of ethanol into the wash medium. PBS treatment was set as control. The ethanol-induced CPT release is significantly higher than acid-induced release. The loading and release mechanisms for hydrophilic cargo (*e.g.* Dox) and hydrophobic cargo (*e.g.* CPT) are different since there is a phase transfer process for hydrophobic cargo during drug loading. Loading capacity is independent of the functional groups that are used, and the cargo can be quickly released by an organic solvent in which the cargo is dissolvable.

DETAILED DESCRIPTION

[0054] Some embodiments of the current invention are discussed in detail below. In describing embodiments, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. A person skilled in the relevant art will recognize that other equivalent components can be employed and other methods developed without departing from the broad concepts of the current invention. All references cited anywhere in this specification are incorporated by reference as if each had been individually incorporated.

[0055] Embodiments of the invention include submicron structures of a silica body defining a plurality of pores and an outer surface between pore openings of said plurality of pores and a cationic polymer on the surface of said silica body. The submicron structure has a maximum dimension less than one micron (μm).

Silica Body

[0056] The submicron structure includes a silica body that defines a plurality of pores therein. For example, the silica body can be a mesoporous silica nanoparticle. The fact that we refer to the body as a silica body does not preclude materials other than silica from also being incorporated within the silica body. In some embodiments, the silica body may be substantially spherical with a plurality of pore openings through the surface providing access to the pores. However, the silica body can have shapes other than substantially spherical shapes in other embodiments of the current invention. Generally, the silica body defines an outer surface between the pore openings, as well as side walls within the pores. The pores can extend through the silica body to another pore opening, or can extend only partially through the silica body such that it has a bottom surface of the pore defined by the silica body.

[0057] In some embodiments, the silica body is mesoporous. In other embodiments, the silica body is microporous. As used herein, “mesoporous” means having pores with a diameter between 2 nm and 50 nm, while “microporous” means having pores with a diameter smaller than 2 nm. In general, the pores may be of any size, but in some embodiments are large enough to contain one or more therapeutic compounds therein. In such embodiments, the pores allow small molecules, for example, therapeutic compound such as anticancer compounds to adhere or bind to the inside surface of the pores, and to be released from the silica body when used for therapeutic purposes. In some embodiments, the pores are substantially cylindrical.

[0058] Some embodiments of the invention include nanoparticles having pore diameters between about 1 nm and about 10 nm in diameter. Other embodiments include nanoparticles having pore diameters between about 1 nm and about 5 nm. Other embodiments include particles having pore diameters less than 2.5 nm. In other embodiments, the pore diameters are between 1.5 and 2.5 nm. Silica nanoparticles having other pore sizes may be prepared, for example, by using different surfactants or swelling agents during the preparation of the silica nanoparticles.

[0059] The submicron structures according to some embodiments of the current invention may be referred to as nanoparticles. The term nanoparticles as used herein is intended to include particles as large as 1000 nm. In general, particles larger than 300 nm become ineffective in entering living cells. In some embodiments, colloidal suspensions may be formed using a plurality of submicron structures according to some embodiments of the invention. In that case, larger particles can tend to settle rather than remaining suspended in Brownian motion. As used herein, size of the submicron structure refers to the size of the primary particles, as measured by transmission electron microscopy (TEM) or similar visualization technique. Particle size does not refer to agglomerates in solution or suspension. Some embodiments include nanoparticles having an average maximum dimension between about 50 nm and about 1000 nm. Other embodiments include nanoparticles having an average maximum dimension between about 50 nm and about 500 nm. Other embodiments include nanoparticles having an average maximum dimension between about 50 nm and about 200 nm. Other embodiments include nanoparticles having an average maximum dimension less than about 500 nm, less than about 300nm, less than about 200nm, or less than about 100nm.

[0060] In some embodiments, the surface of the submicron structure or nanoparticle is unmodified. As used herein, an "unmodified" nanoparticle has had no other functional groups added to the surface after formation of the nanoparticle. Unmodified nanoparticles have an anionic charge due to free silyl hydroxide moieties present on the surface.

Cationic Polymer

[0061] As used herein, the term "polymer" is a macromolecule composed of repeating structural units, usually in a linear or branched sequence.

[0062] The cationic polymer may be any polymer bearing an overall positive charge, such as, for example, poly(ethyleneimine) (PEI), polyamidoamine, polylysine, poly(allylamine) or poly(diallyldimethylammonium chloride). Other cationic polymers will be apparent to those of skill in the art, and may be found, for example, in "Polymer Handbook, 4th Edition, Edited by: Brandrup et al.; John Wiley & Sons, 1999; and De Smedt et al., Pharmaceutical Research, vol. 17, no. 2, pp. 113-126). Other examples include, chitosan, poly(N-isopropyl acrylamide-co-Acrylamide), Poly(N-isopropyl acrylamide-co-acrylic acid), poly(L-lysine), diethylaminoethyl-dextran, poly-(N-ethyl-vinylpyridinium bromide), poly(2-dimethylamino)ethyl methacrylate), poly(ethylene glycol)-co-poly(trimethylaminoethylmethacrylate chloride). Cationic polymer

modified nanoparticles have a positive charge. In some embodiments, the cationic polymer is poly(ethyleneimine) (PEI).

[0063] The cationic polymer may be bound covalently or electrostatically to the surface of the silica body. In some embodiments, the cationic polymer is electrostatically bound to the surface of the silica body. For example, the cationic polymer may bind electrostatically to an unmodified silica body, which has an overall negative charge, or to a surface-modified silica body bearing a plurality of negatively charged surface modifying groups such that the silica body has an overall negative charge. Surface modification of the silica body is described in detail below.

[0064] In some embodiments, the cationic polymer has a weight average molecular weight less than about 30,000, less than about 25,000, less than about 20,000, less than about 15,000, or less than about 10,000. In some embodiments, the cationic polymer has a weight average molecular weight greater than about 600, greater than about 1000, greater than about 1500, or greater than about 1800, greater than about 2000, greater than about 3000, or greater than about 4000, or greater than about 5000. The range of molecular weight may be between any recited endpoints.

Core Structure

[0065] In some embodiments, the silica body may have a core structure. As used herein, a core structure is a metal crystal or nanocrystal in the interior of the silica body. In some embodiments, the silica body does not include a metal nanoparticle or metal nanocrystal as a core structure. In some embodiments, the core structure is superparamagnetic metal, silver, or gold. Other embodiments include submicron structures having more than one core structure in the nanoparticle, such as, for example, a silver and gold nanocrystal in the core structure, or a silver nanocrystal and superparamagnetic iron nanocrystal. In some embodiments, the core structure is a superparamagnetic nanocrystal, such as, for example, an iron oxide nanocrystal. A superparamagnetic nanocrystal core makes the particles visible using magnetic resonance imaging (MRI). The nanoparticle may be used as MRI contrast agents in addition to any transfection or anti-cancer activity. The superparamagnetic nanocrystal in the core of the nanoparticle also allows the particles to be manipulated or collected by a magnetic field, for example. In other embodiments, the core structure is a gold nanocrystal.

Surface Modification

[0066] Other embodiments include submicron structures as described above, which further include a surface modification. As used herein, "surface modification" means attaching or appending molecules or other materials to the surface of the silica body, in addition to the cationic polymer. Surface modification also modifies the surface of the pores of the silica body. The surface modification may be covalent, electrostatic or a combination of both. For example, the surface may include a covalent surface modification and an electrostatic surface modification on the same nanoparticle. In some embodiments, the surface modification may be further derivatized, for instance, by further covalent or electrostatic bonds. Surface modifications, as described herein, may be used on any silica body having an unreacted silica surface, including nanodevices having stoppers, impellers or valves, as described below.

[0067] In some embodiments, the surface modification comprises a plurality of anionic or electrostatic molecules attached to an outer surface of said silica body, wherein the anionic or electrostatic molecules provide hydrophilicity or aqueous dispersability to the nanoparticle and are suitable to provide repulsion between other similar submicron structures. Anionic surface modified nanoparticles are described, for example, in International Application No. PCT/US2008/013476, filed December 8, 2008, published as WO 2009/078924, the contents of which are incorporated herein by reference in its entirety.

[0068] In some embodiments, the plurality of anionic molecules include at least one phosphonate moiety. In some embodiments, the plurality of anionic molecules are trihydroxysilylpropyl methylphosphonate. Trihydroxysilylpropyl methyl phosphonate surface modifications are prepared, for example, by treating the silica body with trihydroxysilyl propyl methylphosphonate.

[0069] In some embodiments, the surface modification is covalently bonded to the surface of the silica body. In other words, the surface modification has a functional group covalently bonded to the surface. As used herein, the "functional group" defines a chemical moiety linked to the surface of the nanoparticle, either directly, or via a linker. In some embodiments, the functional group is a phosphonate, amine, sulfhydryl, disulfide, carboxylic acid, epoxide, halide (i.e. fluorine, chlorine, bromine, or iodine), azide, alkyne, or hydrophobic moiety. In some embodiments, the functional group is a phosphonate or an amide. In some embodiments, the functional group may be further bonded, covalently or electrostatically to a further compound.

[0070] In general, any reaction capable of reacting with the silyl hydroxide surface of the silica body may be used to covalently modify the surface. For example, the surface of the silica body may be treated with a trialkoxysilyl compound or trihydroxysilyl compound. The compound reacts with the silyl hydroxide surface of the silica body, forming covalent silicon-oxygen bonds. Trialkoxysilyl and trihydroxysilyl compounds bearing various functional groups may be used to modify the surface of the nanoparticle.

[0071] In some embodiments, the covalent surface modification comprises a phosphonate, amine, sulfhydryl, disulfide, carboxylic acid, epoxide or hydrophobic organic moiety. As discussed above, various functional groups may be present on the surface modification, depending on the reagents used to modify the surface. In some embodiments, the functional group (i.e. phosphonate, amine, sulfhydryl, disulfide, carboxylic acid, epoxide, halide, azide, alkyne, or hydrophobic organic moiety) may be separated from the silica surface by a linker. In some embodiments, the functional group is covalently bonded to the silica surface via a C₁ to C₁₂ alkyl linker. In other words, a C₁ to C₁₂ alkyl group is present between the atom covalently bonded to the surface and the functional group (i.e. phosphonate, amine, sulfhydryl, disulfide, carboxylic acid, epoxide or hydrophobic organic moiety). In other embodiments, the functional group is covalently bonded to the silica surface via a C₁ to C₆ alkyl linker. Nanoparticles bearing a surface modification are called surface-modified nanoparticles.

[0072] As used herein a C₁ to C₁₂ alkyl chain includes linear, branched and cyclic structures having 1 to 12 carbon atoms, and hybrids thereof, such as cycloalkylalkyl. Examples of alkyl chains include methylene (CH₂), ethylene (CH₂CH₂), propylene (CH₂CH₂CH₂), and so forth.

[0073] As used herein, surface modification having a phosphonate (also known as phosphonate-modified nanoparticles) have at least one phosphonic acid (-P(O)(OH)₂) group or phosphinic acid (-P(O)(OH)R, where R is an C₁ to C₁₂ alkyl group). The phosphonic or phosphinic acid may be charged or uncharged, depending on the pH. At physiological pH, phosphonic acids and phosphinic acids are negatively charged, or anionic. Phosphonate modifications may be prepared, for example, by treating the silica body surface with a phosphonate bearing trialkylsiloxane compound or phosphonate-bearing trihydroxysilyl compound, such as (trihydroxysilyl)propyl methylphosphonate.

[0074] In some embodiments, the surface modification has a phosphonate (i.e. phosphonic acid or phosphinic acid) group. Functionalization of the particle surface with a

phosphonate group provides electrostatic binding of positively charged (i.e. cationic) hydrophilic therapeutic compounds (e.g. doxorubicin) to the porous interior, from where the drug could be released by acidification of the medium under abiotic and biotic conditions. In addition, phosphonate modification also improves exterior coating with the cationic polymer, PEI, which endows the MSNP with the ability to contemporaneously bind and deliver siRNA.

[0075] As used herein, surface modifications having an amine (also known as amine modified nanoparticles) will have at least one primary ($-NH_2$), secondary ($-NHR$), tertiary ($-NR_2$) or quaternary amine. An amine-modified surface may be charged or uncharged, depending on the amine and pH. Amine modifications may be prepared, for example, by treating the silica body surface with an amine bearing trialkoxysilane compound, such as aminopropyltriethoxysilane, 3-(2-aminoethylamino)propyl-trimethoxysilane, or 3-trimethoxysilylpropyl ethylenediamine. An amine modified silica body may have an overall negative charge at certain pH, and when combined with anionic surface modifications, as discussed above.

[0076] As used herein, surface modifications having a sulfhydryl (or thiol) group will have at least one $-SH$ moiety. Such a modification may be prepared, for example, by treating the surface of the nanoparticle with a sulfhydryl bearing trialkoxysilane compound, such as 3-mercaptopropyltriethoxysilane.

[0077] As used herein, surface modifications having a disulfide group will have at least one $-S-S-$ moiety. Such a modification may be prepared, for example, by treating the surface of the nanoparticle with a disulfide bearing trialkoxysilane compound, or by treating a sulfhydryl modified surface with 2,2'-dithiodipyridine or other disulfide.

[0078] Surface modifications having a carboxylic acid group will have at least one $-CO_2H$, or salt thereof. Such a modification may be prepared, for example, by treating the surface with a carboxylic acid bearing trialkoxysilane compound, or by treating the surface with a trialkoxysilane compound bearing a functional group that may be converted chemically into a carboxylic acid. For example, the surface may be treated with 3-cyanopropyltriethoxysilane, followed by hydrolysis with sulfuric acid.

[0079] Surface modifications having an epoxide will have at least one epoxide present on the surface of the nanoparticle. Such a modification may be prepared, for example, by treating the surface with an epoxide bearing trialkoxysilane compound, such as glycidopropyltriethoxysilane.

[0080] Surface modifications having a hydrophobic moiety will have at least one moiety intended to reduce the solubility in water, or increase the solubility in organic solvents. Examples of hydrophobic moieties include long chain alkyl groups, fatty acid esters, and aromatic rings.

[0081] Different surface modifications may be combined. For example, an anionic surface modification (such as, for example, a phosphonate modification) may be combined with an amine modification, thiol modification, or hydrophobic modification if desired. In some embodiments, the modified silica body has an overall negative charge before being combined with a cationic polymer. If the silica body has an overall negative charge, a cationic polymer may bind electrostatically to the surface of the silica body.

Surface Derivatization

[0082] Any of the covalent surface modifications described above may be further derivatized, for example, by further covalent or electrostatic bonds. In some embodiments, the surface modification is further covalently bonded to another compound, such as a light-emitting molecule, targeting compound, polymer, peptide, protein, nucleic acid, sugar, oligosaccharide, or polysaccharide. Light emitting molecules include compounds which emit light by either fluorescence or phosphorescence. Light emitting molecules include dyes, such as fluorescent dyes. Examples of light emitting molecules include fluorescent dyes such as fluorescein, and rhodamine B. Light emitting molecules may be covalently bonded to the surface modified silica body by any useable method. For example, amine-modified nanoparticles having a free NH_2 group may be reacted with fluorescent dyes bearing amine-reactive groups such as isocyanates, isothiocyanates, and activated esters, such as N-hydroxysuccinimide (NHS) esters. Examples of fluorescent dyes bearing amine reactive groups include, for example, fluorescein isothiocyanate, N-hydroxysuccinimide-fluorescein, rhodamine B isothiocyanate, or tetramethylrhodamine B isothiocyanate. Other dyes will be apparent to those of skill in the art. Nanoparticles bearing light-emitting molecules may be used, for example, for fluorescence imaging, for instance when the nanoparticles interact with the surface of a microbe.

[0083] In some embodiments, the surface modification is further bonded to a polymer, such as, for example, polyethylene glycol (PEG). Polymers covalently bonded to the surface modification should be covalently bonded at only one location to prevent crosslinking. For

example, the surface may be modified with poly(ethylene glycol) methyl ether, which has only one reactive end.

[0084] In some embodiments, the surface modification is further bonded to a peptide or protein. Peptides include polypeptides having at least 2 amino acids. Various amino acid residues on peptides or proteins may form a covalent bond with surface-modified nanoparticles. For example, carboxylic acid residues (from aspartic acid and glutamic acid) may react with amine-modified nanoparticles bearing a free NH_2 group. Likewise, amine residues on proteins (i.e. from lysine) may react with carboxylic acid bearing surface modifications or with epoxide bearing surface modifications. Sulfhydryl surface modifications may react with disulfide bonds (e.g. from cystine residues) in the protein via thiol exchange. Disulfide surface modifications, such as 2-thiopyridine disulfides may react with free thiols (e.g. from cysteine residues) in the protein to form a covalent bond with the protein. Other suitable methods for conjugating the proteins to the surface-modified nanoparticles will be evident to those of skill in the art.

[0085] In some embodiments, the polymer, protein, peptide, oligonucleotide, sugar, oligosaccharide, or polysaccharide is covalently attached to the surface modifying group via a linker. Various bifunctional crosslinkers are known to those in the art for covalently bonding to proteins, any of which may be used to covalently link a surface modified nanoparticle to a protein. For example, heterodifunctional crosslinkers such as succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) and melaimidobutyryloxysuccinimide ester (GMBS) may be used to react with amine-modified nanoparticles (via the succinimide esters), and then form a covalent bond with a free thiol in the protein (via the maleimide). Other crosslinkers, such as succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) may react with amine-modified nanoparticles (via the succinimide ester), and form a covalent bond with a free thiol in the protein via thiol exchange. Other difunctional crosslinkers include suberic acid bis(N-hydroxysuccinimide ester), or disuccinimidyl carbonate which can react with amine-modified, hydroxyl-modified, or unmodified nanoparticles, and free amines or hydroxyl groups on the polymer (such as poly(ethyleneglycol) methyl ether) or protein (e.g. from lysine residues). Other bifunctional and heterobifunctional crosslinkers useable with various surface modifications will be evident to those of skill in the art.

[0086] In some embodiments, the surface modifying material is a polymer, protein, peptide, nucleic acid, sugar, oligosaccharide, polysaccharide, or combination thereof.

[0087] Surface-modified nanoparticles bearing a protein are also called protein-modified nanoparticles. The protein may be bonded covalently (directly to the surface modification or via a linker) or may be electrostatically bonded to the modified or unmodified nanoparticles as discussed above. The protein may be a targeting protein or an antibody. A "targeting protein" as used herein, means a protein which binds to a particular surface feature of a cell. Antibodies and peptides are also used to bind to particular surface features of cells, and may be used to modify the nanoparticles of the invention. Protein-modified nanoparticles may be used to selectively target specific cells, by interacting specifically or selectively to a cell of interest.

[0088] In some embodiments, the surface modification is electrostatically bonded to the surface. As used herein "electrostatically bonded" means bonded based on the attraction of opposite charges. An unmodified nanoparticle has a negative charge, due to the presence of free silyl hydroxide residues on the surface of the nanoparticle. The particle may also bear a surface modification having a negative charge (such as a phosphonate modification), such that the overall charge of the surface is negative. The surface may be modified with material bearing a positive charge, which will bind to the surface electrostatically.

[0089] In other embodiments, in addition to the cationic polymer, a protein may bind to the surface electrostatically. A protein having a net positive charge will bind electrostatically to unmodified nanoparticles or surface modified nanoparticles bearing a negative charge. For example, proteins such as Bovine Serum Albumin (BSA) and protein solutions such as Fetal Bovine Serum (FBS) bind electrostatically to unmodified or negatively charged nanoparticles.

[0090] A protein having a net negative charge will bind electrostatically to modified nanoparticles having a positive charge, such as amine-modified nanoparticles, or nanoparticles modified by cationic polymers. For example, negatively charged proteins bind electrostatically to nanoparticles bearing a cationic polymer, as described herein.

Oligonucleotides

[0091] In some embodiments, the submicron structure further includes an oligonucleotide. The oligonucleotide binds electrostatically to the cationic polymer and outside surface of the silica body.

[0092] As used herein, an oligonucleotide is a nucleic acid polymer, and may be a ribonucleic acid polymer (RNA) or deoxyribonucleic acid polymer (DNA). DNA may be double stranded, and may be, for example a plasmid. RNA may be single stranded or double

stranded and may be, for example a siRNA. Other oligonucleotides include, for example, microRNA (miRNA) and small hairpin RNA (shRNA).

[0093] Without wishing to be bound by theory, the cationic polymer envelopes and protects the oligonucleotide from degradation. Oligonucleotide-containing structures according to the invention may, for example, deliver oligonucleotides to the interior of a cell when the submicron structure enters the cell. Genes or therapeutic oligonucleotides (such as siRNA) may thus be successfully delivered into a cell. In other words, oligonucleotide-containing submicron structures may be used for transfection.

Therapeutic Compounds

[0094] In some embodiments, the submicron structure further includes a therapeutic compound within the silica body or pores of the silica body. As used herein, a therapeutic compound is a small molecule used to treat a disease or disorder. In principle any type of therapeutic compound may be incorporated into the pores of the silica body. In some embodiments, the therapeutic compound is an anticancer compound (i.e. used to treat cancer). In some embodiments, the therapeutic compound is hydrophobic. Examples of hydrophobic therapeutic compounds include, for example paclitaxel and camptothecin and related compounds. In some embodiments, the therapeutic compound is cationic. Cationic compounds electrostatically bind to the surface of the silica body. Even in the presence of a cationic polymer on the surface of the silica body, cationic compounds may bind to the interior of the silica body or within the pores of the silica body. Examples of cationic therapeutic compounds include, for example, daunomycin, doxorubicin or related compounds. In some embodiments, the therapeutic compound is anionic. Anionic compounds may bind in pores or surface of the silica body, for example, by binding electrostatically to amine-modified surfaces or to cationic-polymer bound silica bodies.

Oligonucleotide-Therapeutic compound combinations

[0095] In some embodiments, the submicron structure includes both an oligonucleotide and therapeutic compound. The benefits of both the oligonucleotide and therapeutic compound may be realized. In some embodiment, the oligonucleotide may be a DNA plasmid. In some embodiments, the oligonucleotide may be an RNA polymer. In some embodiments, the oligonucleotide may be a small interfering RNA (siRNA). In some embodiments, the

oligonucleotide may be a siRNA that decreases translation of a gene that produces drug resistance. In some embodiments, the siRNA decreases translation of a gene that produces drug resistance in a cancer cell, and the therapeutic compound is an anticancer compound. By co-delivering siRNA and therapeutic compounds, the siRNA may decrease the resistance of the cell to the therapeutic compound. In the context of cancer, for example, the siRNA may reduce translation of the p-glycoprotein (pgp), implicated in multiple drug resistance (MDR). Other resistance genes that may be suppressed by siRNA include, for example, MRP1 (ABC transporters), breast cancer resistance protein (BCRP, ABC transporters), glucosylceramide synthase (GCS) and c-Myc (oncogene regulating MDR1 expression). Other resistance genes will be apparent to one of ordinary skill in the art.

[0096] The therapeutic compound, may be, for example doxorubicin. As a result, a cancer cell that is normally resistant to doxorubicin may become susceptible to doxorubicin activity. In this way, multiple drug resistant cancers may be treated.

Additional structural features

[0097] In some embodiments, the submicron structure described above further includes a stopper assembly attached to the silica body. The stopper assembly has a blocking unit arranged proximate at least one pore and has a structure suitable to substantially prevent material from entering or being released when the blocking unit is arranged in a blocking configuration. The stopper assembly is responsive to the presence of a predetermined stimulus such that the blocking unit is released in the presence of the predetermined stimulus to allow material to enter or be released. The predetermined stimulus is a predetermined catalytic activity that is suitable to cleave, hydrolyze, oxidize, or reduce a portion of the stopper assembly. Examples of stopper assemblies are described, for example, in International Application No. PCT/US2009/031891, filed January 23, 2009, now published as WO 2009/094580 and incorporated herein by reference in its entirety.

[0098] In some embodiments the stopper assembly can include a thread onto which the blocking unit can be threaded. The thread has a longitudinal length that is long relative to a transverse length and is suitable to be attached at one longitudinal end to the silica body. The stopper assembly can also have a stopper attached to a second longitudinal end of the thread in some embodiments. The stopper can be selected among a wide range of possible stoppers based on the type of environment

[0099] For example, according to some embodiments, a synthetic strategy can involve the use of a snap-top “precursor”. The assembly of the snap-top precursors can be performed step-wise from the silica nanoparticle surfaces outward. For instance, the silica nanoparticles are treated with aminopropyltriethoxysilane (APTES) to achieve an amine-modified nanoparticle surface. An azide terminated tri(ethylene)glycol thread is attached to the amine-modified nanoparticles. The precursor is completed through the addition of α -cyclodextrin as the blocking unit at 5°C, which complexes with the threads at the low temperature. The precursor can enable the preparation of many different systems based on a common general structure in which different stoppers can be attached depending on the specific desired application. For example, stoppers may be selected that respond to enzymes (for example, ester linked or peptide linked), pH (for example, vinyl ether linked), and redox (for example disulfide linked) stimulation. However, the broad concepts of the current invention are not limited to only these specific examples. There are a wide range of possible stoppers that may be selected according to the particular application.

[00100] Other embodiments include submicron structures further including an impeller attached to the silica body. Silica bodies modified by impellers are described, for example in International Application No. PCT/US2009/031871, filed January 23, 2009, published as WO 2009/094568, the contents of which are incorporated herein in their entirety. The term “impeller” as used herein is intended to have a broad meaning to include structures which can be caused to move and which can in turn cause molecules located proximate the impeller to move in response to the motion of the impeller.

[00101] In operation, the impellers are driven by an energy transfer process. The energy transfer process can be, but is not limited to, absorption and/or emission of electromagnetic energy. For example, illuminating with light at an appropriate wavelength can cause the plurality of impellers to wag back and forth between two molecular shapes. The motion of the plurality of impellers causes motion of molecules (for example, peptides, proteins, ions, drugs or antibiotics) of interest into and/or out of the silica body. On the other hand, in the absence of excitation energy, the plurality of impellers can remain substantially static, at least for time periods long enough for the desired application, to act as impediments to block molecules from exiting and/or entering the storage chamber.

[00102] The impellers can be, but are not limited to, azobenzenes according to some embodiments of the current invention. For example, the azobenzenes can include the following:

1) One phenyl ring derivatized with a functional group that enables attachment directly to the silica surface or to a modified silica surface as described later. The list of suitable functional groups contains but is not limited to: alcohols, (-ROH), anilinium amines (-NH₂) primary amines (-RNH₂), secondary amines (-R₁R₂NH), azides (N₃), alkynes (RC≡CH), isocyanates (-RNCO), isothiocyanates (-RNCS), acid halides (RCOX), alkyl halides (RX) and succinimidyl esters. 2) other functional groups on the other phenyl ring (which is the moving end of the machine). The list of these functional groups includes but is not limited to: -H (here the phenyl ring is underivatized), esters (-OR), primary and secondary amines, alkyl group, polycyclic aromatics, and various generations of dendrimers. The bulkiness of these functional groups can be designed for specific systems. For example, large dendritic functionalities might be required when very large pore openings or very small guest molecules are employed.

[00103] When illuminated or irradiated with light of a particular wavelength, the azobenzene undergoes photoisomerization, causing the second phenyl group to move.

[00104] In other embodiments, impellers are based on redox of copper complexes. The copper complexes can include bifunctional bidentate stators that contain diphosphine and/or diimine bidentate metal chelators on one end of the stator, while at the other end functionalities such as alkoxysilanes (for immobilization on silica and silicon substrates) and thiols (for immobilization on gold substrates) are present. The copper complexes can contain a rotator that is a rigid bidentate diimine metal chelator, which rotates and changes the shape of the overall molecule upon redox or photons. These copper complexes exist in two oxidation states, each of which corresponds to a specific shape. Copper (I) is tetrahedral while copper (II) is square planar. The different oxidation states, and hence different shapes that are caused by a 90° rotation of the rotator, can be generated in three ways: Reduction and oxidation (1) using electrodes and an electric current (2) by use of chemical reducing and oxidizing agents, and (3) by the photo- excitation of light of the appropriate wavelength.

[00105] Some embodiments include submicron structure further including a valve assembly attached to the silica body. Porous nanoparticles having valves are described, for example, in International Application No. PCT/US2009/032451, filed January 29, 2009, published as WO 2009/097439, the contents of which are incorporated herein in their entirety. In some embodiments, the valve assembly is operable in an aqueous environment. The valve assembly has a valve arranged proximate the at least one pore and has a structure suitable to substantially prevent material from entering or being released while the valve is arranged in a

blocking configuration. The valve assembly is responsive to a change in pH such that the valve moves in the presence of the change in pH to allow the material to enter or be released from the silica body.

[00106] According to some embodiments of the current invention, the pH-responsive valve assembly relies on the ion-dipole interaction between cucurbit[6]uril (CB[6]) and bisammonium stalks, and that can operate in water. CB[6], a pumpkin-shaped polymacrocyclic with D_{6h} symmetry consisting of six glycouril units strapped together by pairs of bridging methylene groups between nitrogen atoms has received considerable attention because of its highly distinctive range of physical and chemical properties. Of particular interest in the field of supramolecular chemistry is the ability of CB[6] to form inclusion complexes with a variety of polymethylene derivatives, especially diaminoalkanes: the stabilities of these 1:1 complexes are highly pH-dependent. The pH-dependent complexation-decomplexation behavior of CB[6] with diaminoalkanes has enabled the preparation of dynamic supramolecular entities which can be controlled by pH. In some embodiments, [2]pseudorotaxanes having bisammonium stalks and CB[6] rings, may be constructed on the surface of the mesoporous silica nanoparticles, and the pH-dependent binding of CB[6] with the bisammonium stalks is exploited to control the entry or release of molecules from the silica nanoparticles. At neutral and acidic pH values, the CB[6] rings encircle the bisammonium stalks tightly, blocking the nanopores efficiently when employing suitable lengths of tethers. Deprotonation of the stalks upon addition of base results in spontaneous dethreading of the CB[6] rings and unblocking of the pores.

Pharmaceutical Compositions

[00107] Embodiments include pharmaceutical compositions comprising any of the submicron structures according to the invention.

[00108] In certain embodiments, the composition may be in any suitable form such as a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. The composition may include suitable parenterally acceptable carriers and/or excipients.

[00109] In certain embodiments, the compositions may be in a form suitable for administration by sterile injection. To prepare such a composition, the nanoparticles are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles

and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl or n-propyl p-hydroxybenzoate). For parenteral formulations, the carrier will usually comprise sterile water, though other ingredients, for example, ingredients that aid solubility or for preservation, may be included. Injectable solutions may also be prepared in which case appropriate stabilizing agents may be employed.

[00110] Formulations suitable for parenteral administration usually comprise a sterile aqueous preparation, which may be, for example, isotonic with the blood of the recipient (e.g., physiological saline solution). Such formulations may include suspending agents and thickening agents and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose form.

[00111] Parenteral administration may comprise any suitable form of systemic delivery or localized delivery. Administration may for example be intravenous, intratumoral, intra-arterial, intrathecal, intramuscular, subcutaneous, intramuscular, intra-abdominal (e.g., intraperitoneal), etc., and may be effected by infusion pumps (external or implantable) or any other suitable means appropriate to the desired administration modality.

Methods

[00112] Embodiments include methods for using the submicron structures according to any embodiment of the invention for treatment of a disease or disorder by administering to a subject in need of treatment an effective amount of a submicron structure of the invention.

[00113] The submicron structures may be administered, for example, as a pharmaceutical composition. Administration may be achieved by any suitable means. In certain embodiments, the compositions may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Routes of administration include, for example, subcutaneous, intravenous, intraperitoneal, intramuscular, or intradermal injections that provide continuous, sustained levels in the patient. Administration to human patients or other animals is generally carried out using a physiologically effective amount of a compound of the invention in a physiologically-acceptable carrier. Suitable carriers and their

formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin.

[00114] The composition may be administered parenterally by injection, infusion or implantation in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and/or adjuvants. In some embodiments, the compositions are added to a retained physiological fluid, such as cerebrospinal fluid, blood, or synovial fluid. The compositions of the invention can be amenable to intravenous (i.v.) injection and direct injection (i.e. intratumoral injection), application or infusion at a site of disease or injury.

[00115] By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, organ or subject.

[00116] By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated subject. The effective amount of an active therapeutic agent for the treatment of a disease or injury varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending clinician will decide the appropriate amount and dosage regimen.

[00117] By "subject" is meant an animal. In some embodiments, a subject may be a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

[00118] As used herein, the terms "treat," treating, "treatment," "therapeutic" and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

[00119] Embodiments of the invention include methods of treating drug resistant cancer by administering to a patient in need a submicronstructure according to the invention having an siRNA that reduces the translation of a gene responsible for the drug resistance, and an anticancer compound. The anticancer compound is a compound to which the cancer is resistant but becomes susceptible to treatment upon co-administration with an siRNA that reduces the translation of a resistance gene.

[00120] In addition to being used for the delivery of chemical therapeutic agents, MSNP have the potential as hybrid organic-inorganic materials that can act as carriers for nucleic acids and therefore potentially useful for the delivery of small interfering RNAs (siRNAs) and other

forms of gene therapy (Park et al., *Int. J. Pharm.*, vol. 359, pp. 280-284, 2008; Radu et al., *J. Am. Chem. Soc.*, vol. 126, pp. 13216-13217, 2004; Torney et al., *Nat. Nanotechnol.*, vol. 2, pp. 295-300, 2007). As opposed to other gene delivery systems based on inorganic materials, the porous structure of MSNP allows both the binding of nucleotides on the surface as well as the encapsulation of small molecules within the particles. It is even possible to combine these modules to achieve dual delivery of drugs and nucleic acids (Torney et al., *Nat. Nanotechnol.*, vol. 2, pp. 295-300, 2007). To maximize the delivery of negatively charged nucleic acids to cells, the silica surface may be converted into positive charge in order to bind DNA and siRNA. Some of the methods for introducing cationic charge on inorganic materials, which include silica, iron oxide, and gold, typically involve surface grafting with amine groups and coating with cationic polymers (e.g. polyethyleneimine, polyamidoamine, polylysine) through either covalent or electrostatic association (Radu et al., *J. Am. Chem. Soc.*, vol. 126, pp. 13216-13217, 2004; Bharali et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, pp. 11539-11544, 2005; Bonoiu et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, pp. 5546-5550, 2009; Elbakry et al., *Nano Lett.*, vol. 9, pp. 2059-2064, 2009; Fuller et al., *Biomaterials*, vol. 29, pp. 1526-1532, 2008; Kneuer et al., *Bioconjugate Chem.*, vol. 11, pp. 926-932, 2000; McBain et al., *J. Mater. Chem.*, vol. 17, pp. 2561-2565, 2007; Zhu et al., *Biotechnol. Appl. Biochem.*, vol. 39, pp. 179-187, 2004). The gene delivery capabilities of these surface modified materials have been demonstrated and may prove useful as alternatives to traditional viral vectors.

[00121] The effects of polyethyleneimine (PEI) coating on the MSNP in terms of cellular uptake, cytotoxicity, and efficiency of nucleic acid delivery were studied. PEIs are synthetic cationic polymers that compact DNA and siRNA into complexes that are effectively taken up in cells to make nucleic acid delivery and gene therapy possible (Boussif et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, pp. 7297-7301, 1995; Godbey et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, pp. 5177-5181, 1999; Urban-Klein et al., *Gene Ther.*, vol. 12, pp. 461-466, 2005). Although the polymer itself is used as a delivery vehicle, PEI can also be attached to nanoparticle surfaces through covalent and electrostatic interactions to achieve the same goal (Park et al., *Int. J. Pharm.*, vol. 359, pp. 280-284, 2008; Elbakry et al., *Nano Lett.*, vol. 9, pp. 2059-2064, 2009; Fuller et al., *Biomaterials*, vol. 29, pp. 1526-1532, 2008; McBain et al., *J. Mater. Chem.*, vol. 17, pp. 2561-2565, 2007; Liong et al., *Adv. Mater.*, vol. 21, pp. 1684-1689, 2009). Complexing the polymer with the nanoparticles has the potential advantage of facilitating DNA and siRNA delivery by a multifunctional platform that also allows imaging, targeting and concurrent drug

delivery. PEI was chosen as the polymer coating to enhance the particle uptake into cells and facilitate endosomal escape for the nucleotide delivery (Duan et al., *J. Am. Chem. Soc.*, vol. 129, pp. 3333-3338, 2006). It is documented that while low MW PEI is not cytotoxic, these polymers are ineffective at transfecting nucleotides in contrast to the high MW PEI. In this regard, it has been demonstrated that the size (MW), compactness and chemical modification of PEI affect the efficacy and toxicity of this polymer (Florea et al., *AAPS PharmSci.*, vol. 4, p. E12, 2002; Neu et al., *J. Gene. Med.*, vol. 7, pp. 992-1009, 2005). Therefore, several PEI polymer sizes ranging from MW of 0.6 to 25 KD were investigated in order to balance the efficiency of nucleic acid delivery and cellular toxicity, which proceed by the proton sponge effect that involves proton sequestration on the polymer surface, heightened activity of the proton pump, osmotic swelling lysosomal injury, intracellular Ca^{2+} flux and mitochondrial damage (Xia et al., *Nano Lett.* Vol. 6, pp. 1794-1807, 2006; Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). The large number of amine groups of PEI also allows the polymer-coated particles to move across cell membranes through rapid endocytosis. Cellular uptake of PEI-coated MSNP may be enhanced by at least two orders of magnitude compared to that of the MSNP which are unmodified (silanol surface) or coated with phosphonate or poly(ethylene glycol) groups. Furthermore, with coating of PEI MW 10 KD, it is possible to achieve both increased cellular uptake and high transfection efficacy while minimizing the toxicity normally observed with higher molecular weight PEI.

[00122] In some embodiments, surface functionalization with polyethyleneimine (PEI) polymers may enhance oligonucleotide (i.e. DNA, RNA, shRNA or siRNA) binding. The tight complexing between PEI and nucleic acids on the particle surface protects these cargo molecules from enzymatic degradation. The positive charge of PEI-coated nanoparticles may also lead to strong electrostatic interaction with the negatively charged cell surface membrane, which may facilitate particle cellular uptake and lysosomal release via a proton sponge mechanism. In addition, the molecular weight of PEI polymer may be carefully selected in order to reduce possible cytotoxicity.

[00123] Surface coating with PEI yields cationic MSNP with therapeutically useful nucleic acid delivery properties that include high binding avidity of DNA and siRNA as well as a high rate of cellular uptake. siRNA complexed to the MSNP-PEI surface is quite effective to achieve green fluorescent protein (GFP) knockdown in transduced HEPA-1 cells, while plasmid DNA delivery is comparable to a commercially available transfection agent. The facilitated cellular uptake of cationic particles may enhance the ability of MSNP to deliver hydrophobic

chemotherapeutic agents, such as paclitaxel, to pancreatic cancer cells. A potential downside of cationic functionalization to achieve drug or nucleic delivery is induction of cytotoxicity, best demonstrated by the use of MSNP-PEI-25 KD. However, this toxicity could be reduced or eliminated by attaching shorter length polymers that retain nucleic acid and drug delivery capabilities. Thus, the therapeutic use of the MSNP platform can be extended to delivery of DNA and siRNA constructs.

[00124] Packaging siRNA on the surface of cationic MSNP may provide several benefits. First, the MSNP surface can be functionalized to enhance siRNA binding through the attachment of PEI polymers, for example, which in their own right have been used as effective siRNA compacting and transducing agents. The tight complexing between PEI and nucleic acids on the particle surface protects these molecules from enzymatic degradation as demonstrated for DNA. Furthermore, the positive charge of PEI-coated nanoparticles leads to strong electrostatic interaction with the negatively charged cell surface membrane, leading to facilitated particle wrapping and cellular uptake. This is in agreement with the recent demonstration that PEI-coated nanoparticles is taken up into cells at high efficiency (Rosenholm et al., *ACS Nano*, vol. 3, pp. 197-206, 2009; Duan et al., *J. Am. Chem. Soc.*, vol. 129, pp. 3333-3338, 2006). However, the latter study did not look at nucleic acid delivery but did show that the attachment of the ligand such as folic acid further enhance uptake in cancer cells (Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008; Rosenholm et al., *ACS Nano*, vol. 3, pp. 197-206, 2009). While PEI can be used in various ways to make siRNA delivery complexes, coating it onto the surface of MSNP utilizes this therapeutic platform as a carrier with large surface area, which is inexpensive and simple to synthesize, and which can be decorated with functional groups and fluorescent tags and can be used for magnetic resonance imaging through the inclusion of superparamagnetic iron oxide nanocrystals (Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008). Not only may PEI coating enhance siRNA delivery, but the particles also are capable of anticancer compound such as paclitaxel delivery, which constitutes a significant advance for the use of MSNP as a therapeutic platform. One can envisage using siRNA and drug delivery simultaneously, e.g., delivery of siRNA that knocks down the expression of the P-glycoprotein (Pgp) drug exporter at the same time as delivering a chemotherapeutic agent that is exported by Pgp (Ludwig et al., *Cancer Res.*, vol. 66, pp. 4808-4815, 2006). This may be an effective strategy for the treatment of cancers that have developed a drug resistance due to the activity of this exporter.

[00125] Several advantages of MSNP-PEI for the delivery of siRNA also hold true for plasmid DNA transduction. In fact, a number of modifications of the PEI polymer, including various polymer sizes, degrees of branching, thiol cross-linking and covalent attachment have been used as gene transfer agents since the 90's and are enjoying increasing popularity because of the high transfection efficiency of plasmid DNA and siRNA into cells and live animals (Boussif et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, pp. 7297-7301, 1995; Godbey et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, pp. 5177-5181, 1999; Urban-Klein et al., *Gene Ther.*, vol. 12, pp. 461-466, 2005; Kircheis et al., *J. Gene. Med.*, vol. 1, pp. 111-120, 1999). An advantage of using PEI for gene transfer lies in the simplicity with which the polymer can be mixed with DNA/siRNA without involving covalent attachment. Moreover, PEI protects the nucleic acids from degradation by nucleases. In all of these therapeutic applications, however, one has to keep in mind that high MW polymer could cause toxicity (Florea et al., *AAPS PharmSci.*, vol. 4, p. E12, 2002). As demonstrated by the present invention, it is now possible to deal with a toxicity problem while maintaining effective nucleic acid delivery by using intermediate length polymers. These intermediate length polymers may provide effective cellular uptake and siRNA/plasmid DNA delivery. Moreover, this approach may produce transfection of > 70% cells in the population, which may increase effectiveness from the perspective of gene therapy.

[00126] Other than mediating its effect through high cellular uptake, the efficient gene transduction ability of PEI proceed via the proton sponge effect, implying that the primary amines buffer the protons being pumped into the lysosomal compartment by the v-ATPase (proton pump) (Boussif et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, pp. 7297-7301, 1995; Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008; Yezhelyev et al., *J. Am. Chem. Soc.*, vol. 130, pp. 9006-9012, 2008). This results in heightened pump activity, leading to the accumulation of a Cl⁻ and a water molecule for each proton that is retained; ultimately this leads to osmotic rupture of the endosome (Sonawane et al., *J. Biol. Chem.*, vol. 277, pp. 5506-5513, 2002). While effective for delivering the nucleic acid cargo to the cytosol, the effect of PEI or cationic particles on the proton pump is a major contributory factor in cationic cytotoxicity (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). Cationic nanoparticle toxicity can lead to clinically significant adverse health effects (Clottens et al., *Occup. Environ. Med.*, vol. 54, pp. 376-387, 1997; Hoet et al., *Toxicol. Appl. Pharmacol.*, vol. 175, pp. 184-190, 2001; Hoet et al., *Toxicol. Sci.*, vol. 52, pp. 209-216, 1999). Methods to reduce cationic toxicity while maintaining effective cargo delivery are therefore desired (Nel et al., *Nat. Mater.*, vol. 8, pp. 543-557, 2009). Experimental examples of

how PEI toxicity has been reduced include neutralizing the cationic charge by anhydride, differential ketalization, altering PEI crosslinking through adjustment of disulfide content and using shorter polymers (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008; Shim et al., *J. Control. Release.*, vol. 133, pp. 206-213, 2009; Veisheh et al., *Biomaterials*, vol. 30, pp. 649-657, 2009; Hobel et al., *Eur. J. Pharm. Biopharm.*, vol. 70, pp. 29-41, 2008 Peng et al., *Bioconjugate Chem.*, vol. 20, pp. 340-346, 2009). The latter principal can also be applied to PEI-coated MSNP, cytotoxicity may be reduced or eliminated while maintaining efficient nucleic acid delivery. Thus, by attaching a 10 KD polymer, it was possible at particle doses < 50 µg/ml to obtain highly efficient transduction without any cell death. Preliminary data also indicate that the PEI-coated MSNP can be intravenously injected into mice without causing acute toxicity. It appears, therefore, that cationic toxicity can be controlled to achieve a therapeutically useful outcome. While the gene transduction efficiency is significantly reduced with lower MW (0.6-1.8 KD) polymers, the facilitated MSNP uptake is good enough for delivering paclitaxel (**Figure 8**).

[00127] To use the MSNP delivery system, the first step is to conduct toxicity test in mice. Results show that *i.v.* injection of MSNP in mice has no obvious toxic effects to the major organs or systems of the animals. It is interesting that MSNP-PEI 25 KD showed toxicity to cultured cells while no toxicity *in vivo*. This may be because of the dilution of the MSNP in the blood system, the huge number of cells in the body, and strong defense/clearance capability in living organisms. Until now, there are only limited *in vivo* data regarding the toxicity, biodistribution, and biopersistence of mesoporous silica nanoparticles (Kim et al., *Angew. Chem., Int. Ed.*, vol. 47, pp. 8438-8441, 2008; Barb et al., *Adv. Mater.*, vol. 16, pp. 1959-1966, 2004; Taylor et al., *J. Am. Chem. Soc.*, vol. 130, pp. 2154-2155, 2008; Wu et al., *ChemBioChem*, vol. 9, pp. 53-57, 2008). Several groups used MRI agent to coat mesoporous silica nanoparticles for *in vivo* imaging, they found that MSNP were in the blood vessel 30 min after administration, then MSNP tended to accumulate more in the RES systems such as liver and spleen tissue than in kidney, lung, and heart; in fact MSNP appeared to have cleared kidney after 2 h (Kim et al., *Angew. Chem., Int. Ed.*, vol. 47, pp. 8438-8441, 2008; Wu et al., *ChemBioChem*, vol. 9, pp. 53-57, 2008). Long-term MRI tracking study revealed that the MRI signal gradually disappeared after 90 days, which means that MSNP were resistant to decomposition and not easily excreted from the body, suggesting a potential candidate for long-term liver/spleen MRI monitoring and targeted drug delivery (Wu et al., *ChemBioChem*, vol. 9, pp. 53-57, 2008). Another interesting

finding is MSNP accumulate preferentially at tumor sites through their enhanced permeability and retention (EPR) effect in tumor targeting (Kim et al., *Angew. Chem., Int. Ed.*, vol. 47, pp. 8438-8441, 2008). To achieve the full potential of MSNP, more *in vivo* tests may be done regarding to their toxicity, biodistribution, pharmacokinetics, and targeting.

[00128] Due to its unique structure and ease with which the surface can be functionalized, mesoporous silica nanoparticles (MSNP) constitute a multi-functional platform that can be used for drug (Lu et al., *Small*, vol. 4, pp. 421-426, 2008; Coti et al., *Nanoscale*, vol. 1, pp. 16-39, 2009; Slowing et al., *Adv. Drug Deliv. Rev.*, vol. 60, pp. 1278-1288, 2008; Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008) and nucleic acid delivery (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). For therapeutic purposes, nanoparticles that contain a phosphonate surface coating may be beneficial because of the ease of particle dispersal, good bio-safety index as well as the ability to adsorb cationic polyethylenimine (PEI) polymers for complexing and delivery of DNA and siRNA (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). Since the polymer attachment leaves the porous interior free for drug binding and delivery, it establishes the potential to achieve simultaneous drug and nucleic acid delivery (Astrid et al., *J. Am. Ceram. Soc.*, vol. 84, pp. 806-812, 2001). It was envisaged that there some disease conditions such as multidrug resistant cancer might benefit from the delivery of both agents. Dual delivery might, therefore achieve simultaneous drug and siRNA delivery, leading to a synergistic therapeutic outcome.

[00129] Significant uncertainties remained, however. For example, it was uncertain whether the cationic polymer would interfere with a therapeutic compound binding to the porous interior of the silica body. For example, binding a cationic polymer to the surface might block access to the interior of the silica body. Particularly for cationic therapeutic compounds, a cationic polymer might displace any cationic therapeutic compound bound to the silica body, whether in the interior or on the surface. It was unknown whether a therapeutic compound could even be loaded into the silica body with the cationic polymer pre-bound. Assuming a therapeutic compound could bind to the interior of the silica body in the presence of the cationic polymer, it was unclear whether the cationic polymer would prevent drug release inside a cell.

[00130] Through experimentation, the inventors successfully met these challenges and prepared a dual delivery system. As demonstrated in the present invention, for example, a cationic polymer, such as PEI, will not interfere in drug binding to the porous interior. Furthermore, as demonstrated herein, cationic therapeutic compounds, such as doxorubicin can

be loaded into the silica body, but only after a cationic polymer, such as PEI is coated on the surface of the silica body. Furthermore, loading therapeutic compounds, such as doxorubicin or paclitaxel did not disturb siRNA binding, as demonstrated herein. Finally, as demonstrated herein, the dual delivery system could be preferentially taken up by drug resistant cancer cells and was capable of acting synergistically to overcome drug resistance. In other words, the cationic polymer did not prevent release of the therapeutic compound in the cell.

[00131] In order to test the utility of MSNP as a dual delivery platform, drug-resistant squamous carcinoma cell line, KB-V1, was investigated to see if Pgp knockdown restores doxorubicin (Dox) sensitivity. KB-V1 cells exhibit MDR as a result of Pgp overexpression (Ludwig et al., *Cancer Res.*, vol. 66, pp. 4808-4815, 2006). In order to effectively engineer particles to deliver Dox as well as Pgp siRNA, particle functionalization by the attachment of negative (phosphonate) as well as positive (PEI) surface groups are functionally effective. Moreover, because PEI delivery of siRNA constructs to the cytosol may require intermediary lysosomal processing, it was uncertain whether this endocytic route was appropriate for doxorubicin (Dox) delivery. As shown in the current invention, Dox can be stably attached to the porous interior by a proton-sensitive electrostatic binding interaction that allows effective drug release from the acidifying LAMP-1-positive compartment. Pgp siRNA co-delivery increases intracellular Dox concentrations with improved cytotoxic killing. The improvement of Dox resistance provides proof-of-principal testing that MSNP can be engineered to provide contemporaneous drug and siRNA delivery by effective use of charge and the state of protonation or deprotonation at the particle surface.

[00132] As shown herein, MSNP can be functionalized to deliver a chemotherapeutic agent as well as Pgp siRNA to a drug-resistant cancer cell line. The functionalization of the particle surface with a phosphonate group allows electrostatic binding of Dox to the porous interior, from where the drug could be released by acidification of the medium under abiotic and biotic conditions. In addition, phosphonate modification also allows exterior coating with the cationic polymer, PEI, which endows the MSNP with the ability to contemporaneously bind and deliver Pgp siRNA. The dual delivery of Dox and siRNA in KB-V1 cells was capable of increasing the intracellular as well as intranuclear drug concentration to levels exceeding that of free Dox or the drug being delivered by MSNP without siRNA co-delivery. This reflects the ability of the siRNA to effectively knock down Pgp expression and therefore interfering in drug efflux as one of the resistance mechanisms in KB-V1 cells. Unlike hydrophobic cargo (**Figure**

32), Dox can be released from the lysosome by a proton-sensitive mechanism. While clearly effective at improving cytotoxic killing through its dual delivery capabilities, siRNA-PEI-MSNPs could not restore Dox sensitivity in KB-V1 to the level seen in drug-sensitive KB-31 cells. This may be due to the extremely high levels of Pgp expression, as confirmed in **Figure 23**.

[00133] Two of the major problems in cancer chemotherapy are toxic side effects as well as development of MDR in cancer cells. Nanoparticle drug delivery is capable of overcoming both problems through tumor cell targeting as well as the capability to overcome drug resistance (Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008; Ferrari et al., *Nat. Rev. Cancer*, vol. 5, pp. 161-171, 2005). MDR can basically be divided into two distinct categories, namely pump and non-pump resistance (Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008; Saad et al., *Nanomedicine*, vol. 3, pp. 761-776, 2008). Pump resistance refers to the inducible formation of membrane-bound channels or pores that actively expel a series of structural and functionally distinct chemotherapeutic agents from the cell. Drug efflux significantly decreases the intracellular concentration that limits their cytotoxic potential. The key proteins involved in pump resistance are Pgp and MRP-1, while the major mechanism in non-pump resistance is activation of cellular anti-apoptotic defense pathways, including drug-induced expression of Bcl-2 protein (Saad et al., *Nanomedicine*, vol. 3, pp. 761-776, 2008). Moreover, the pump and non-pump resistance mechanisms could be mutually interactive (Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008). Given this background, a number of nanomaterial design strategies can be used to overcome drug resistance:

[00134] The first is co-delivery of the chemotherapeutic drug with a pharmaceutical agent that interferes in pump activity or in non-pump pathways. One example is the use of verapamil as a Pgp inhibitor that have been combined with Dox, aimed at reducing cardiotoxicity of Dox as well as overcoming Pgp-mediated MDR (Wu et al., *J Pharm. Pharmaceut. Sci.*, vol. 10, pp. 350-357, 2007). In Dox-resistant K562 leukemia cells, a liposomal Dox-verapamil complex doubled the cytotoxicity index ($IC_{50}=11.4 \mu M$) of free Dox ($IC_{50}=23.4 \mu M$) (Wu et al., *J Pharm. Pharmaceut. Sci.*, vol. 10, pp. 350-357, 2007). Another example is using a polymeric nanoparticle formulation of poly(ethyleneoxide)-poly(epsilon-caprolactone) to co-administer ceramide that is capable of lowering the apoptotic threshold to paclitaxel, thereby enhancing paclitaxel-induced cytotoxicity in a MDR human ovarian cancer cell line (van Vlerken et al., *Cancer Res.*, vol. 67, pp. 4843-4850, 2007).

[00135] A second strategy is to use nanoparticles to deliver the chemotherapeutic together with siRNA that interfere in key protein expression in pump-dependent or independent drug resistance pathways, as demonstrated herein. There are reports demonstrating that compared to the free drug, dual delivery with a nucleic acid is capable of enhancing the efficacy in a synergistic fashion (Chen et al., *Small*, vol. 5, pp. 2673-2677, 2009; Patil et al., *Biomaterials*, vol. 31, pp. 358-365, 2009). Chen et al showed that MSNP served as a drug delivery vehicle to deliver Dox and Bcl-2 siRNA that can effectively silence the Bcl-2 mRNA and significantly suppress the non-pump resistance, and therefore result in enhanced cell killing capability of Dox in multidrug resistant A2780/AD human ovarian cancer cells (Chen et al., *Small*, vol. 5, pp. 2673-2677, 2009). Another example is paclitaxel that was delivered by poly(d,l-lactide-co-glycolide) nanoparticles along with Pgp siRNA to the MDR murine mammary cancer cell line, JC. The dual delivery system showed significantly higher cytotoxicity *in vitro* and significantly greater inhibition of tumor growth *in vivo* than nanoparticles loaded with paclitaxel alone (Patil et al., *Biomaterials*, vol. 31, pp. 358-365, 2009).

[00136] The third strategy is to deliver the drug together with a combination of siRNA's that interfere in both pump (*e.g.* Pgp) and non-pump (*e.g.* Bcl-2) mechanisms. Such an approach may be necessitated by the co-existence of pump and non-pump resistance mechanisms (Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008; Saad et al., *Nanomedicine*, vol. 3, pp. 761-776, 2008). In this regard, it has been demonstrated that the increase in intracellular drug concentration as a result of the suppression of drug efflux pumps leads to almost proportional activation of anti-apoptotic cellular defense (Gottesman et al., *Annu. Rev. Med.*, vol. 53, pp. 614-627, 2002; Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008). As one example, a cationic liposome carrier system was developed to deliver Dox contemporaneously with two species of siRNA targeting of MRP-1 as well as Bcl-2 (Saad et al., *Nanomedicine*, vol. 3, pp. 761-776, 2008). This triple component (Dox, MRP-1 siRNA, and Bcl-2 siRNA) delivery system demonstrated enhanced Dox cytotoxicity in human MDR H69AR lung cancer cells which showed >100 fold enhancement in cytotoxicity (Saad et al., *Nanomedicine*, vol. 3, pp. 761-776, 2008). Similar attempts in experiments in KB-V1 cells where Bcl-2 was combined with Pgp siRNA and Dox did not improve the cytotoxicity or IC₅₀ significantly (not shown).

[00137] In some embodiments, this invention demonstrates how MSNP can be engineered to effectively deliver a drug together with siRNA. Because Dox (pKa = 8.2) is positively

charged at physiological pH, a number of surface charge modifications were used to demonstrate the utility of using electrostatic charge to bind and deliver Dox from the MSNP surface (**Figure 20A, 21B**). These studies demonstrated that Dox as well as the cationic dye, Hoechst 33342 ($pK_a = 11.9$) (**Figure 27**), are capable of binding to negatively charged surfaces from where both agents could be released by dropping the environmental pH (**Figure 20C-F**). Thus, when the MSNP are suspended in buffered cell culture medium ($pH = 7.4$), the attached phosphonate ($pK_a = 2$) and carboxylate ($pK_a = 5$) groups are de-protonated and assume a negative charge, whereas unmodified OH-MSNP ($pK_a = 7$) is near its isoelectric point and therefore not quite as effective for electrostatic binding (**Figure 20A**). By contrast, the amine groups ($pK_a \sim 9$) are protonated at physiological pH and exhibit a positive charge that prevents electrostatic binding of the same agents (Bagwe et al., *Langmuir* vol. 22, pp. 4357-4362, 2006; Santra et al., *Chem. Commun.*, pp. 2810-2811, 2004). From a therapeutic perspective, phosphonate-MSNP exhibit good particle dispersibility and biocompatibility. The ability to release Dox from the interior surface by proton interference allowed Dox delivery inside the cell in an acidifying compartment (**Figure 20F, upper panel**). The role of the lysosomal proton pump is supported by the finding that NH_4Cl interferes in Dox release to the nucleus (**Figure 20F, lower panel**). Phosphonate attachment also facilitates the binding of cationic PEI to the particle exterior (**Figure 18, right panel**). This binding interaction is sufficiently strong to allow the polymer and attached siRNA to stay on the particle surface until entry into the lysosomal compartment. PEI may play a role in firm cellular attachment and selection of the initial endosomal compartment. Importantly, the polymer is attached to the particle surface to leave the pores accessible to Dox binding (**Figure 20B**).

[00138] It is important to briefly mention the importance of selecting the correct PEI polymer size to prevent particle toxicity (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009; Meng et al., *ACS Nano*, vol. 3, pp. 1620-1627, 2009; Lin et al., *Chem. Mater.*, vol. 21, pp. 3979-3986, 2009). It is well known that PEI exerts cytotoxic effects when used by itself or attached to the nanoparticle surface as a polyplexing agent (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). In this regard, 25 kD PEI polymer as well as high doses of the 10 kD polymer can render the MSNP toxic as a result of the proton sponge effect in the lysosome (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). For these reasons, the particle dose and exposure time may be limited to within safe limits to conduct Dox and siRNA delivery with PEI 10 kD polymer (**Figure 26**). Curiously and somewhat paradoxically, siRNA delivery to the cytosol is also

dependent on the proton sponge effect of the PEI-coated particle and in this case, the lysosome appears to be a key organelle in the dual drug delivery paradigm.

[00139] Above design features extend the utility of MSNP as a drug delivery platform for chemotherapeutic agents. In addition to being able to deliver hydrophobic drugs such as camptothecin and paclitaxel, it is also possible to deliver water soluble drugs by a packaging and release mechanism that is quite different from the phase transition principle involved in hydrophobic drug delivery (see **Figure 32**). While it is not possible to release hydrophobic drugs through an acidification mechanism, camptothecin can be extracted from phosphonate-MSNP pores by ethanol treatment (**Figure 32**). The release characteristics of Dox are exactly opposite, namely a release response to protons but not ethanol (**Figure 20C and D**). Taken together, these results demonstrate the dynamic features of MSNP for obtaining optimal drug packaging and delivery.

[00140] Because Pgp overexpression is one of the major mechanisms of multiple drug resistance (MDR) in cancer cells, knockdown of Pgp gene expression by nanoparticle siRNA delivery could help to restore the intracellular drug levels to the concentrations required for induction of apoptosis and cytotoxicity. Thus, dual drug and siRNA delivery by nanoparticles can be used to overcome drug resistance in MDR cancer cells. The feasibility of the MSNP platform to improve the cytotoxicity of Dox by co-delivery of Pgp siRNA is demonstrated as proof-of-principle. Downregulation of Pgp expression allowed the intranuclear Dox levels to increase above the threshold required for inducing apoptosis and cell death. However, since the KB-V1 cell line expresses very high Pgp level that may go beyond the clinically relevant expression level, it may not be possible to restore drug sensitivity to the level seen in KB-31. It is reasonable to expect that the MSNP delivery platform will be more effective in a lesser Pgp expressing cell type. In addition, the MDR phenotype is quite complex and often involve a combination of drug resistance mechanisms such as increased efflux, blocked apoptosis, decreased drug influx, increased drug metabolism, and increased DNA repair (Gottesman et al., *Annu. Rev. Med.*, vol. 53, pp. 614-627, 2002; Jabr-Milane et al., *Cancer Treat. Rev.*, vol. 34, pp. 592-602, 2008). Preliminary research has revealed that 36 genes were either up- or down-regulated in KB-V1 cells compared to KB-31 cells. These genes can be categorized into several groups, including oxidative stress regulation (HBB, IDH), drug metabolism (HMGCS1, ACAT1), signal transduction (CGA, SGK), tumor suppression (PTEN), *etc* (Wang et al., *Chin. J. Cancer Res.*, vol. 14, pp. 5-10, 2002). It is possible that these gene products could contribute

to MDR besides Pgp overexpression. What additional mechanisms are involved is still uncertain but apparently does not involve Bcl-2 as inclusion of Bcl-2 with Pgp siRNA did not significantly improved cell killing. Understanding of abovementioned MDR mechanisms may provide new opportunity to develop more therapeutic components or combinations to achieve better therapeutic effects.

[00141] In conclusion, MSNP can be functionalized to act as a dual delivery vehicle for Dox as well as Pgp siRNA in a drug-resistant cancer cell line. To improve the drug sensitivity of KB-V1 cells, phosphonate attachment was used to deliver the therapeutic compound as well as the siRNA *via* a lysosomal processing pathway. This dual delivery system increased the intracellular Dox levels to the extent that it improves cytotoxic killing in this KB-V1 MDR cell line. This strategy could be an effective new approach for the treatment of cancers that develop multiple drug resistance.

[00142] From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

[00143] The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

[00144] Terms listed in single tense also include multiple unless the context indicates otherwise.

[00145] Where a range of values is provided in the present application, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The end values of any range are included in the range.

[00146] The examples disclosed below are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, databases, and patents cited herein are hereby incorporated by reference for all purposes.

[00147] Methods for preparing, characterizing and using the compounds of this invention are illustrated in the following Examples. Starting materials are made according to procedures known in the art or as illustrated herein. The following examples are provided so that the invention might be more fully understood. These examples are illustrative only and should not be construed as limiting the invention in any way.

EXAMPLES

Reagents

[00148] Tetraethylorthosilicate (98%), cetyltrimethylammonium bromide (CTAB, 95%), fluorescein isothiocyanate (FITC, 90%), polyethyleneimine (MW 1.2 and 25 KD), poly(ethylene glycol) methyl ether (MW 5 KD), N,N'-disuccinimidyl carbonate (DSC, 95%), 4-(dimethylamino)pyridine (DMAP, 99%), aminopropyltriethoxysilane (APTS, 99%), 3-(trihydroxysilyl)propyl methylphosphonate (42%), succinic anhydride (99%), fluorescamine, paclitaxel, propidium iodide (PI), β -actin antibody, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Polyethyleneimine (MW 0.6, 1.8, and 10 KD) were from Alfa Aesar (Ward Hill, MA). The MTS assay kit was from Promega (Madison, WI). Dulbecco's Modified Eagle's medium (DMEM), penicillin/streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was from Atlanta Biologicals, Inc (Lawrenceville, GA). siRNA for GFP knock down was purchased from IDT Technologies (Coralville, IA). For all experiments and analyses, water was de-ionized and filtered with a 0.45 μ m pore size polycarbonate syringe filter (Millipore, Billerica, MA). All chemicals were reagent grade and used without further purification or modification.

MSNP dispersion and use to perform tissue culture

[00149] All cell cultures were maintained in 25 cm² cell culture flasks in which the cells were passaged at 70-80% confluency every 2-4 days. RAW 264.7, BxPC3, PANC-1, and HEPA-1 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Carlsbad, CA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (complete medium). BEAS-2B cells were cultured in BEGM (Charles City, IA) in type I rat tail collagen-coated flasks or plates. Cells were cultured at 37°C in a humidified

5% CO₂ atmosphere. To disperse MSNP, the stock solution (in water) was sonicated (Tekmar Sonic Disruptor probe) for 15 sec prior to aliquoting. In order to coat the surface of MSNP with bovine serum albumin (BSA), the aliquoted NP suspension (~10 µl) was mixed with an equal volume of 4% BSA. Tissue culture media (1 ml) was added to the BSA coated MSNP suspension. Cell culture media deprived of serum (e.g. BEGM) was modified by addition of BSA at a concentration of 2 mg/ml. The cell culture media containing MSNP at the desired concentration was sonicated for 15 sec and characterized as described before.

Assays for cellular viability and mitochondrial function

[00150] Cellular viability was determined by the MTS assay, which looks at the reduction of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to formazan in viable cells. Briefly, 2×10^4 cells were plated onto 96 multi-well plates (Costar; Corning, NY). After incubation with the indicated dose of MSNP for various lengths of time at 37°C, formazan absorbance was measured at 490 nm. The mean absorbance of non-exposed cells served as the reference for calculating 100% cellular viability.

[00151] Cell death and mitochondrial function were detected using propidium iodide (PI) uptake and JC-1 fluorescence. Fluorescent probes were diluted in DMEM before the addition to cells for 30 min at 37 °C in the dark: (i) 5 µg/ml propidium iodide (PI) in 200 µl DMEM (assessment of cell death); (ii) 5 µM JC-1 (assessment of $\Delta\Psi_m$). Flow cytometry was performed using a LSR (Becton Dickinson, Mountain View, CA). PI was analyzed in FL-2 and JC-1 was analyzed in both FL-1 and FL-2. Forward and side scatter were used to gate out cellular fragments.

Assessment of cellular MSNP uptake by flow cytometry and confocal microscopy

[00152] For the performance of flow cytometry, aliquots of 5×10^4 cells (RAW 264.7, BEAS-2B, PANC-1, and BxPC3) were cultured in 48-well plates in 0.4 ml medium. RITC-labeled MSNP with a phosphonate surface coating were added to the above cultures at a dose of 25 µg/ml for 30 minutes, followed by incubation with the FITC-labeled MSNP-PEI series at final concentrations of 25 µg/ml for 3 hr. All cell types were trypsinized and washed with trypan blue to quench the fluorescence of cell surface attached MSNP. Cells were analyzed in a LSR flow cytometer using mean FL-2 and FL-1 to assess RITC and FITC fluorescence, respectively. Data are reported as fold increase above control (cells without MSNP).

[00153] Cellular uptake of MSNP was performed by adding 25 $\mu\text{g/ml}$ of the various MSNP to 8-well chamber slides (Nunc) in which 5×10^4 cells were cultured in each well containing 0.4 ml culture medium. Cell membranes were stained with 5 $\mu\text{g/ml}$ wheat germ agglutinin (WGA) Alexa Fluor[®] 594 conjugate in PBS for 30 min. Slides were mounted with DAPI (Molecular Probes, Eugene, OR) and visualized under a confocal microscope (Leica Confocal 1P/FCS) in the UCLA/CNSI Advanced Light Microscopy/Spectroscopy Shared Facility. High magnification images were obtained with a $63\times$ objective. Optical sections were averaged 2-4 times to reduce noise. Images were processed using Leica Confocal Software.

EXAMPLE 1 - *Synthesis and surface modification of mesoporous silica nanoparticles MSNP*

[00154] The basic synthesis of MSNP was conducted by mixing the silicate source tetraethylorthosilicate (TEOS) with the templating surfactants cetyltrimethylammonium bromide (CTAB) in basic aqueous solution (pH 11). In a round bottom flask, 100 mg CTAB was dissolved in a round-bottom flask containing solution of 48 ml distilled water and 1.75 ml sodium hydroxide (2 M). The solution was heated to 80°C and stirred vigorously. After the temperature had stabilized, 0.5 ml TEOS was added slowly into the heated CTAB solution. After 15 min, 0.23 mmol of the organosilane solution was added into the mixture. 3-trihydroxysilylpropyl methylphosphonate was used for phosphonate surface modification, and aminopropyltriethoxy silane (APTS) was used for amine surface modification. After 2 hr, the solution was cooled to room temperature and the materials were washed with methanol using centrifugation. In order to incorporate fluorescent dye molecules in the silicate framework, fluorescein-modified silane was first synthesized and then mixed with TEOS. To synthesize fluorescein-modified silane, 2.4 μL APTS was mixed with 1 mg fluorescein isothiocyanate (FITC) in 0.6 ml absolute ethanol, and stirred for 2 hr under inert atmosphere. In another formulation, rhodamine B isothiocyanate (RITC) was used instead of FITC to synthesize rhodamine B-modified APTS. The dye-modified silane was then mixed with TEOS before adding the mixture into the heated CTAB solution. The surfactants were removed from the pores by refluxing the particles in a mixture of 20 ml methanol and 1 ml hydrochloric acid (12.1 M) for 24 hr. The materials were then centrifuged and washed with methanol.

[00155] For the poly(ethylene glycol) modification, 1 g of poly(ethylene glycol) methyl ether (MW 5 KD, mPEG) was dried under vacuum for 30 min and dissolved in 5 ml dioxane (with slight heating). mPEG has only one reactive end that can be attached to the particle surface

and limits the coupling process only to that end, whereas normal PEG has two reactive ends and may cause particle cross-linking. 307.4 mg disuccinimidyl carbonate (DSC) was dissolved in 2 ml anhydrous DMF (with slight heating) and mixed with the mPEG solution. 146.6 mg 4-(dimethylamino)pyridine was dissolved in 2 ml acetone and added slowly into the mPEG solution. The mixture was stirred for 6 hours under an inert atmosphere. The polymer was precipitated by the addition of 30 ml diethyl ether to the solution and separated by centrifugation. After washing the polymer twice with diethyl ether, the activated mPEG was dried under vacuum. 60 mg of amine-modified MSNP was washed and resuspended in 2 ml anhydrous DMF. 300 mg of the activated mPEG was dissolved in 9 ml DMF and mixed with the particles. The mixture was stirred for 12 hr and washed thoroughly with DMF and PBS.

[00156] To perform polyethyleneimine (PEI) modification, 5 mg of phosphonate-modified MSNP were dispersed in a solution of 2.5 mg PEI (MW 25 KD) and 1 ml absolute ethanol. The process to coat the particles with other PEI polymers (MW 0.6, 1.2, 1.8, 10 KD) was carried out similarly. After the mixture was sonicated and stirred for 30 min, the particles coated with PEI were washed with ethanol and PBS. Thermogravimetric analysis showed that the amount of PEI on the particles was approximately 5 weight percent. To succinylate the PEI 25K-coated particles, 1 mg particles were resuspended in 0.25 ml anhydrous DMF and mixed with different amounts of succinic anhydride (0.15 mg, 0.075 mg, and 0.015 mg). The mixture was sonicated and stirred overnight. The succinylated particles were washed with DMF and resuspended in PBS. To fluorescently label PEI (MW 25 KD), 60 mg of PEI 25 KD was dissolved in 10 ml carbonate buffer (pH 9) and mixed with 1 mg rhodamine B isothiocyanate dissolved in 1 ml DMSO. The mixture was stirred for 24 hr at 4°C and dialyzed against distilled water. The rhodamine B-labeled PEI 25K was attached to the particles by using similar procedure for the unlabeled PEI.

EXAMPLE 2 - *Physicochemical characterization of the NP*

[00157] MSNP were synthesized according to a modified procedure previously described (Radu et al., *J. Am. Chem. Soc.*, vol. 126, pp. 13216-13217, 2004; Cai et al., *Chem. Mater.*, vol. 13, no. 2, pp. 258-263, 2001). All MSNP were characterized for size, size distribution, shape, and charge (**Table 1**).

Table 1. Size distribution of MSNPs in aqueous solutions.

	Size (nm)				Zeta Potential (mV)
	H ₂ O	DMEM 10% serum	BEGM	BEGM 2mg/ml BSA	H ₂ O/DMEM+serum/BEGM+BSA
MSNP-OH	1966	408	1096	416	-10.5/-6.8/-7.2
MSNP-Phosphate	1975	306	867	439	-8.9/-6.5/-5.8
MSNP-PEG	2675	405	1215	542	-10.4/-5.9/-4.5
MSNP-PEI 0.6 KD	1689	415	1243	474	+29.5/-7.8/-6.5
MSNP-PEI 1.2 KD	1684	452	1298	502	+38.7/-6.5/-7.1
MSNP-PEI 1.8 KD	1053	510	1087	550	+36.9/-5.4/-3.2
MSNP-PEI 10 KD	614	702	917	684	+34.1/-7.5/-6.9
MSNP-PEI 25 KD	1473	1043	1544	825	+30.8/-5.9/-4.0

Particle size and zeta potential in solution were measured by a ZetaSizer Nano (Malvern). DMEM = Complete Dulbecco's Modified Eagle Media, which contains 10% fetal calf serum (FCS). BEGM = Bronchial Epithelial Growth Medium, which includes growth factors, cytokines, and supplements (no serum).

[00158] The shape and structure were characterized using a transmission electron microscope (JEOL JEM 2010, JEOL USA, Inc., Peabody, MA). Microfilms for TEM imaging were made by placing a drop of the respective particle suspension onto a 200-mesh copper TEM grid (Electron Microscopy Sciences, Washington, PA) and then drying at room-temperature overnight. A minimum of 5 images of each sample was collected to obtain representative views. Particle size and zeta potential in solution were measured by ZetaSizer Nano (Malvern Instruments Ltd., Worcestershire, UK). This instrument measures the light scattering (DLS) from a suspension at an angle of 173°. Size measurements were performed on dilute suspensions in water or complete cell culture media at pH 7.4. The ZetaSizer Nano was also used to measure the electrophoretic mobility of the MSNP suspended in solution. Electrophoretic mobility is used as an approximation of particle surface charge and can be used to calculate zeta potential. The Helmholtz-Smoluchowski equation was used to recalculate electrophoretic mobility into zeta potential.

[00159] The primary particle size is in the 100-130 nm range with a uniform pore size ~2.5 nm as shown by TEM (**Figure 1**). To conduct biological experimentation, particle size and zeta potential were measured in water as well as tissue culture media (Xia et al., *ACS Nano*, vol. 2, pp. 2121-2134, 2008). For the purposes of this study DMEM supplemented with 10% fetal calf serum (FCS) or BEGM was used as is or supplemented with 2 mg/ml BSA (**Table 1**). The addition of protein leads to improved particle dispersal by countering the colloidal forces that promote particle aggregation in salt containing media (**Figure 9**). While all of the non-PEI coated particles exhibited a negative zeta potential, PEI-coated particles showed a positive

charge (**Table 1**). However, with the addition of FCS or BSA, all PEI-coated particles assumed a negative zeta potential.

EXAMPLE 3 - *Differences in the cytotoxic potential of anionic vs cationic MSNP*

[00160] To screen for particle hazard, the MTS assay was used, which reflects dehydrogenase activity in healthy cells (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). While most of the MSNP did not interfere in MTS activity in the PANC-1 and BxPC3 pancreatic cancer cell lines, particles coated with the 25 KD PEI polymer showed decreased cellular viability (**Figure 1**). The particles coated with the 25 KD polymer also induced toxicity in macrophage (RAW 264.7) and bronchial epithelial (BEAS-2B) cell lines staining (**Figure 10**). The toxicity was confirmed by propidium iodide (PI) staining, which showed that the rate of cell death was progressive over 15 hour period (**Figure 10**). Similar to previous results with cationic polystyrene nanoparticles (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008), the toxicity of cationic MSNP involves an effect on mitochondria as determined by JC-1 fluorescence (**Figure 10**). JC-1 measures mitochondrial membrane potential (MMP). In contrast, phosphonate-coated nanoparticles (MSNP-Phos) were non-toxic and did not perturb the mitochondrial function (**Figure 10**).

[00161] To confirm that PEI toxicity is due to cationic charge, succinic anhydride was used to convert the primary NH_2 to COOH groups (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). **Figure 11A** demonstrates that this conversion reduced the toxicity of MSNP-PEI-25 KD in a dose-dependent fashion. Depletion of the primary NH_2 groups was confirmed by fluorescamine, which yields green fluorescence when it reacts with primary amines (**Figure 11B**). Thus, succinic anhydride reduced the mean fluorescence intensity (MFI) in a dose-dependent fashion. The stability of PEI attached to the MSNP surface was confirmed by coating FITC-labeled MSNP with rhodamine-B labeled PEI. Confocal microscopy confirmed that both labels co-localize as shown by the composite yellow fluorescent spots in the cell (**Figure 12**).

EXAMPLE 4 - *Differences in the cellular uptake of anionic versus cationic MSNP*

[00162] Cellular toxicity of cationic polystyrene nanoparticles involves a high rate of cellular uptake due to tight surface membrane binding, which facilitates particle wrapping and cellular uptake (Xia et al., *Nano Lett.* Vol. 6, pp. 1794-1807, 2006; Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). Cellular uptake of FITC-labeled MSNP was assessed by flow cytometry and

confocal microscopy (Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). FITC-labeled MSNP were incubated with PANC-1, BxPC3, RAW 264.7, and BEAS-2B cells. This resulted in a clear shift in the MFI of the different cellular populations following treatment with particles coated with 1.2 and 25 KD PEI polymers as compared to the phosphonate-coated MSNP (**Figure 2 and 3; Figure 13**). When expressed as fold-increase in the MFI compared to untreated cells, the relative abundance of MSNP-PEI-25 KD and MSNP-PEI-1.2 KD uptake was two orders of magnitude higher than the phosphonate or PEG-coated particles (**Figure 2 and 3; Figure 13**). In contrast, there was no difference in the uptake of RITC-labeled MSNP in the same cells treated with the FITC-labeled particles. This confirms that the cationic charge is responsible for high cellular uptake. The flow data was corroborated by confocal studies that showed a significant increase in the number of PEI-coated particles in all cell types (**Figure 2, 3 and 13**). Please notice that a large fraction of the MSNP-PEI-25 KD particles localized at the cell membrane as demonstrated by the composite yellow fluorescence in pancreatic cancer cells stained with the red-fluorescent wheat germ agglutinin (**Figure 2 and 3**). It is possible that this membrane binding might contribute to the toxicity of these particles.

Reducing PEI polymer length maintains effective nucleic acid delivery but eliminates cellular toxicity

[00163] While PEI is quite effective for complexing and delivering nucleic acids, polymer-based delivery of DNA and siRNA often leads to cytotoxicity due to damage to the surface membrane, lysosomes and mitochondria (Boussif et al., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, pp. 7297-7301, 1995; Florea et al., *AAPS PharmSci.*, vol. 4, p. E12, 2002; Xia et al., *ACS Nano*, vol. 2, pp. 85-96, 2008). However, based on the observation that decoration of MSNP with a 1.2 KD polymer resulted in a non-toxic particle, a range of PEI polymers were explored in terms of achieving cellular delivery versus reduction of toxicity. MSNP were coated with 0.6, 1.2, 1.8, 10 and 25 KD polymers and their cytotoxic potential assessed in various cell types. This included the use of HEPA-1 cells for which there is a commercial variant expressing green fluorescent protein (GFP) for the purposes of assessing siRNA knockdown. No toxicity was seen with particles coated with 0.6, 1.2 and 1.8 KD PEI polymers (**Figure 4 and 14**). While MSNP-PEI-10 KD exerted toxic effects at the highest dose (50 µg/ml) tested, MSNP-PEI-25 KD was responsible for the decline in MTS activity at doses ≥ 12.5 µg/ml (**Figure 4 and 14**). This demonstrates that it is possible to adjust MSNP toxicity according to the polymer length

used and is the first demonstration that the choice of the PEI polymer length can be used to modify the toxicity of MSNP while still maintaining a useful function.

Preparation of PEI-MSNP-pDNA/siRNA polyplexes and agarose gel retardation

[00164] Agarose gel retardation assay was used to determine the DNA/siRNA binding ability of PEI-coated MSNP. 0.1 µg plasmid DNA (pEGFP) or siRNA in aqueous solution was used to mix with PEI-coated nanoparticles to obtain particle to pDNA ratios (N/P) ratios of 5-600. The mixture was incubated at room temperature for 30 min for complex formation. 10 µL of the polyplex solution mixed with 2 µL of 6× loading buffer was electrophoresed on 1 % agarose gel containing 0.5 µg/ml ethidium bromide (EB) with Tris-boric acid (TBE) running buffer (pH 8) at 100 V for 30 min. DNA/RNA bands were visualized by an UV (254 nm) illuminator and photographed with a Bio-Rad imaging system (Hercules, CA). The binding capacity was expressed by the N/P ratio that shows total retardation of DNA or siRNA migration (as reflected by the disappearance of DNA/RNA bands on the gel).

DNase I protection assay:

[00165] Particle/pDNA complexes were prepared at a MSNP/pDNA ratio of 100 with 100 ng pDNA in 10 µl total volume. The complex solutions were incubated with 1 µl DNase I (2.7 U/µl) in 50 mM Tris-Cl, 10 mM MgCl₂, pH 7.4. at 37°C for 30 min. The DNase I was inactivated by adding 1 µl of 100 mM ethylenediaminetetraacetic acid (EDTA). The pDNA was then released from the complex by adding 1% sodium dodecyl sulfate (SDS), and analyzed by 1% agarose gel electrophoresis.

[00166] To assess the efficacy of different PEI-coated MSNP in terms of siRNA and plasmid DNA delivery, the nucleic acid binding capacity of the particles was determined using a gel retardation assay. The data show high siRNA and plasmid DNA binding capacity that was mostly independent of the PEI MW (**Figure 5**). All available DNA and siRNA molecules were bound to the cationic particle surface at particle-to-nucleic acid ratios of 10-100 (**Figure 5B**). Noteworthy, the high binding efficacy of MSNP was accompanied by protection of DNA to DNase I degradation (**Figure 5C**). By contrast, phosphonate-coated particles did not show effective DNA binding.

Plasmid DNA and siRNA transfection with the use of MSNP-PEI

[00167] Plasmid DNA (pDNA) containing a GFP insert was used to transfect HEPA-1 cells cultured either in Nunc chamber slides for performance of confocal microscopy or in 48-well plates for assessment by flow cytometry. Cells were plated at a density of 2×10^4 cells per well in 0.4 ml medium. pDNA/MSNP complexes were prepared by mixing 100 ng/ml DNA with 25 μ g/ml MSNP for 30 min prior to cellular incubation for 24 hr. Cells were fixed for confocal microscopy as described above. Harvested cells were used to conduct flow cytometry on FL-1 channel.

[00168] In the next set of experiments the efficacy of DNA delivery was assessed by transfecting a GFP plasmid into HEPA-1 cells. While plasmid delivery by the 10 and 25 KD PEI-coated particles resulted in abundant cellular fluorescence, only faint fluorescence was observed when the carrier particle was coated with a shorter length polymer (**Figure 7**). The transfection efficiency with the longer length polymers compares favorably to the results with Lipofectamine 2000. Moreover, while this commercial agent only transduced a fraction of the cells in the population, MSNP-PEI-10 KD transfected >70% cells in the population (**Figure 7B**). This was confirmed by the magnitude of the MFI increase with the particles versus Lipofectamine (**Figure 7A**). Although also efficient for DNA delivery, MSNP-PEI-25 KD did result in toxicity as explained previously. This may not constitute a problem when stable transfections are being performed because one selects for viable and proliferating cells containing the expressed gene.

[00169] To perform siRNA experiments, HEPA-1 cells were prior transfected with a GFP plasmid and then sorted in the FL-1 channel to select stable GFP expressing cells. The sorted cells were plated at a density of 5×10^4 cells per well containing 0.4 ml culture medium in chamber slides for performance of confocal microscopy and in 48-well plates for performance of flow cytometry. MSNP/siRNA complexes were prepared by incubating 500 ng/ml siRNA with 25 μ g/ml of MSNP-PEI for 30 min in serum-free DMEM. Cells were then exposed to the complexes for 3 hr. DMEM + 10% FCS was then added to bring the final volume to 400 μ l for 48 hr. Cells were fixed and prepared for confocal microscopy as described above. For flow cytometry cells were harvested and analyzed for fold decrease in GFP expression (FL-1 channel).

[00170] To test whether siRNA delivery to its GFP-expressing HEPA-1 cells could provide knockdown of the expression of this fluorescent protein, 100 ng GFP-siRNA was

complexed with MSNP-PEI and incubated with the cells for 48 hr. Comparison of the MFI of transfected versus control cells showed that particles coated with 10 and 25 KD polymers were capable of knocking down GFP expression by 55% and 60%, respectively (**Figure 6A**). This was comparable to the transfection efficiency of commercially available transfection agent, Lipofectamine 2000. By contrast, scrambled siRNA delivery did not have a GFP knockdown effect (**Figure 6A**). Moreover, siRNA delivery by 0.6, 1.2 and 1.8 KD PEI-coated particles did not exert an effect on GFP expression. GFP knockdown was confirmed by confocal microscopy (**Figure 6B**) and immunoblotting for GFP protein expression (**Figure 15**). Please notice that although the toxicity of the 25 KD polymer may contribute to decreased GFP expression, the 10 KD polymers is not toxic at this dose (**Figure 4**). Scrambled siRNA delivery had no effect on GFP expression (**Figure 6B**). Confocal studies using Texas Red-labeled siRNA was performed to track the intracellular fate of the particles (**Figure 6B**). This confirmed that the particles coated with longer polymers were taken up in larger numbers than the shorter range polymers in HEPA-1 cells (**Figure 6B, 15B**).

EXAMPLE 5 – Paclitaxel delivery

Cationic MSNP efficiency in delivering paclitaxel to pancreatic cancer cells

[00171] MSNP are capable of delivering water-insoluble drugs to cancer cells (Liong et al., *ACS Nano*, vol. 2, pp. 889-896, 2008). In light of the high cellular uptake of PEI-coated particles, it was logical to ask whether in the traditional use of the MSNP, the polymer attachment still allows effective delivery of the hydrophobic cancer drug, paclitaxel, to PANC-1 and BxPC3 cells.

Drug loading of paclitaxel

[00172] Paclitaxel was loaded into MSNP-PEI-1.2 KD and -25 KD in DMSO, followed by washing in an aqueous buffer to entrap the hydrophobic drug in the particle pores. Briefly, the modified materials were loaded with paclitaxel by incubating 10 mg of the nanoparticles in a solution of 1 mg of paclitaxel and 0.25 ml of DMSO for 6 hours. After the drug-laden nanoparticles were removed from the suspension by centrifugation and the supernatant removed completely, the materials were dried under vacuum. The drug-laden nanoparticles were washed and sonicated with PBS.

[00173] In order to determine the amount of paclitaxel that partitioned to the MSNP, the aqueous particle suspension were incubated for 6 hours at 4 °C before centrifugation. Methanol was used to release paclitaxel from MSNP to determine the loading capacity. The drug-laden MSNP pellet was resuspended and sonicated in methanol on three occasions and the supernatants were combined to measure the release of the drug by UV absorption at 230 nm. 50 µg/ml particles contained about 1 µM of paclitaxel. Release of the drug from the pores by using methanol confirmed that equal amounts of paclitaxel were loaded into MSNP irrespective of the polymer MW (**Figure 16**).

Paclitaxel delivery by MSNP-PEI

[00174] PANC-1 and BxPC3 cells were plated at 2×10^4 cells per well in a 96 well plate. MSNP particles, loaded with paclitaxel, were incubated with the cells at doses of 10-50 µg/ml for 48-72 hrs. Free paclitaxel corresponding to the amount loaded into MSNP particles was suspended or dissolved in PBS and DMSO to serve as controls. MTS assays were performed after 48 hours to determine the cell viability after treatment.

[00175] Subsequent assessment of the impact of paclitaxel on the viability of PANC-1 and BxPC3 cells showed the efficacy of PEI-coated MSNP in drug delivery (**Figure 8**). Thus, paclitaxel delivery by MSNP-PEI-1.2 KD induced a rate of cytotoxicity that is superior to the drug delivery by an aqueous suspension of paclitaxel and as efficient as the drug suspended in a DMSO carrier (**Figure 8**). While MSNP-PEI-25 KD showed intrinsic particle-related toxicity at doses > 25 µg/ml, the effect was comparable to MSNP-PEI-1.2 KD (**Figure 8**). These data demonstrate that the enhanced cellular uptake of non-toxic cationic MSNP is capable of enhancing the delivery of hydrophobic cancer drugs. This is also a novel demonstration for the delivery function of MSNP.

EXAMPLE 6 - *In vivo* toxicity test of MSNP

[00176] Animal experiment protocols were reviewed and approved by the The Chancellor's Animal Research Committee (ARC) at UCLA. Animal experiments were performed in accordance with UCLA guidelines for care and treatment of laboratory animals and the NIH Guide for the Care and Use of Laboratory Animals in Research (DHEW78-23). The mice were randomly divided into three groups: MSNP-phosphonate, MSNP-PEI 25 KD and the control group. Six mice were used per group, since this number has enough statistical power to

discern differences in the toxic responses. Forty mg/kg particles were used for *i.v.* injection through tail vein once a week for two weeks. Animal weight was monitored after particle injections. Animals were sacrificed later to obtain blood and organs.

Biochemical assay of serum

[00177] The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. The biochemical parameters were assayed by UCLA Division of Laboratory Animal Medicine (DLAM) diagnostic laboratory services.

Histology of major organs

[00178] A small piece of liver, kidney, spleen, lung, heart, and brain was fixed by 10% formalin and then embedded into paraffin, sectioned for 5 μ m thick, and mounted on the glass microscope slides by UCLA Division of Laboratory Animal Medicine (DLAM) diagnostic laboratory services. The sections were stained with hematoxylin-eosin and examined by light microscopy.

Results

[00179] To use MSNP for nucleic acid and drug delivery, it is necessary to first make sure the particle is safe in animals. And for drug carriers, *i.v.* injection is the most used route. *In vivo* toxicity test of MSNP were conducted in mice by *i.v.* injection of 20 mg/kg nanoparticles once a week for two weeks. Before the injection, the particle size was characterized in saline, which is commonly used in *in vivo* assays. However, the average size of MSNP-phosphonate and MSNP-PEI 25 KD is 984 and 842 nm in saline, respectively. Recent research showed that protein corona, the protein coating on the particle surface, can stabilize nanoparticle suspension and serum is high in proteins that can be used as a dispersing agent for nanoparticles. 2% mouse serum was added to saline and used to suspend the MSNP. The serum-containing saline substantially decreased the average size of MSNP-phosphonate and MSNP-PEI 25 KD to 249 and 278 nm, respectively.

[00180] After two weeks, animals were sacrificed. Blood and major organs such as liver, kidney, spleen, lung, heart, and brain were collected to do serum biochemistry assays and histology, respectively. The serum chemistry tests showed no significant changes in all the tested parameters between MSNP-treated and control mice (**Table 2**).

Table 2. Serum biochemistry tests (Avg $\pm$ SD, n = 6)

	Control (Saline)	MSNP-Phos	MSNP-PEI 25 KD
CHOL (mg/dL)	162.5 $\pm$ 8.5	153.8 $\pm$ 15.4	144.4 $\pm$ 6.8
CK (U/L)	3000.3 $\pm$ 2285.3	2690.5 $\pm$ 1129.3	3280.9 $\pm$ 1512.7
ALT (U/L)	59.5 $\pm$ 6.4	55.7 $\pm$ 8.4	46.1 $\pm$ 22.6
AST (U/L)	184.2 $\pm$ 70.2	182.2 $\pm$ 21.1	217.2 $\pm$ 65.6
ALP (U/L)	105.2 $\pm$ 8.6	102.1 $\pm$ 17.5	88.6 $\pm$ 48.9
TBILI (mg/dL)	0.7 $\pm$ 0.1	0.9 $\pm$ 0.3	0.6 $\pm$ 0.1
TPROT (mg/dL)	6.2 $\pm$ 0.2	6.0 $\pm$ 0.4	5.9 $\pm$ 0.3
GLU (mg/dL)	140.8 $\pm$ 16.5	141.2 $\pm$ 29.2	140.8 $\pm$ 34.6
PHOS (mg/dL)	8.4 $\pm$ 0.7	7.4 $\pm$ 1.0	8.3 $\pm$ 0.6
CA (mg/dL)	8.9 $\pm$ 1.7	9.2 $\pm$ 0.4	9.4 $\pm$ 0.2
CO2_LC (mEq/L)	10.1 $\pm$ 2.2	13.3 $\pm$ 3.8	11.3 $\pm$ 1.1
BUN (mg/dL)	20.8 $\pm$ 2.1	22.3 $\pm$ 9.3	22.6 $\pm$ 2.6
CREAT (mg/dL)	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0
DBILI (mg/dL)	0.9 $\pm$ 0.2	1.0 $\pm$ 0.5	0.7 $\pm$ 0.2
ALB (g/dL)	3.3 $\pm$ 0.1	3.2 $\pm$ 0.2	3.2 $\pm$ 0.2
AGR (N/A)	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.1 $\pm$ 0.1
B_CREA (mg/dl)	75.3 $\pm$ 16.2	79.2 $\pm$ 30.0	82.0 $\pm$ 12.4
AMYL (U/L)	1252.2 $\pm$ 215.1	1266.0 $\pm$ 189.5	1139.4 $\pm$ 122.0
LDH (U/L)	681.5 $\pm$ 152.8	638.8 $\pm$ 133.3	611.0 $\pm$ 141.6
MG (mg/dL)	2.0 $\pm$ 0.5	2.2 $\pm$ 0.2	2.3 $\pm$ 0.2
TRIG (mg/dL)	201.2 $\pm$ 43.8	178.2 $\pm$ 65.3	156.0 $\pm$ 28.5

Biochemical parameters includes Cholesterol (CHOL), Creatine Kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), total bilirubin (TBILI), total protein (TPROT), Glucose (GLU), Inorganic phosphorus (PHOS), Calcium (CA), Carbon Dioxide (CO2_LC), Blood Urea Nitrogen (BUN), Creatinine (CREAT), Direct bilirubin (DBILI), Albumin (ALB), Albumin-globulin ratio (AGR), Blood Creatinine (B_CREA), Amylase (AMYL), Lactate dehydrogenase (LDH), Magnesium (MG), Triglycerides (TRIG).

[00181] Histology of major organs also showed no major changes among the groups (representative liver, spleen and kidney sections were shown in **Figure 17**. These results show MSNP-phosphonate and MSNP-PEI 25 KD do not have acute toxicity to major organs or systems in mice.

EXAMPLE 7 - Dual Deliverity siRNA and anticancer compound**Materials**

[00182] Tetraethylorthosilicate (TOES, 98%), cetyltrimethylammonium bromide (CTAB, 95%), fluorescein isothiocyanate (FITC, 90%), doxorubicin hydrochloride (Dox, >98%), camptothecin (CPT, 95%), vinblastine, polyethylenimine (PEI, branched, MW 25 kD), bafilomycin A ($\geq 95\%$), ammonium chloride, β -actin antibody, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Polyethylenimine (branched, MW 1.8 and 10 kD) was purchased from Alfa Aesar. 3-trihydroxysilylpropyl methylphosphonate, cyanoethyltriethoxysilane, and aminopropyltriethoxysilane were purchased from Gelest. Dulbecco's Modified Eagle's medium (DMEM), penicillin/streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was from Atlanta Biologicals, Inc (Lawrenceville, GA). LAMP-1 antibody was obtained from Abcam (Cambridge, MA). siRNA for Pgp knockdown was purchased from IDT Technologies (Coralville, IA). Tariquidar (>97%) was purchased from MedKoo Biosciences, Inc. For all experiments and analyses, water was de-ionized and filtered with a 0.45 μm pore size polycarbonate syringe filter (Millipore, Billerica, MA). All chemicals were reagent grade and used without further purification or modification.

Cell Lines

[00183] KB-31 and KB-V1 cells lines were confirmed as Dox-sensitive and Dox-resistant cell lines, with IC_{50} values of 0.21 and 53.0 $\mu\text{g/ml}$, respectively (**Figure 23**). Immunoblotting analysis confirmed that the Pgp expression in KB-V1 was > 1,000 times that of KB-31 cells (**Figure 23B**).

[00184] KB-31 and KB-V1 cells were plated at 1×10^4 cells per well in 96 well plate. The cells were treated with free Dox in solution in doses ranging from 0.05-3.2 $\mu\text{g/ml}$ (KB-31) and 0.1-64 $\mu\text{g/ml}$ (KB-V1) for 72 hrs. The cell viability was determined by the MTS assay. The treated cells were incubated with MTS working solution for 2-3 hrs before measurement. The mean absorbance of non-exposed cells served as the reference value for calculating 100% cellular viability. To view Pgp expression, the cells were washed in PBS and the pellets lysed in a buffer containing Triton X-100 and protease inhibitors after centrifugation. The protein content of the supernatants was determined by the Bradford method. 100 μg total protein was electrophoresed by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking, the

membranes were incubated with 1:1000 dilution of primary monoclonal antibody to MDR1 (anti-Pgp C219, Abcam). The membranes were overlaid with goat anti-mouse secondary antibody (1:1000 dilution) before the addition of the HRP-conjugated streptavidin-biotin complex. The proteins were detected using ECL reagent according to the manufacturer's instructions.

Statistical analysis

[00185] Data represent the mean $\pm$ SD for duplicate or triplicate measurements in each experiment, which was repeated at least 3 times. Differences between the mean values were analyzed by two-sided Student's t test or one way ANOVA and results were considered statistically significant at $p < 0.05$.

PEI coated MSNP

[00186] An important consideration in the use of PEI is its potential cytotoxicity (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). In this regard, the polymer size plays an important role in the cytotoxicity that results from proton sequestration by unsaturated PEI amines in the lysosomal compartment (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). Thus, before the performance of Dox-induced cytotoxicity, it was necessary to compare the cytotoxic potential of MSNP coated with different PEI polymer lengths as shown in the **Figure 26**. In brief, the findings were that MSNP coated with the 25 kD polymer showed considerable toxicity in the MTS assay when the particle concentration is $> 25 \mu\text{g/ml}$. By contrast, particles coated with 10 kD PEI have no toxic effects if the concentration is kept $< 100 \mu\text{g/ml}$ and the exposure time limited to $\leq 24\text{hr}$ (**Figure 26**). Consequently, MSNP was used at doses $< 100 \mu\text{g/ml}$ and for exposure time periods $< 24\text{ hrs}$ in the drug delivery studies to avoid effects on cell viability. Although no cytotoxicity was seen with the 1.8 kD PEI polymer (**Figure 26A**), this particle is relatively ineffective for siRNA delivery and Pgp knockdown (**Figure 19B, 19C and 19D**).

[00187] Because of the potential toxicity of PEI, it was necessary to assess the cell viability of KB-V1 cells incubated with MSNP coated with different PEI polymers. Briefly, 1×10^4 cells were plated onto 96 multi-well plates (Costar; Corning, NY). After incubation with the indicated amounts of the various PEI coated MSNP for 24 hrs, the MTS assay was performed as described in materials and methods section. MSNP coated with the 10 kD polymer was examined for time periods ranging from 1~48 hrs, aiming to chose an optimized time period for

treatment that avoids PEI toxicity. The cells were treated with 100 µg/ml PEI-MSNPs for 1, 4, 8, 16, 24, and 48 hrs, and the old medium was replenished by fresh medium for another 71, 68, 56, 48, and 24 hrs culture. The cytotoxicity assay was checked at 72 hrs after particle treatment.

Particle Synthesis, PEI coating and interior surface functionalization

[00188] The MSNPs were synthesized according to previously published sol-gel procedure (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009; Jie et al., *Small*, vol. 3, pp. 1341-1346, 2007). Briefly, for the synthesis of unmodified MSNP (OH-MSNPs), 100 mg of CTAB was dissolved in a solution of 48 mL water and 0.35 mL sodium hydroxide (2 M) and heated to 80 °C. One half mL of TEOS was added into the aqueous solution containing CTAB surfactants. For the phosphonate modification, 3-trihydroxysilylpropyl methylphosphonate was added to the mixture 15 minutes after the addition of TEOS. The CTAB surfactants were then removed from the pores by heating the particles in acidic ethanol. To perform PEI coating, 5 mg of phosphonate-modified MSNP were dispersed in a solution containing 2.5 mg PEI (1.8 kD, 10 kD, 25 kD) in 1 mL absolute ethanol. After sonication and stirring for 30 min the PEI coated particles were washed with PBS. The amount of polymer coated onto the particle surface was approximately 5 weight percentage.

[00189] Because Dox is positively charged under physiological pH, it was necessary to demonstrate that phosphonate or other anionic surface groups are effective for drug binding, including in particles that have been coated with PEI. To demonstrate this principle, particle surfaces were decorated with carboxylate (COOH) and amine groups in addition to phosphonate and silanol (OH groups). The surface functionalization was achieved by mixing organoalkoxysilanes (made up in ethanol) with TEOS before adding the mixture into the CTAB solution (Lim et al., *J. Am. Chem. Soc.*, vol. 119, pp. 4090-4091, 1997). For carboxylate modification, 50 µL cyanoethyltriethoxysilane was mixed with 500 µL ethanol and 500 µL TEOS, then added into the surfactant solution. After the surfactant removal process, the particles were further heated in a solution of 50% sulfuric acid to hydrolyze the cyanide groups into carboxylic groups. For amine modification, 50 µL of aminopropyltriethoxysilane was first mixed with 500 µL ethanol and 500 µL TEOS before adding to the surfactant solution. After 2 hrs, the solution was cooled to room temperature and the materials were washed with methanol before the surfactant removal process.

Physicochemical characterization of MSNP

[00190] PEI-coated phosphonate MSNP were characterized for size, zeta potential, and shape, respectively. The shape and porous structure were characterized using transmission electron microscopy (JEOL JEM 2010, JEOL USA, Inc., Peabody, MA). Microfilms for TEM imaging were made by placing a drop of the respective MSNP suspensions onto a 200-mesh copper TEM grid (Electron Microscopy Sciences, Washington, PA) and then drying at room-temperature overnight. A minimum of 5 images for each sample was captured and representative images included in **Figure 18**. Particle size and zeta potential in pure water, after stabilization with 1 mg/mL BSA in water, or in cell culture medium were measured by ZetaSizer Nano (Malvern Instruments Ltd., Worcestershire, UK). All the measurements were performed in 40 µg/ml MSNP suspensions in filtered water or filtered complete cell culture media at pH 7.4. The analysis was also studied on Dox loaded particles. Similar analysis was also performed on cargo (Dox and siRNA) loaded PEI-MSNP samples.

[00191] In order to determine if Pgp overexpression in KB-V1 cells is impacted by Pgp siRNA delivery, PEI polymers in the size range 1.8-25 kD were electrostatically bound to the phosphonate-MSNP surface. Polymer binding to 100-120 nm size MSNP, exhibiting uniform pore sizes of 2-2.5 nm was confirmed by TEM (**Figure 18A, left**). The TEM image of the particles decorated with the 10 kD polymer shows that the surface coating (arrows) did not occupy the porous interior (**Figure 18A, right**). To optimize the particle dispersal for biological experimentation, the PEI-coated particles were further treated with 1 mg/ml BSA before transfer to the complete cell culture medium. BSA coating significantly improves particle dispersal in water as well as salt-containing complete DMEM (**Figure 18B**). While the PEI-coated particles exhibit a high positive zeta potential value in water, this value changes to slightly negative upon dispersal in complete DMEM containing FCS (**Figure 18B**). Note that similar analysis was also performed on the Dox loaded PEI-MSNP samples with or without siRNA binding, and that no significant changes were found with or without cargo loading (**Table 3**).

Table 3. Comparison of particle size and zeta potential of PEI 10 kD MSNP before or after cargo loading. Size and zeta potential remain the same with or without cargo loading.

Particle	Size in H ₂ O (nm)	Size in BSA (nm)	Size in DMEM (nm)	Zeta Potential in H ₂ O / CDMEM (mV)
PEI 10 kD-MSNP	758	241	261	+34.1/-3.8
PEI 10 kD-Dox-MSNP	711	198	225	+28.1/-5.2
siRNA-PEI 10 kD-Dox-MSNP	832	234	278	+25.4/-7.3

PEI-MSNP coating with Pgp siRNA and use of agarose gel electrophoresis to determine N/P ratios

[00192] An agarose gel retardation assay was used to determine the siRNA binding to PEI-MSNP. 0.1 µg siRNA was mixed with 0.4–6.4 µg amount PEI-MSNP in aqueous solution to obtain particle/nuclei acid (N/P) ratios of 4–64. 10 µL of the polyplex solution was mixed with 2 µL of 6× loading buffer and electrophoresed in a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide (EB) at 100 V for 30 minutes in Tris-boric acid (TBE) running buffer (pH = 8). Nucleic acid bands were detected by UV light (254 nm) and the photos were captured in a Bio-Rad imaging system (Hercules, CA). The results were used to calculate the threshold N/P ratios for subsequent experiments. The threshold is defined as the lowest N/P ratio value that prevents free siRNA from entering the gel. To determine the influence of Dox loading, the gel electrophoresis assay was also performed.

[00193] In order to determine the optimal N/P ratios for Pgp siRNA delivery, agarose gel electrophoresis was used to evaluate binding of various amounts of siRNA to MSNP coated with the different polymer lengths as shown in **Figure 19A**. This demonstrated that binding capacity increases with increasing size of PEI polymers, indicating that all siRNA was bound at a N/P ratio >16 (PEI 1.8kD), >16 (PEI 10 kD) and >8 (PEI 25 kD). Based on this result, N/P ratios of 80, 80 and 10 were used for polymer lengths of 1.8, 10 or 25 kD, respectively for siRNA knockdown experiments. It is worth mentioning that Dox trapped in the pores of MSNP does not influence siRNA binding to the particle surface (**Figure 24**) and also that siRNA binding does not significant change the zeta potential of MSNP (**Table 3**).

Cell culture

[00194] All cell cultures were maintained in 25 cm² or 75 cm² cell culture flasks in which the cells were passaged at 70-80% confluency every 2-4 days. The drug-sensitive KB-31 line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Carlsbad, CA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (complete DMEM medium). The MDR cell line, KB-V1 (kindly provided by Dr. Michael M. Gottesman from National Cancer Institute, NIH, Bethesda, Maryland), was maintained in 1 µg/ml of vinblastine (made up from 10 mg/ml stock in DMSO) in complete DMEM (Ludwig et al., *Cancer Res.*, vol. 66, pp. 4808-4815, 2006). While the doubling time of the parental line was approximately 14-18 hrs, the resistant cell line doubled every 25-30 hrs.

[00195] *Assessment of cellular uptake of dual fluorescent labeled siRNA-PEI-MSNP by confocal microscopy*

[00196] 200 µg FITC-labeled PEI coated MSNP were incubated with 2.5 µg Texas red-labeled Pgp siRNA for 30 minutes to yield a N/P ratio of 80. Cellular uptake of MSNP was performed by adding 20 µg/ml of the dual-labeled particles to 8-well chamber slides. Each well contained 5×10^4 cells in 0.4 ml culture medium. Cell membranes were co-stained with 5 µg/ml Alexa Fluor 633-conjugated wheat germ agglutinin (WGA) in PBS for 30 min. Slides were mounted with Hoechst 33342 and visualized under a confocal microscope (Leica Confocal 1P/FCS) in the UCLA/CNSI Advanced Light Microscopy/Spectroscopy Shared Facility. High magnification images were obtained with the 100× objective. The signal intensity of the red channel, reflecting siRNA abundance, was calculated by Image J software (version 1.37c, NIH).

[00197] Subsequent performance of confocal microscopy utilizing dual-labeled particles (Texas red-labeled-siRNA adsorbed to FITC-labeled MSNP) demonstrated a high rate of cellular uptake in KB-V1 cells in accordance with polymer length (**Figure 19B**). Image J analysis confirmed a significant increase in siRNA uptake for particles coated with the 10 and 25 compared to the 1.8 kD polymer (**Figure 19C**). The reason for this high uptake is due to the positively charged amines, which facilitates strong PEI binding to and wrapping by the surface membrane (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). Please notice that the Pgp siRNA (red) and FITC-MSNP (green) co-localize (yellow, merged) in the cell, demonstrating that the nucleic acid is stably attached to the PEI-coated particle surface (**Figure 19B**). It has

previously been demonstrated that nucleic acids bound to the PEI-MSNP surface are resistant to enzymatic cleavage (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009).

siRNA-PEI-MSNP exposure and assessment of PGP expression

[00198] Pgp siRNA-PEI-MSNP complexes were freshly prepared as described above. The siRNA duplex consists of 5'-r(CGGAAGGCCUAAUGCCGAA) dTdT (sense) and 5'-r(UUCGGCAUUAGGCCUCCG) dTdG (antisense) strands. First, 5 µl of 250 ng/µl Pgp siRNA or scrambled siRNA was added to 100 µg PEI 1.8 kD-MSNP (N/P ratio=80), 100 µg PEI 10 kD-MSNP (N/P ratio=80), or 12.5 µg PEI 25 kD-MSNP (N/P ratio=10), in 5 µl aqueous solution. After incubation at room temperature for 30 min, the complexes were stabilized by 1 mg/mL BSA and then transferred into 1 ml complete DMEM. KB-V1 cells were plated at 2×10^5 cells per well, then exposed to the complexes for 16 hrs. Sixteen hours later, the medium was replaced with fresh DMEM containing 10% FCS and cultured for a further 56 hrs. Cells then were harvested for immunoblotting.

[00199] Briefly, KB-31 and KB-V1 cells were plated at 1×10^4 cells per well in 96 well plate. The cells were treated with free Dox in solution in doses ranging from 0.05-3.2 µg/ml (KB-31) and 0.1-64 µg/ml (KB-V1) for 72 hrs. The cell viability was determined by the MTS assay. The treated cells were incubated with MTS working solution for 2-3 hrs before measurement. The mean absorbance of non-exposed cells served as the reference value for calculating 100% cellular viability. To view Pgp expression, the cells were washed in PBS and the pellets lysed in a buffer containing Triton X-100 and protease inhibitors after centrifugation. The protein content of the supernatants was determined by the Bradford method. 100 µg total protein was electrophoresed by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking, the membranes were incubated with 1:1000 dilution of primary monoclonal antibody to MDR1 (anti-Pgp C219, Abcam). The membranes were overlaid with goat anti-mouse secondary antibody (1:1000 dilution) before the addition of the HRP-conjugated streptavidin-biotin complex. The proteins were detected using ECL reagent according to the manufacturer's instructions.

[00200] A commercially available cationic liposomal transfection agent (Lipofectamine 2000) was used as a positive control. Protein abundance was quantified by densitometric scanning using a laser Personal Densitometer SI and Image Quant software (Amersham Biosciences).

[00201] To assess the efficacy of Pgp siRNA delivered by PEI-coated MSNP, Pgp expression was followed by western blotting (**Figure 19D**). This demonstrated 80 or 90% reduction, in MDR-1 expression in cells treated with MSNP coated particles that contain the 10 and 25 kD polymers, respectively. This efficacy was maintained in Dox-loaded MSNP (**Figure 25**). Scrambled siRNA-PEI-MSNP (**marked as “X” in Figure 19D**) was used as a negative control to rule out any impact by the siRNA delivery method. No Pgp knockdown was seen in the scrambled siRNA-MSNP group. The PEI-coated particles were also more effective than the commercially available transfection agent, Lipofectamine 2000, which is widely used in molecular biology.

Optimizing Dox loading and release via a proton-sensitive mechanism in vitro

[00202] Based on its cationic charge at pH 7.4, it was uncertain whether Dox ($pK_a=8.2$) would bind to the negatively charged porous interior, including under circumstances where the exterior surface was occupied by PEI. 10 mg of MSNP functionalized by OH, COOH or phosphonate attachments were mixed with 1 mg of Dox in 0.25 mL water for 12 hrs. As control, positively charged amine-MSNP was used. Subsequently, the particles were collected by centrifugation and washed with water. To corroborate the electrostatic binding hypothesis, the cationic fluorescent dye, Hoechst 33342 ($pK_a=11.9$) was used as model cargo in the same particles. This procedure was repeated in phosphonate MSNP that were coated with 10 and 25 kD PEI polymers. In order to compare the loading yields of above particles, 1 mg Dox-loaded MSNP pellet was resuspended and sonicated in 1 mL of a heated HCl solution ($pH = 5.0$) for 15 minutes. After centrifugation, another 1 mL fresh HCl aqueous solution was added. All the supernatants were combined until the MSNP became colorless. At this point the pH was readjusted to 7.0 by 1 M NaOH and the fluorescence spectrum of Dox measured at excitation and emission wavelength of 485/550 nm in a microplate reader (SpectraMax M5 Microplate Reader, Molecular Device, USA). The procedure was also repeated to determine Dox loading in MSNP coated with 10 kD and 25 kD PEI polymers.

[00203] The effects of acidification and ethanol extraction of Dox-loaded phosphonate MSNP were assessed. 1 mg Dox-loaded MSNP was suspended into 3 ml phenol red-free DMEM medium acidified to pH 5.0 or replenished with 10% (v/v) ethanol at 37 °C. The supernatants were collected at various time points and cleared by centrifugation for measurement of Dox fluorescence. Similar experiments were carried out in siRNA bound

phosphonate-MSNP coated with PEI polymers. To see if the release profile will be influenced by siRNA binding, release after 12 hrs was studied using siRNA-PEI-Dox-MSNP.

[00204] MSNP are capable of loading and releasing water-insoluble drugs (paclitaxel and camptothecin) by a phase transfer mechanism that can be reversed by ethanol washing of the particles to demonstrate the role of hydrophobicity in MSNP drug entrapment (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009; Jie et al., *Small*, vol. 3, pp. 1341-1346, 2007). A key question was whether similar effective packaging of water-soluble Dox, is possible at physiological pH, where the drug ($pK_a = 8.2$) carries a positive charge. One conceivable approach is electrostatic attachment to the negatively charged MSNP surface. To test this possibility, Dox was loaded in MSNP decorated with OH, COOH or phosphonate groups. These particles were also compared to positively charged particles decorated with amine groups. After washing of the drug-bound particles and quantification of Dox release by HCl, the loading capacities of OH-, COOH- and phosphonate-MSNP were 1.2%, 4.2% and 8.4% (w/w), respectively (**Figure 20A**). By contrast, amine-decorated particles showed low ($<0.1\%$, w/w) Dox binding capacity (**Figure 20A**). The principle of electrostatic binding to a negatively charged particle surface was also demonstrated through the use of a cationic dye, Hoechst 33342, which bound to negative but not a positive MSNP surface (**Figure 27**). Importantly, electrostatic binding of Dox to the phosphonate surface was not negated by when the particles were also coated with 10 or 25 kD PEI polymers; these particles demonstrated equivalent loading capacity to the uncoated phosphonate particles (**Figure 20B**).

[00205] Importantly, Dox could be released in a time-dependent manner from the phosphonate-MSNP or PEI-phosphonate-MSNP surface by lowering of the solution pH (**Figure 20C and 20D**). The Dox release profile was not be affected by siRNA binding to PEI on exterior surface of the particle (**Figure 28**). This establishes the possibility that intracellular drug release should be possible if the particles are capable of gaining entrance to acidifying cellular compartments.

[00206] To illustrate the drug loading/release mechanism is different between water soluble drug and water insoluble drug, the effect of surface charge on binding and release of the hydrophobic chemotherapeutic agent, CPT was evaluated. 10 mg of surfactant-extracted particles was mixed in a solution of 1 mg of CPT and 0.25 mL DMSO for 12 hrs. The CPT-loaded particles were collected by centrifugation, dried under vacuum, and washed with water.

The loading yield, release profile was studied based on the fluorescent readout of CPT ($\lambda_{\text{ex/em}}=370/429$ nm) in a microplate reader (**Figure 32**).

Confocal microscopy to determine MSNP lodging in an acidifying cellular compartment, including the effects of inhibiting lysosomal acidification on Dox release

[00207] KB-V1 cells grown on chamber slides were fixed, permeabilized, and labeled with standard immunocytochemistry protocol (Xia et al., *ACS Nano*, vol. 3, pp. 3273-3286, 2009). LAMP-1 staining was performed by using a 1:500 dilution of mouse-anti-human mAb (H4A3, Abcam, USA) for 16 hrs at 4 °C. This was followed by a 1:500 diluted TRITC-conjugated goat-anti-mouse secondary antibody (Santa Cruz, USA) for 1 hr at room temperature. Cell membranes and nuclei were stained with WGA 633 and Hoechst 33342, respectively. Slides were visualized under a confocal microscope (Leica Confocal 1P/FCS). Since the Dox release is a proton-sensitive process, the effect of neutralizing the lysosomal pH with NH_4Cl was also investigated in KB-31 cells. These cells were initially treated with 40 $\mu\text{g/ml}$ FITC-labeled MSNP that were simultaneously coated with the 10 kD PEI polymer with or without the addition of 20 mM NH_4Cl . All the images of the particles and fluorescent Dox release were captured by the same confocal microscopy applying same parameter setting over a 72 hour time period.

[00208] In order to determine whether this is possible in KB-V1 cells, the intracellular localization of FITC-labeled MSNP was measured in relation to lysosomal co-staining by fluorescent labeled anti-LAMP-1 antibody. Indeed, confocal microscopy confirmed >55% co-localization of the green-labeled particles with the red-labeled lysosomes (**Figure 20E**). Moreover, in a subsequent confocal study released Dox from PEI-coated particles after entrance into the lysosome could reach the nucleus, which was brightly stained by the fluorescent drug (**Figure 20F, upper panel**). Some of the drug was retained in particles localized in the perinuclear region. This visual image changed completely when cells were treated with NH_4Cl ; most Dox remained confined to the lysosomal compartment with little or no nuclear staining (**Figure 20F, lower panel**). This suggests that the interference in lysosomal acidification by NH_4Cl prevents effective Dox release to KB-V1 nuclei.

Determination of intracellular Dox concentration

[00209] Dox uptake in KB-V1 cells was quantitatively evaluated in a microplate reader at 72 hrs. 5×10^4 cells were placed into a 96 wells plate and treated with 2 $\mu\text{g/ml}$ free Dox or the equivalent amount of drug loaded into MSNP before or after PEI coating, with or without attachment of Pgp siRNA. Following the washing of the cells in cold PBS, the intracellular Dox fluorescence was detected at excitation and emission wavelength of 485/550 nm in a microplate reader (SpectraMax M5 Microplate Reader, Molecular Device, USA). Moreover, confocal images were captured at the end of experiment. Image J software (version 1.37c, NIH) was used to analyze the nuclear fluorescence.

[00210] The relative inefficiency of free Dox to induce KB-V1 cytotoxicity may be a result of the rapid rate by which the drug was being exported by the overexpressed Pgp. Intracellular Dox concentration can be determined by measuring cellular Dox fluorescence intensity in a microplate reader (**Figure 21A**). The comparatively low drug uptake after treatment with free Dox was slightly improved by delivering the drug *via* the phosphonate-MSNP. While the total amount of intracellular drug increased when being delivered by particles coated with the 10 kD PEI polymer (**Figure 21A**), little of the drug reached the nucleus as determined by confocal imaging (**Figure 21B**). Interestingly, the intracellular Dox concentration increased significantly in the presence of siRNA (**Figure 21A**) so that there was also a significant increase in nuclear Dox staining by 72 hr (**Figure 21B**). This contrasts with most of the Dox being confined to the endosomal compartment in cells not receiving siRNA, suggesting that although PEI-MSNP may take more Dox into to cells, any released drug is rapidly extruded from the cell before reaching the nucleus (**Figure 21B**). This suggests that knocking down Pgp expression (**Figure 25**) allows a sufficient quantity of the drug being slowly released from the particle to enter the nucleus where it induces cytotoxicity. A quantitative measurement of Dox release to the nucleus through the use of Image J software confirmed a statistically significant increase of the drug in KB-V1 nuclei after delivery by siRNA-PEI-Dox-MSNP as compared to free Dox or Dox loaded into the particles without siRNA (**Figure 21C**). Dox delivered by PEI-MSNP in the presence of siRNA significantly enhance intranuclear Dox concentration when compared to free Dox or Dox delivered by MSNP or PEI-MSNP without siRNA.

Assessment of cytotoxicity and apoptosis in KB-V1 cells treated with Dox-loaded particles in the absence and presence of Pgp siRNA

[00211] To measure cytotoxicity of the different Dox formulations, KB-V1 cells were treated with free Dox, Dox-MSNP, PEI-Dox-MSNP and siRNA-PEI-Dox-MSNP, respectively. For the latter two particle types, incubation time was for 16 hrs before replenishment of the old medium with fresh complete DMEM and performance of a MTS assay at 72 hrs. Based on the absorption readout at 490 nm, the IC₅₀ of free and Dox-loaded MSNP were calculated. The induction of apoptosis at 72 hours was assessed through the use of Annexin V-SYTOX Blue. Briefly, 5×10^5 cells were harvested and stained by FITC-Annexin V- SYTOX Blue working solution (Annexin V, Trevigen; SYTOX Blue, Invitrogen) at room temperature for 15 min. The cells were washed in binding buffer before performance of flow cytometry (Becton Dickinson, Mountain View, CA). Data analysis was performed by BD CellQuest. To confirm the flow data in which there may be a minor overlap of Dox with FITC-Annexin V, a TUNEL detection kit was used according to the manufacturer's instructions to confirm the induction of apoptosis. Briefly, 72 hrs following treatment with free Dox or Dox loaded particles, cells were washed, fixed, and permeabilized before TUNEL staining. The number of TUNEL-positive cells was assessed under a fluorescent microscope (200×). At least 3 fields were counted by the same investigator to calculate the percentage of TUNEL positive cells.

[00212] In order to reconcile these findings with improved cell killing, the MTS assay was used to compare KB-V1 cytotoxicity under incremental Dox concentrations (**Figure 22A**). Based on the calculated IC₅₀ values of the various formulations, it was possible to rank the killing efficiency as follows: siRNA-PEI-Dox-MSNP > PEI-Dox-MSNP $\approx$ Dox-MSNP > free Dox. Moreover, the IC₅₀ value of the siRNA-delivering MSNP was approximately 2.5 times lower than the IC₅₀ of free Dox or other Dox-loaded particles. This suggests an additive effect between the drug and the siRNA that are being delivered by MSNP. Although it was not possible to restore drug sensitivity to the level seen in KB-31 cells, a much higher percentage of apoptotic KB-V1 cells were observed after treatment with Dox-loaded MSNP that co-delivers siRNA as compared to free Dox or Dox delivered by non-siRNA delivering particles (**Figure 22B**). Flow cytometry data was confirmed through the use of another apoptosis assay using TUNEL staining (**Figure 29**), which showed an identical trend. Failure to fully restore drug sensitivity could be due to extremely high levels of Pgp expression and/or additional drug resistant pathways in KB-V1 that were not targeted in the current research. Because of the

duration of time it takes for the siRNA to exert its effect, cell viability was analyzed after longer treatment periods (*e.g.* 96 hrs) but did not observe any improvement in cytotoxicity (**Figure 30**). In addition, KB-V1 cells were treated by co-administration of Dox and Tariquidar, which is a potent and effective Pgp inhibitor in human clinical trial, to see if KB-V1 sensitivity can be restored to that seen in KB-31 cells. While this combination significantly improves cell killing capability in KB-V1 cells, it is incapable of completely restoring Dox sensitivity to the level seen in KB-31 cells (**Figure 31**).

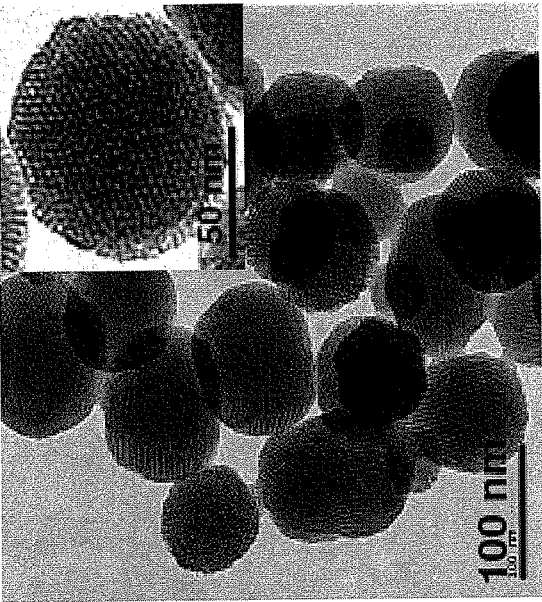
[00213] The embodiments illustrated and discussed in this specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention. Figures are not drawn to scale. In describing embodiments of the invention, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. The above-described embodiments of the invention may be modified or varied, without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. It is therefore to be understood that, within the scope of the claims and their equivalents, the invention may be practiced otherwise than as specifically described.

WE CLAIM:

1. A submicron structure, comprising:
a silica body defining a plurality of pores and an outer surface between pore openings of said plurality of pores,
a cationic polymer on the surface of said silica body, and
wherein said submicron structure has a maximum dimension less than one micron.
2. The submicron structure of claim 1, further comprising a cationic therapeutic compound and an oligonucleotide electrostatically bound to the cationic polymer wherein the oligonucleotide is an siRNA that reduces translation of a protein causing resistance in a cell.
3. The submicron structure of claim 2, wherein the siRNA reduces translation of a protein that causes resistance to said therapeutic compound in the cell.
4. The submicron structure of claim 2, wherein the siRNA reduces translation of p-glycoprotein.
5. The submicron structure of claim 2, wherein the cationic therapeutic compound is doxorubicin.
6. The submicron structure of claim 1, wherein the cationic polymer is electrostatically bound to the silica body.
7. The submicron structure of claim 1, wherein the cationic polymer is selected from the group consisting of polyethyleneimine, polyamidoamine, polylysine, poly(allylamine), and poly(diallyldimethylammonium chloride).
8. The submicron structure of claim 1, wherein the cationic polymer is polyethyleneimine.
9. The submicron structure of claim 1, comprising an oligonucleotide electrostatically bound to said cationic polymer.

10. The submicron structure of claim 9, wherein the oligonucleotide is DNA.
11. The submicron structure of claim 9, wherein the oligonucleotide is RNA.
12. The submicron structure of claim 11, wherein the RNA is siRNA.
13. The submicron structure of claim 1, comprising a therapeutic compound within the silica body or pores of the silica body.
14. The submicron structure of claim 13, wherein the therapeutic compound is hydrophobic.
15. The submicron structure of claim 13, wherein the therapeutic compound is cationic.
16. The submicron structure of claim 13, wherein the therapeutic compound is anionic.
17. The submicron structure of claim 1, wherein the silica body is mesoporous.
18. The submicron structure of claim 1, wherein the pores are substantially cylindrical pores having an ensemble average diameter between about 1 nm and about 10 nm.
19. The submicron structure of claim 1, wherein the silica body is substantially spherical having a diameter between about 50 nm and about 1000 nm.
20. The submicron structure of claim 1, wherein the silica body is substantially spherical having a diameter between about 100 nm and about 500 nm.
21. The submicron structure of claim 1, further comprising a plurality of anionic molecules attached to an outer surface of said silica body.
22. A submicron structure according to claim 21, wherein said plurality of anionic molecules comprise a phosphonate moiety.

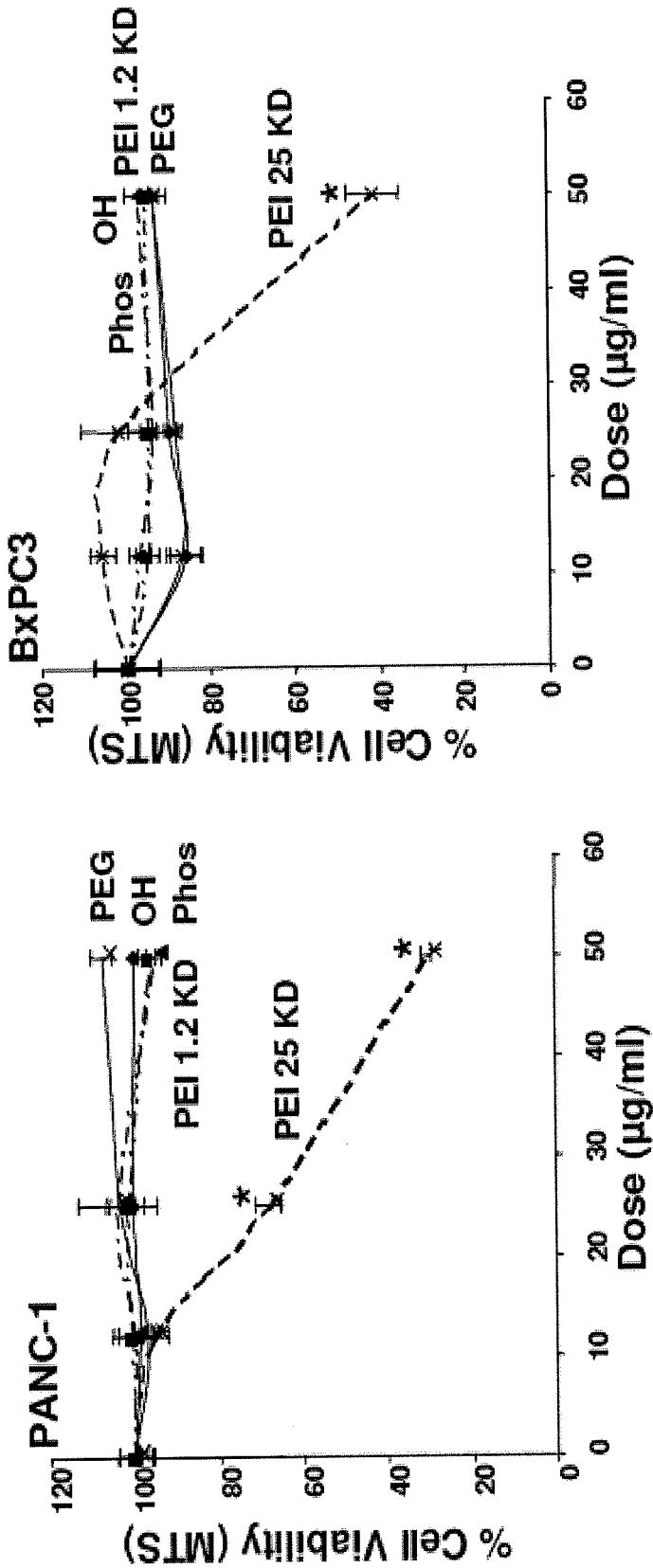
23. A submicron structure according to claim 21, wherein said plurality of anionic molecules are trihydroxysilylpropyl methylphosphonate.
24. The submicron structure according to claim 1, further comprising a light-emitting compound, peptide, protein, oligonucleotide, sugar, oligosaccharide, or polysaccharide covalently bonded to the surface of the silica body.
25. The submicron structure according to claim 1, further comprising a light-emitting compound covalently bonded to the surface of the silica body.
26. The submicron structure of claim 1 further comprising a core structure within said silica body.
27. The submicron structure of claim 26, wherein said core structure is a superparamagnetic nanocrystal, silver nanocrystal, or gold nanocrystal.
28. The submicron structure of claim 27, wherein the superparamagnetic nanocrystal is an iron oxide nanocrystal.
29. A therapeutic method comprising administering to a subject in need of treatment an effective amount of a submicron structure according to any one of claims 1-28.
30. The method of claim 29 for treating drug resistant cancer wherein the submicron structure comprises an siRNA that reduces translation of a protein causing resistance in the drug resistant cancer.
31. A method for transfecting a cell comprising administering to the cell a submicron structure according to any one of claims 1-28 comprising an oligonucleotide.



1A

Figure 1

1B



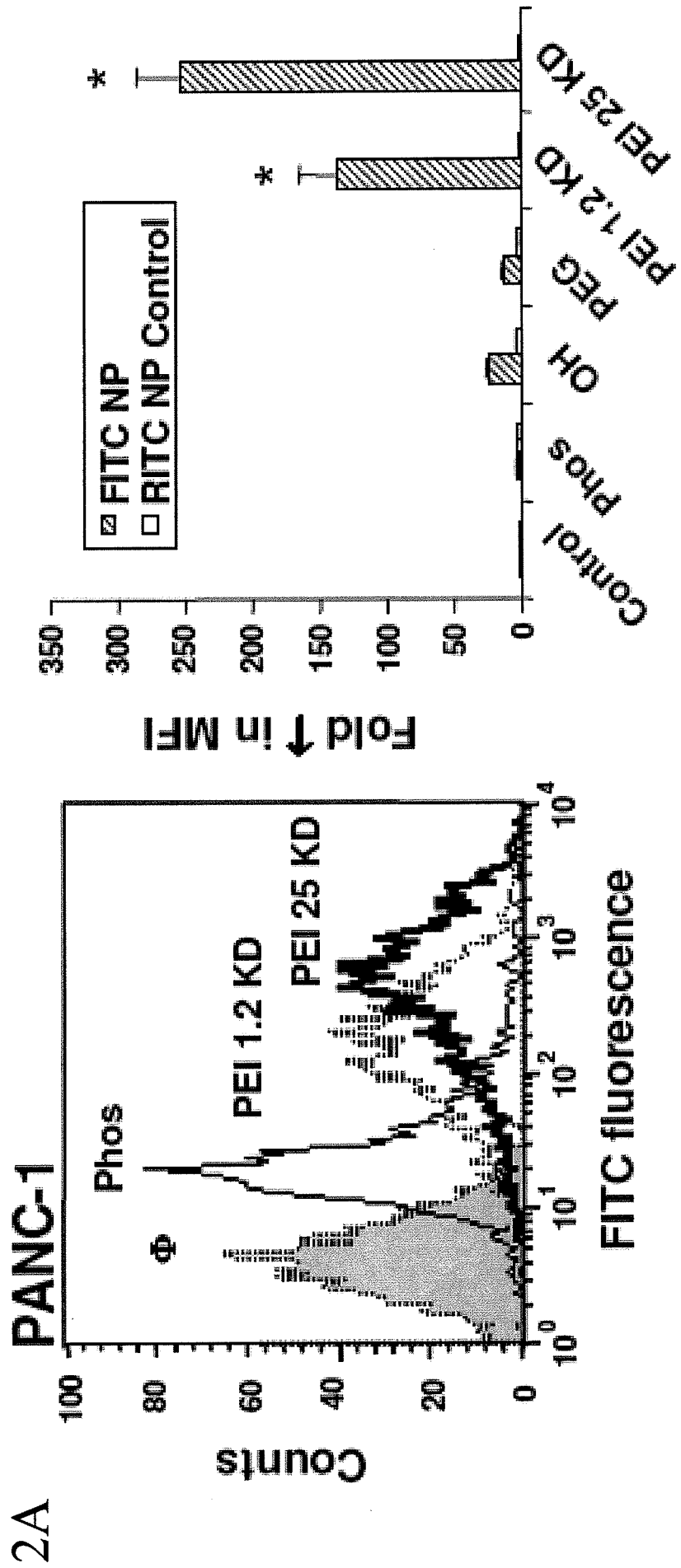


Figure 2

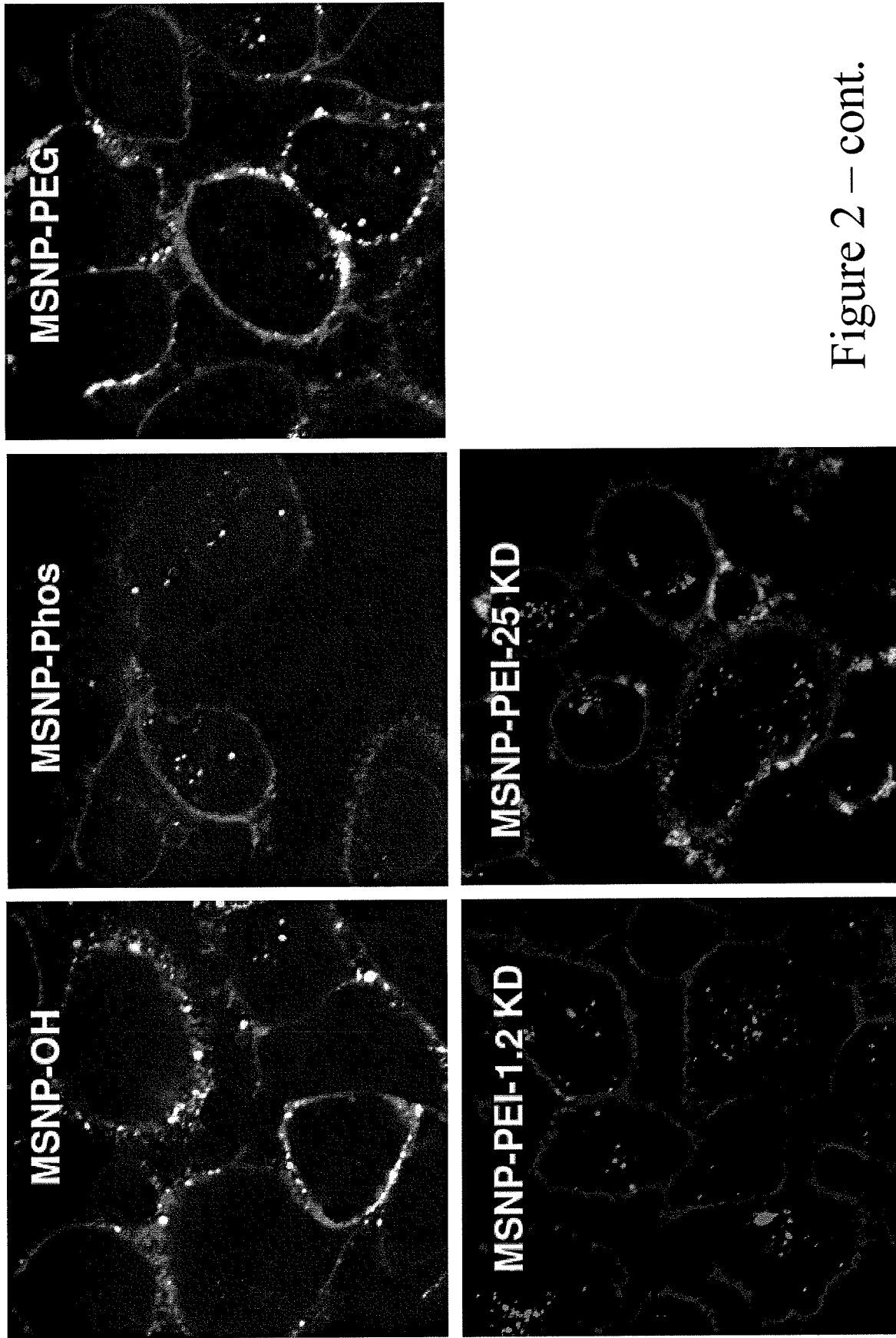


Figure 2 – cont.

PANC-1

2B

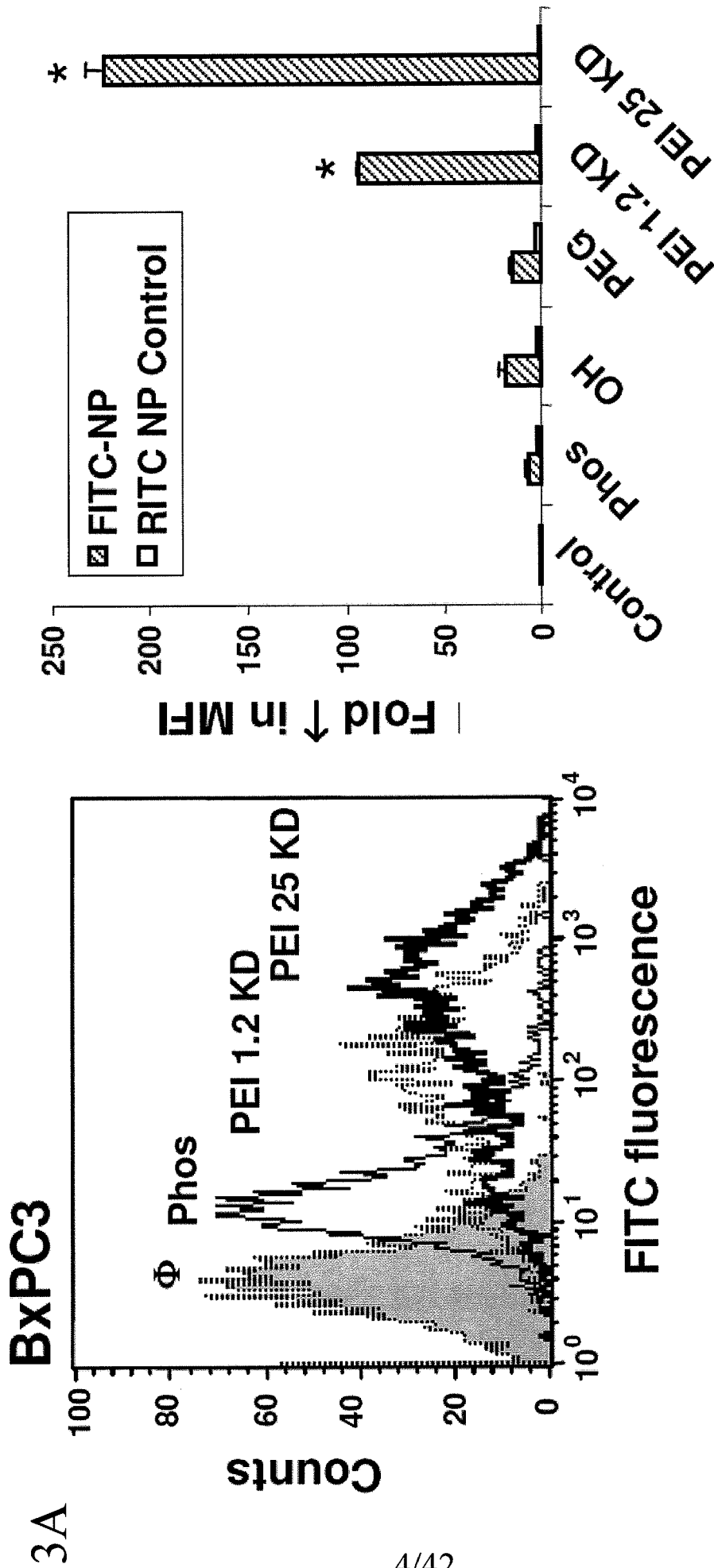


Figure 3

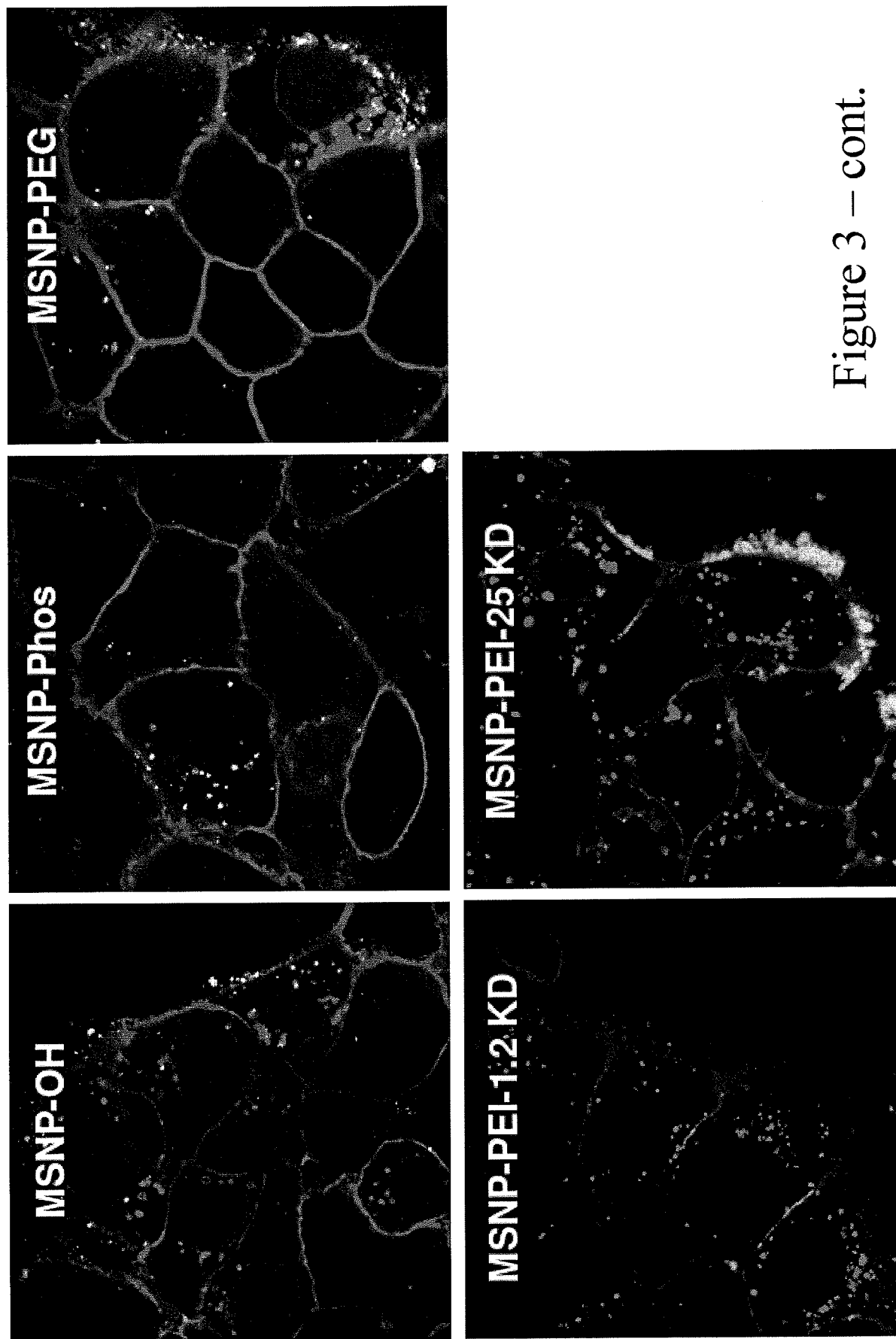
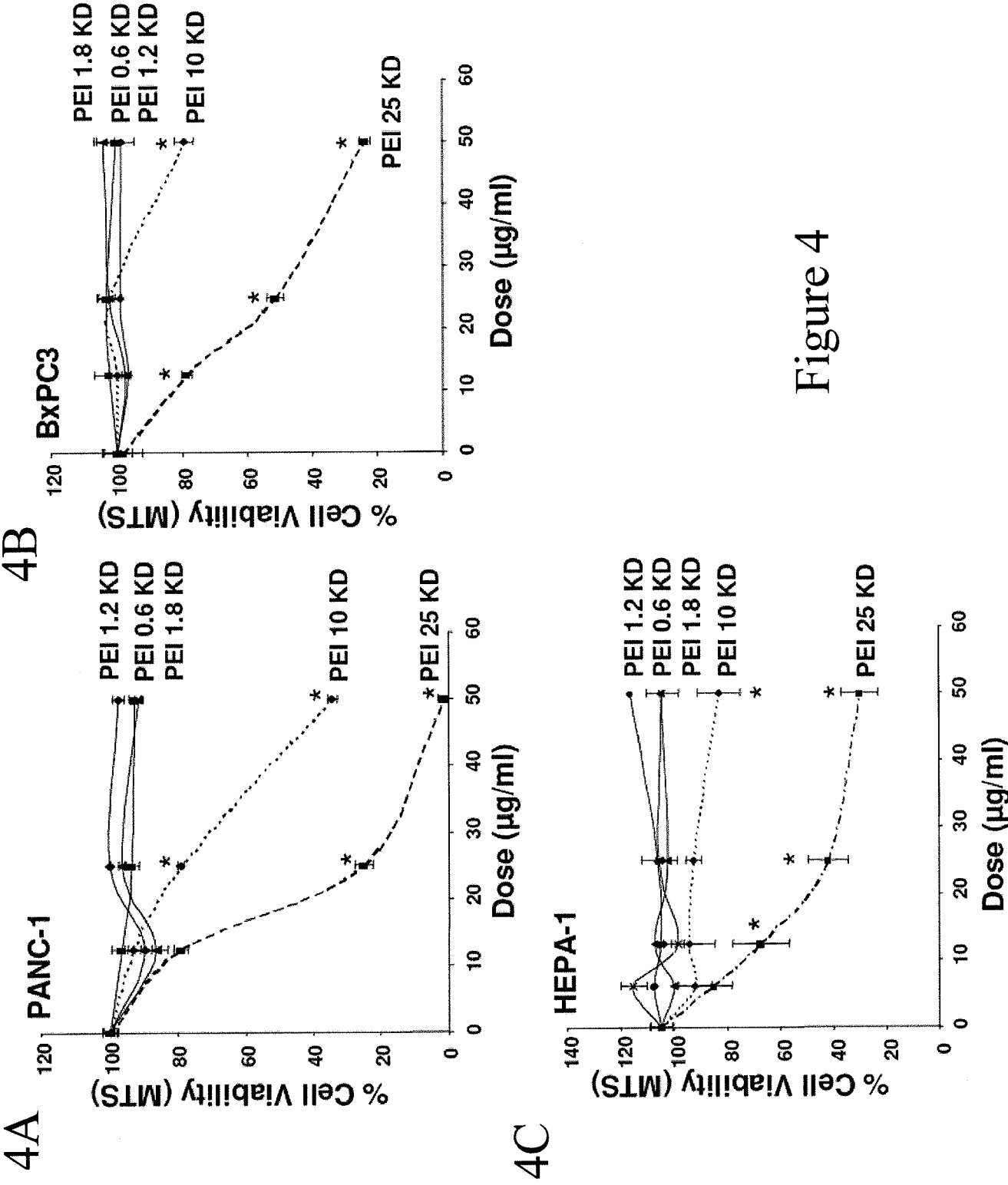


Figure 3 – cont.

3B

BxPC3



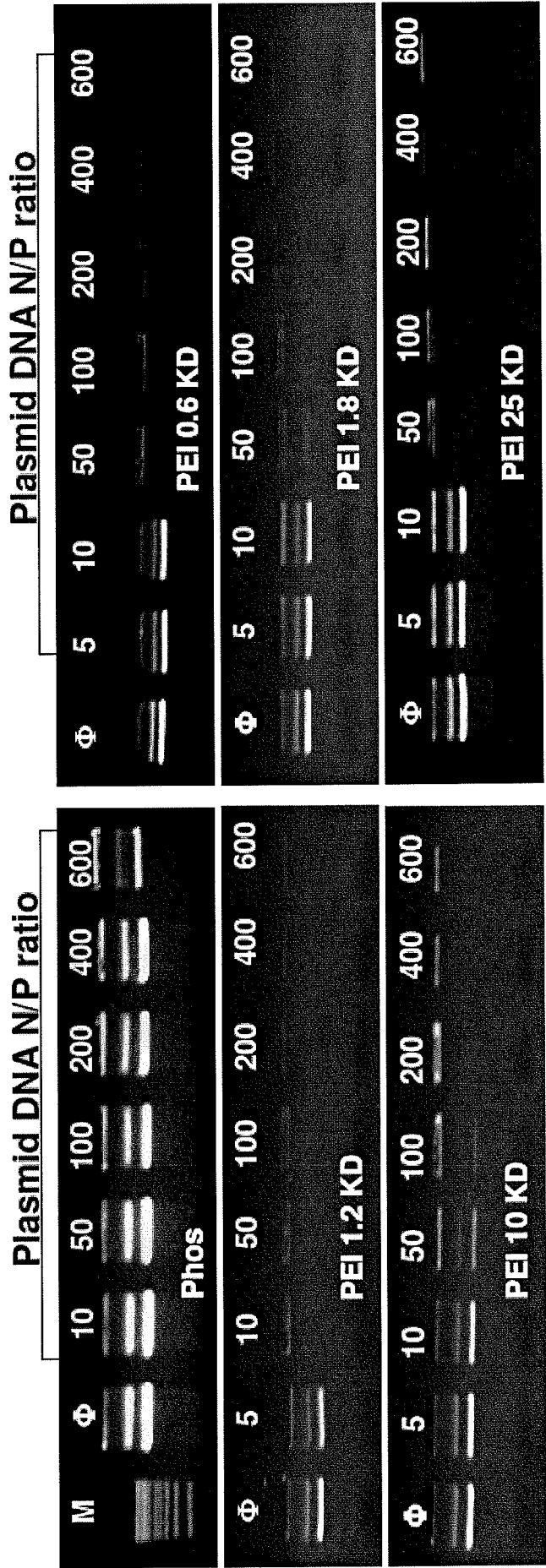


Figure 5

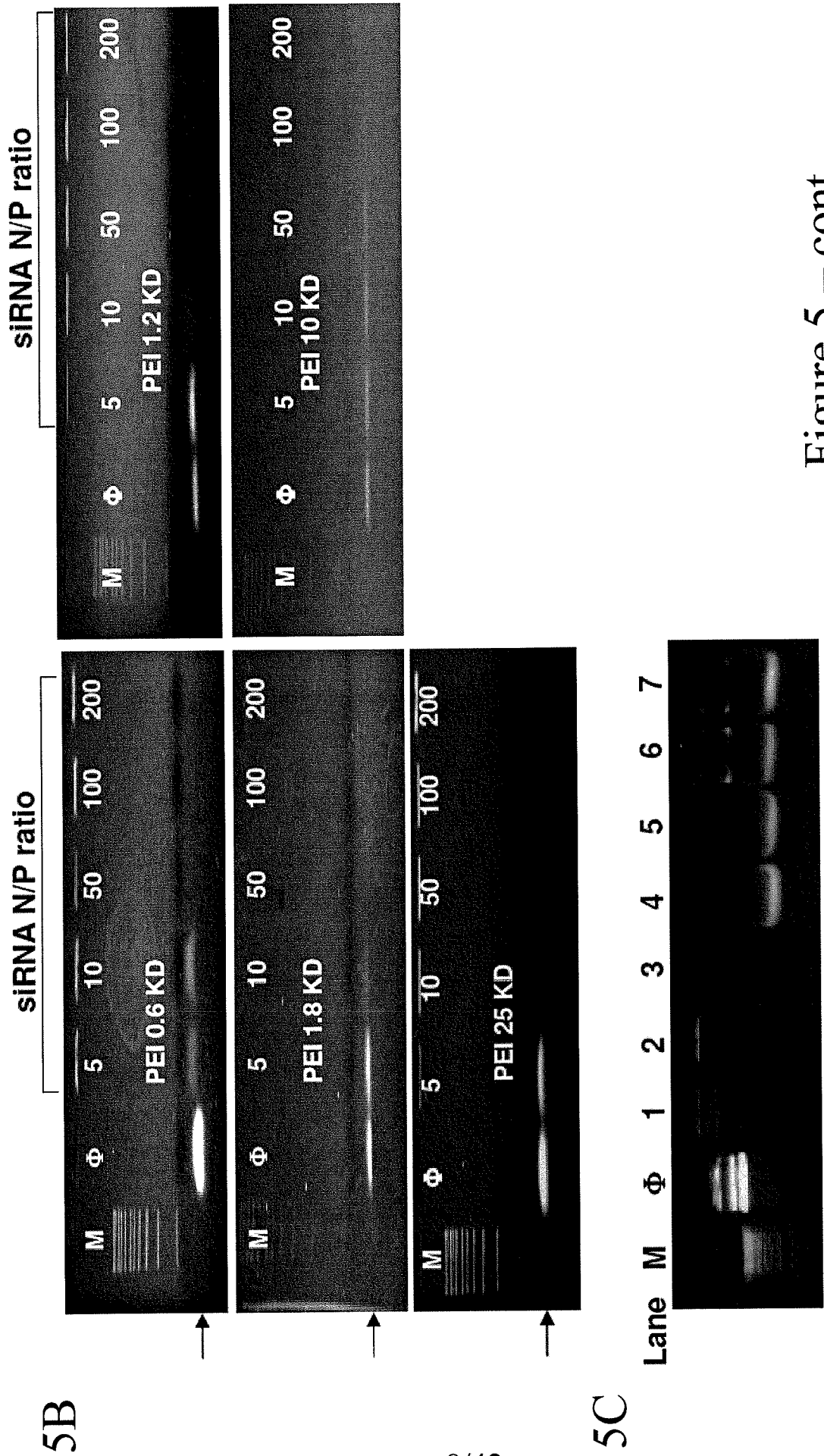


Figure 5 – cont.

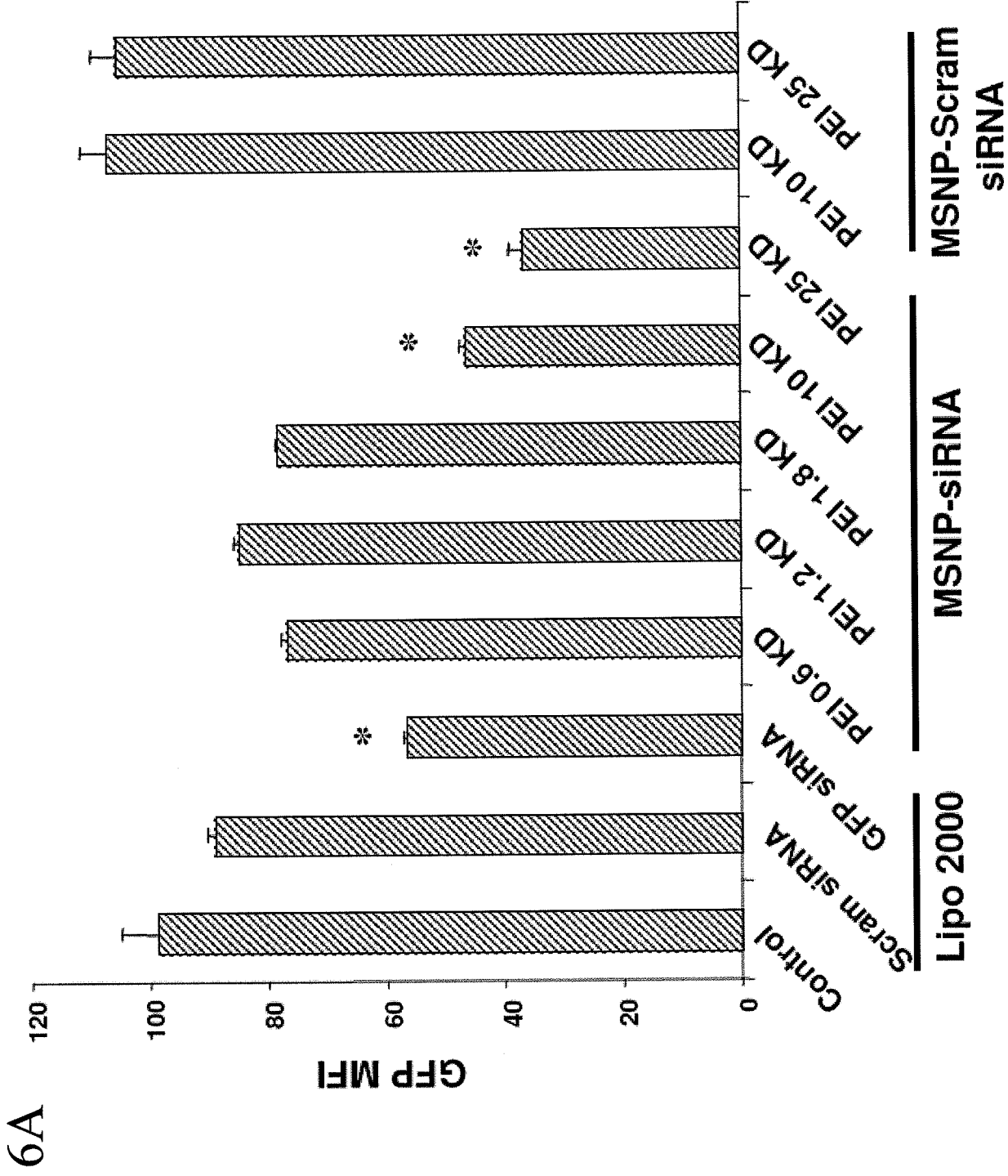


Figure 6

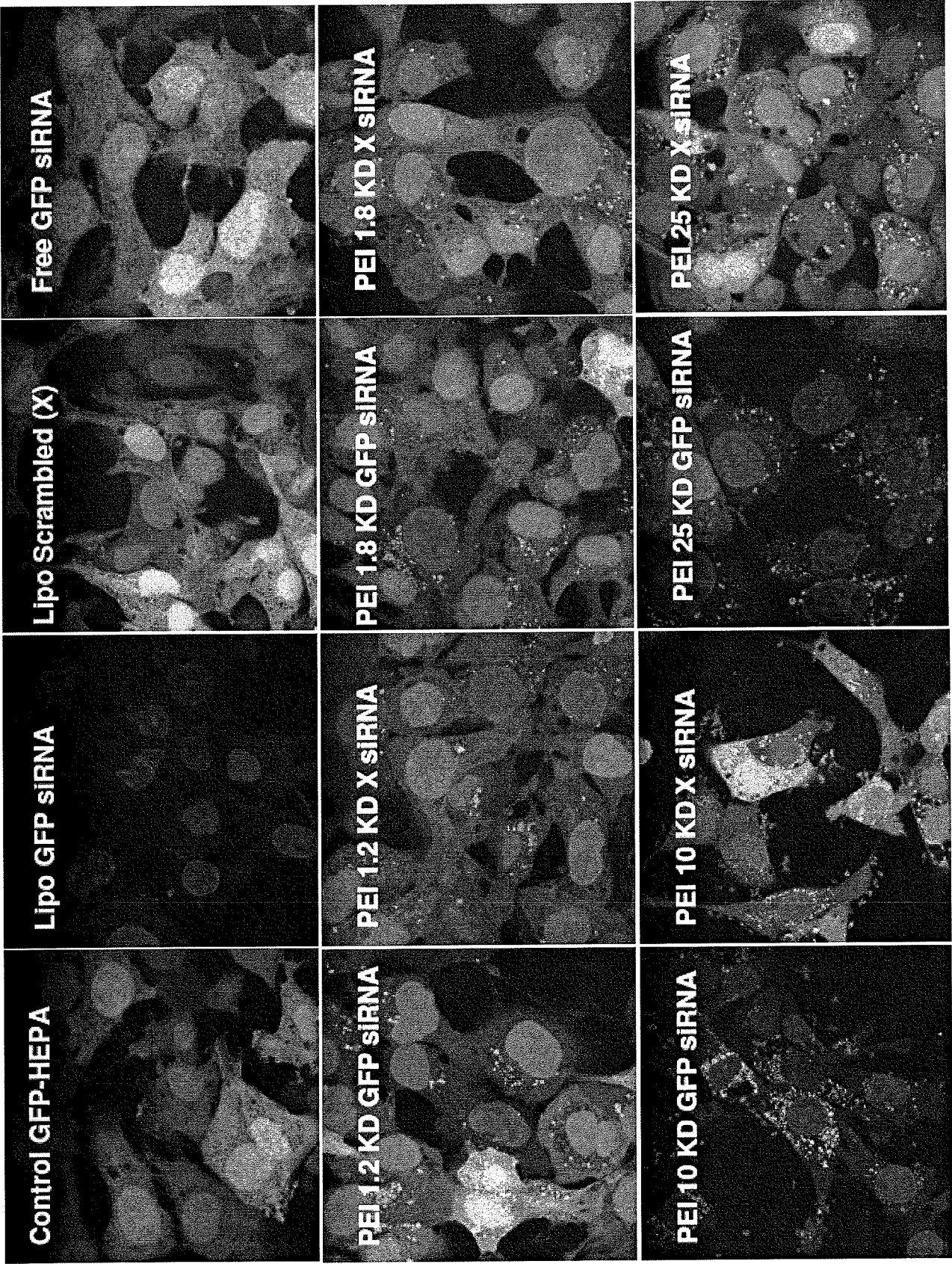
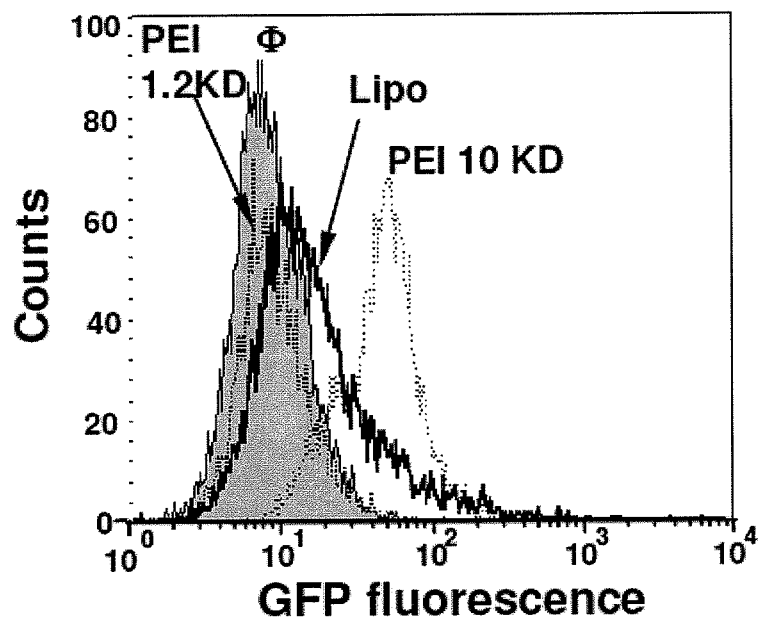


Figure 6 – cont.

7A



7B

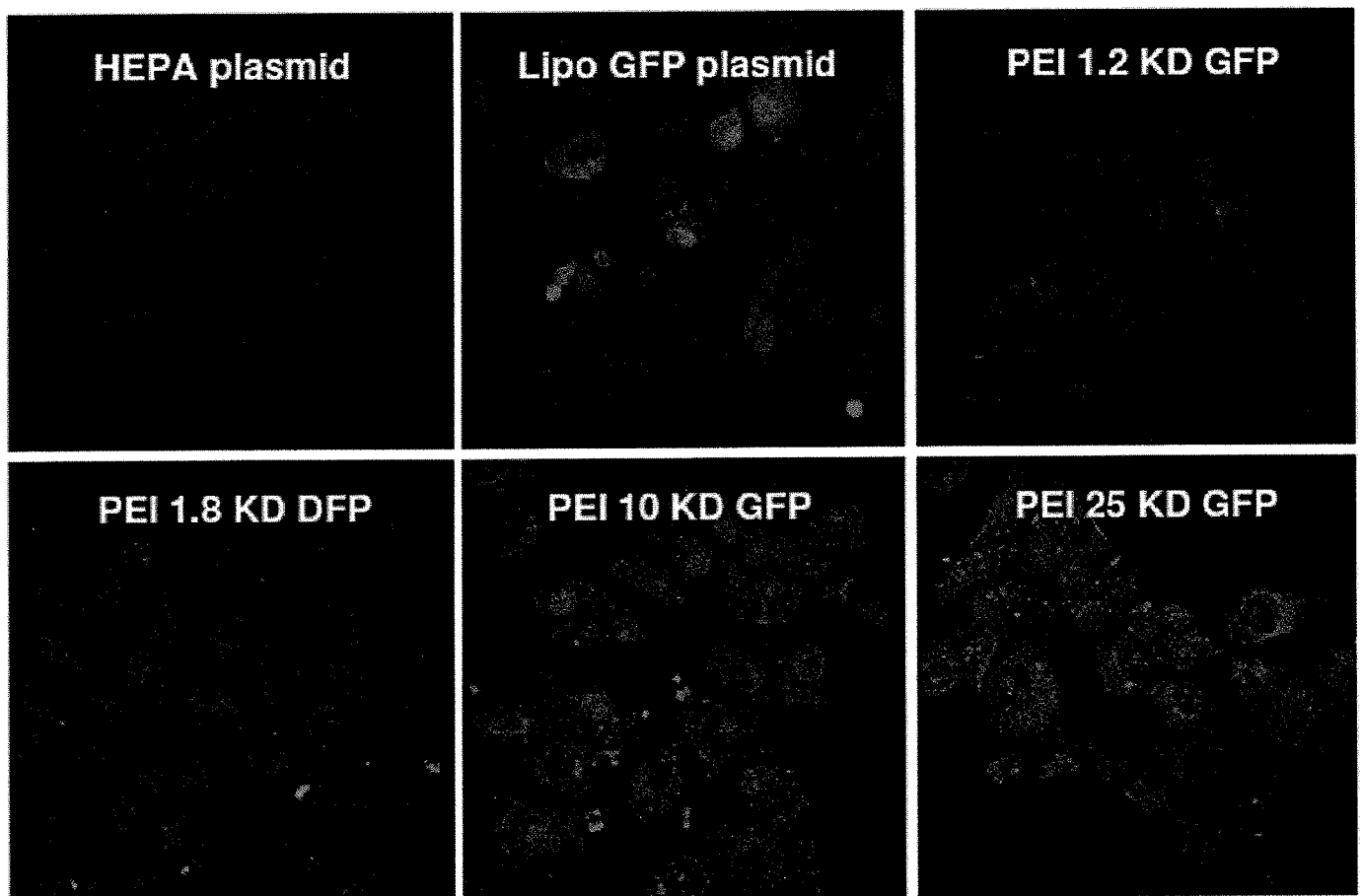


Figure 7

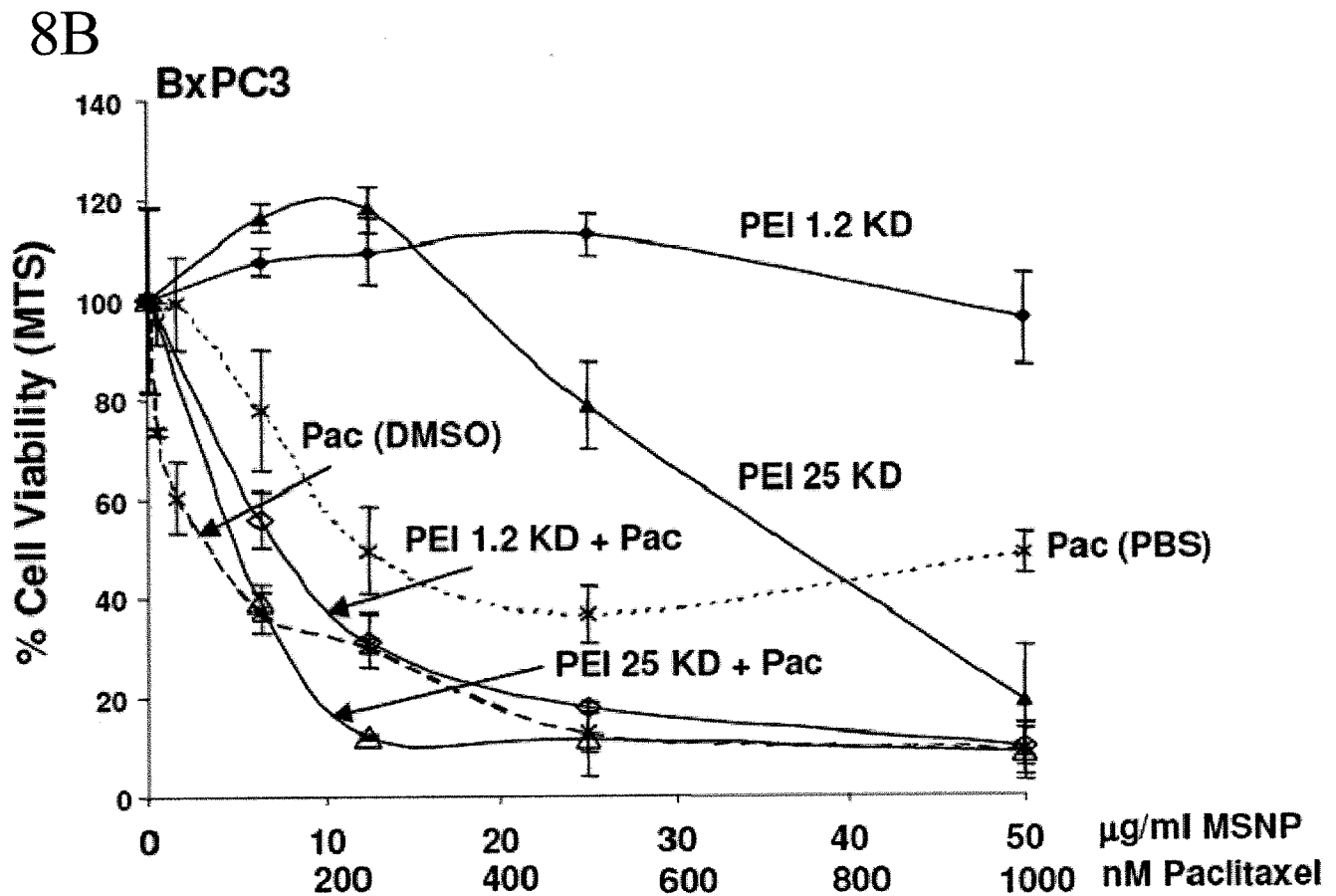
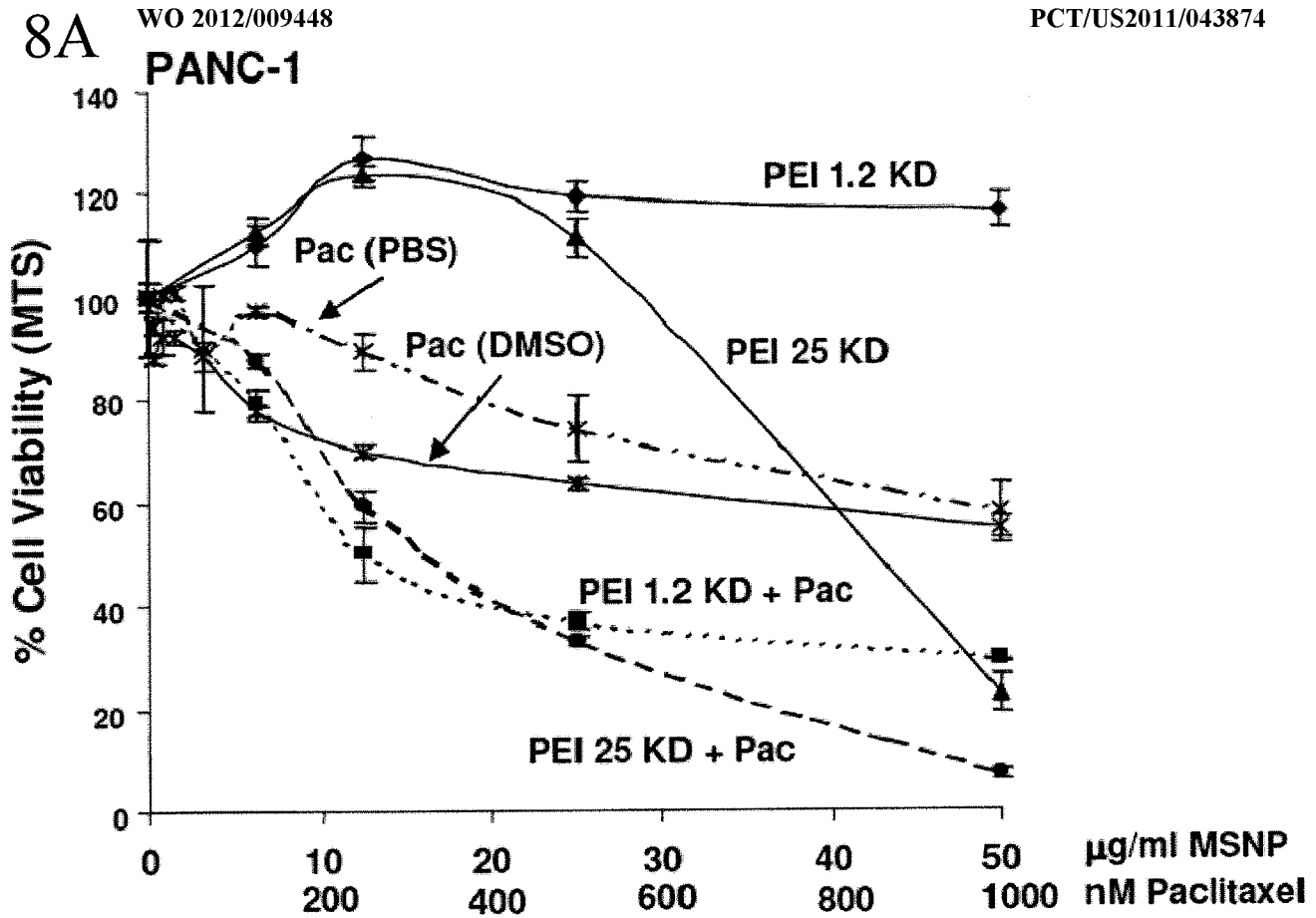


Figure 8

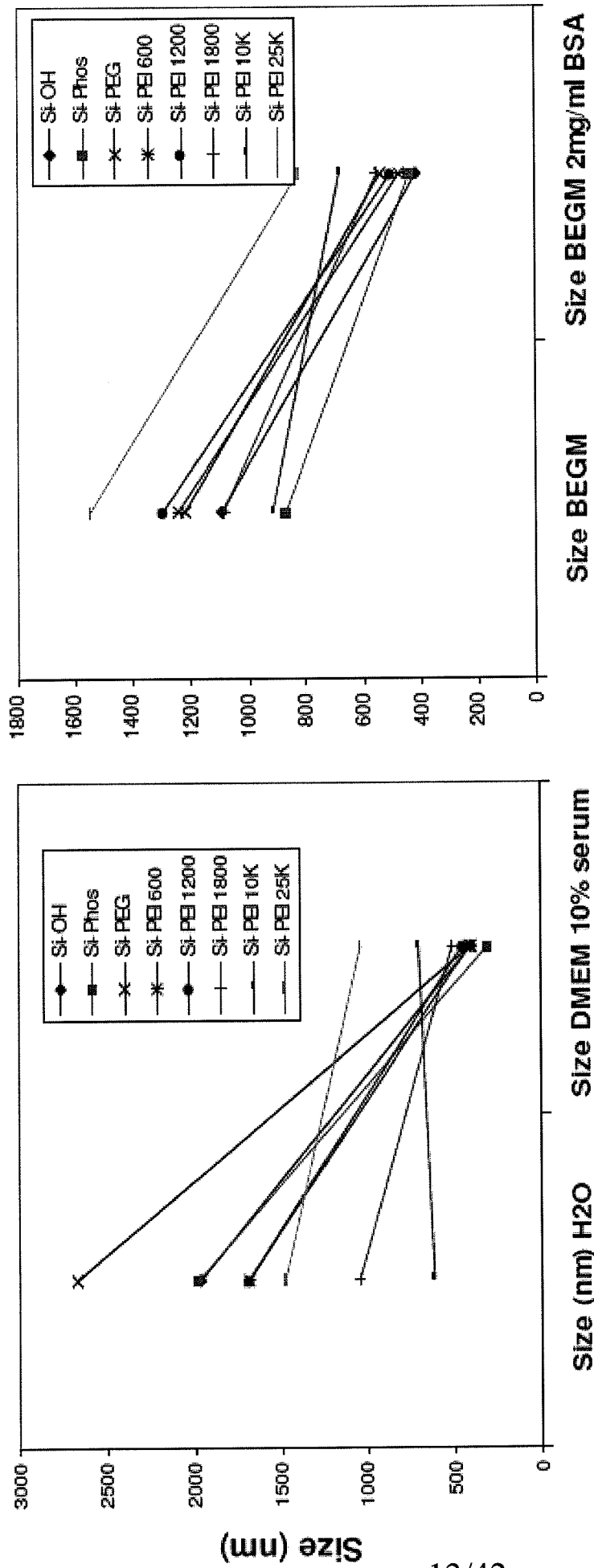


Figure 9

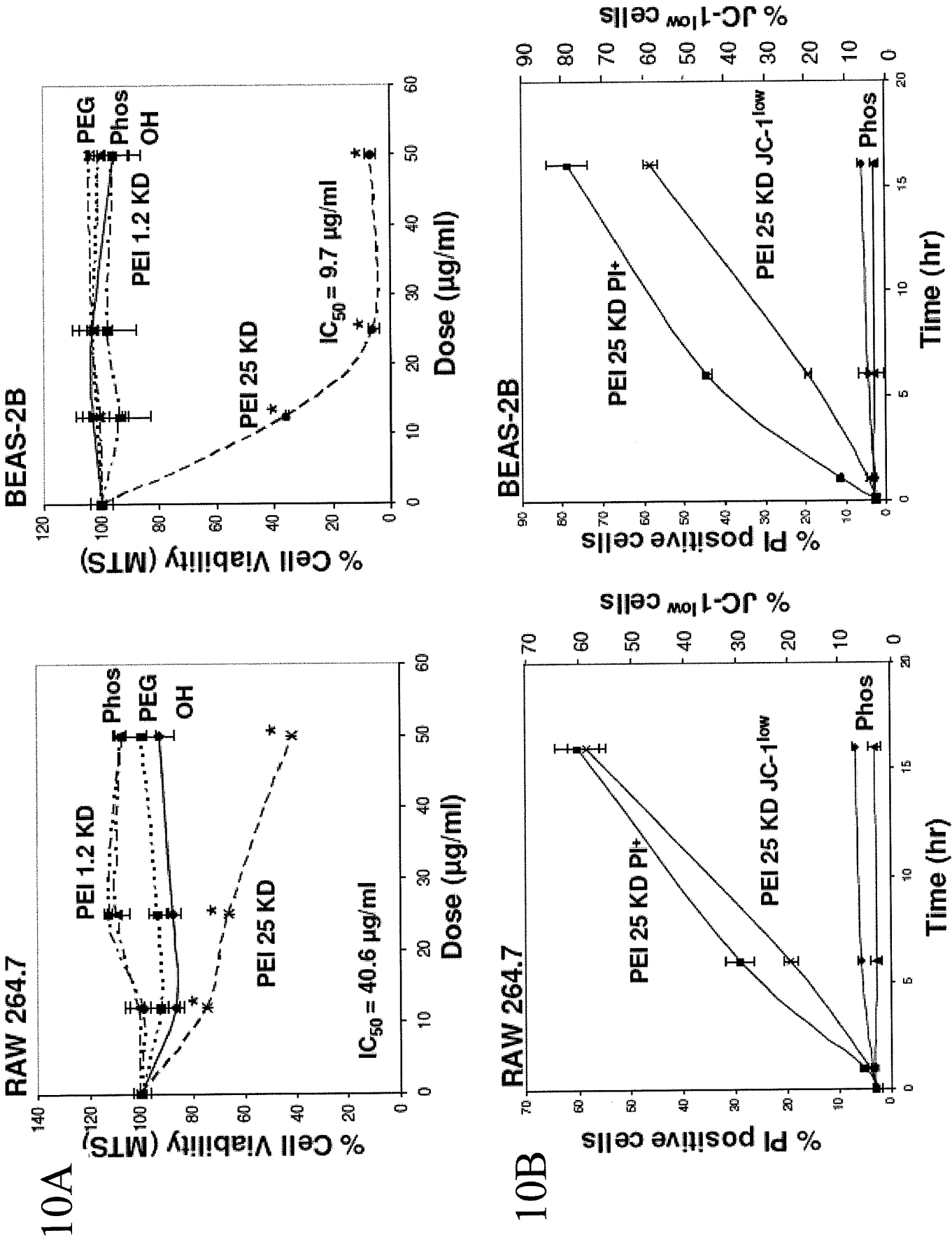


Figure 10

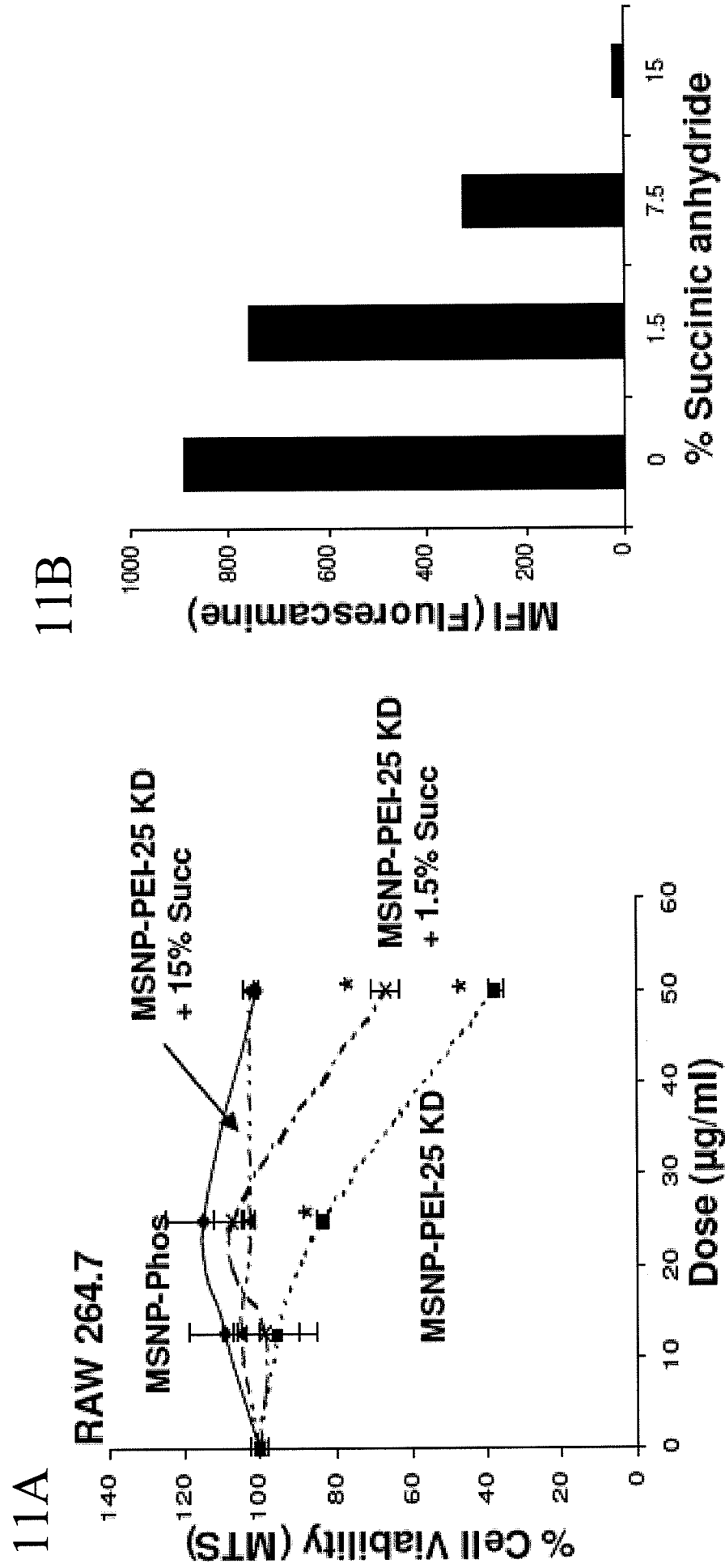


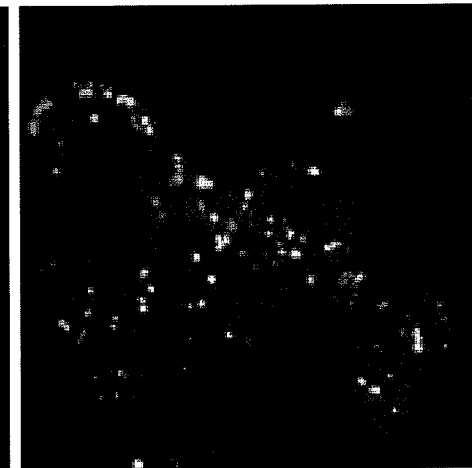
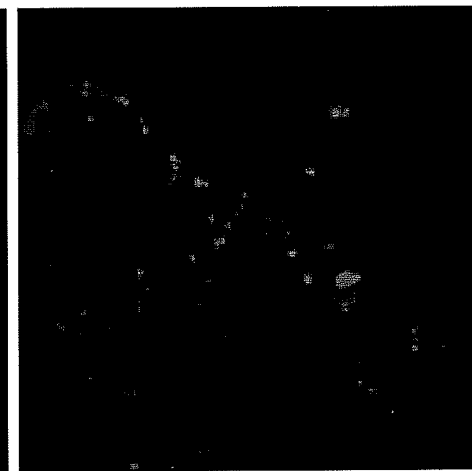
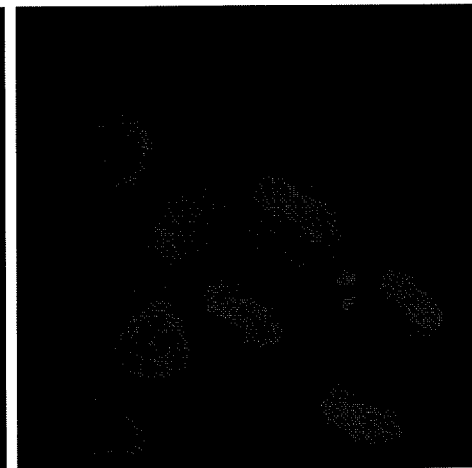
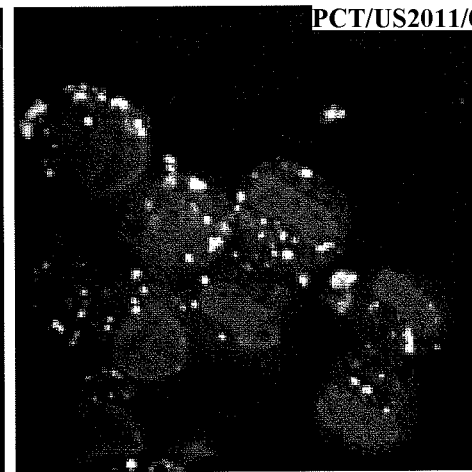
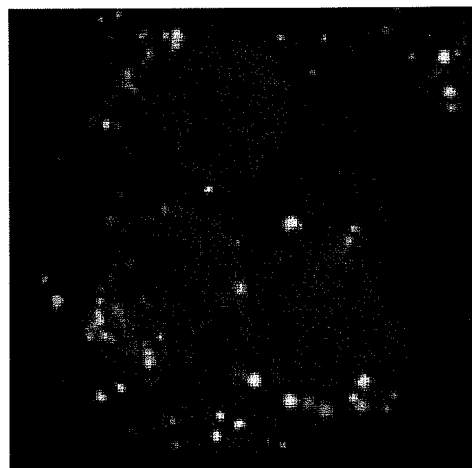
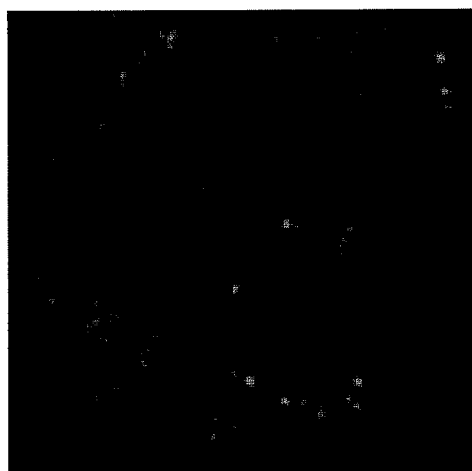
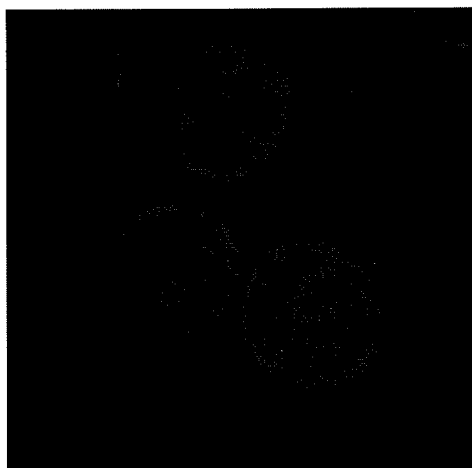
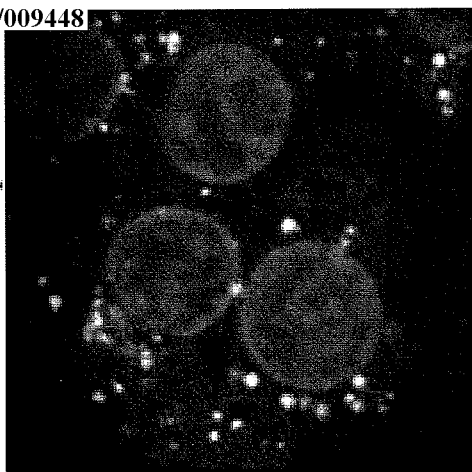
Figure 11

Overlay

Blue: Nucleus

Red: PEI

Green: MSNP



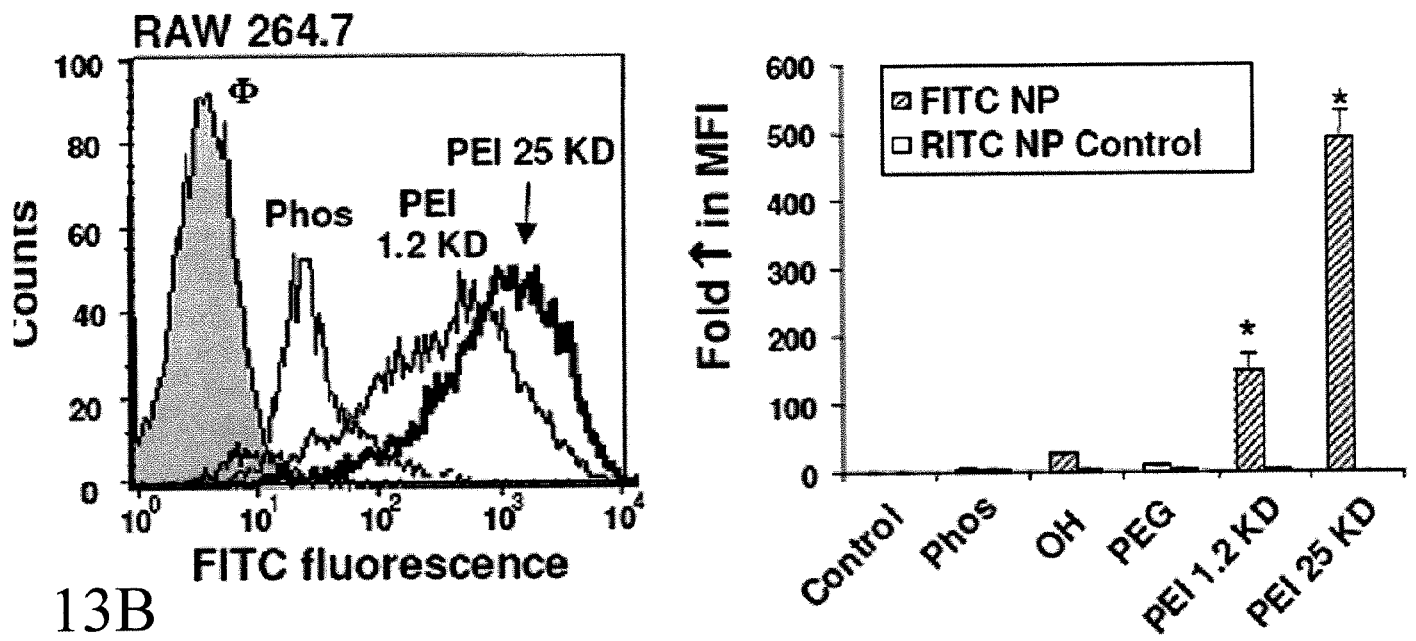
3 hr

16/42

6 hr

Figure 12

13A



13B

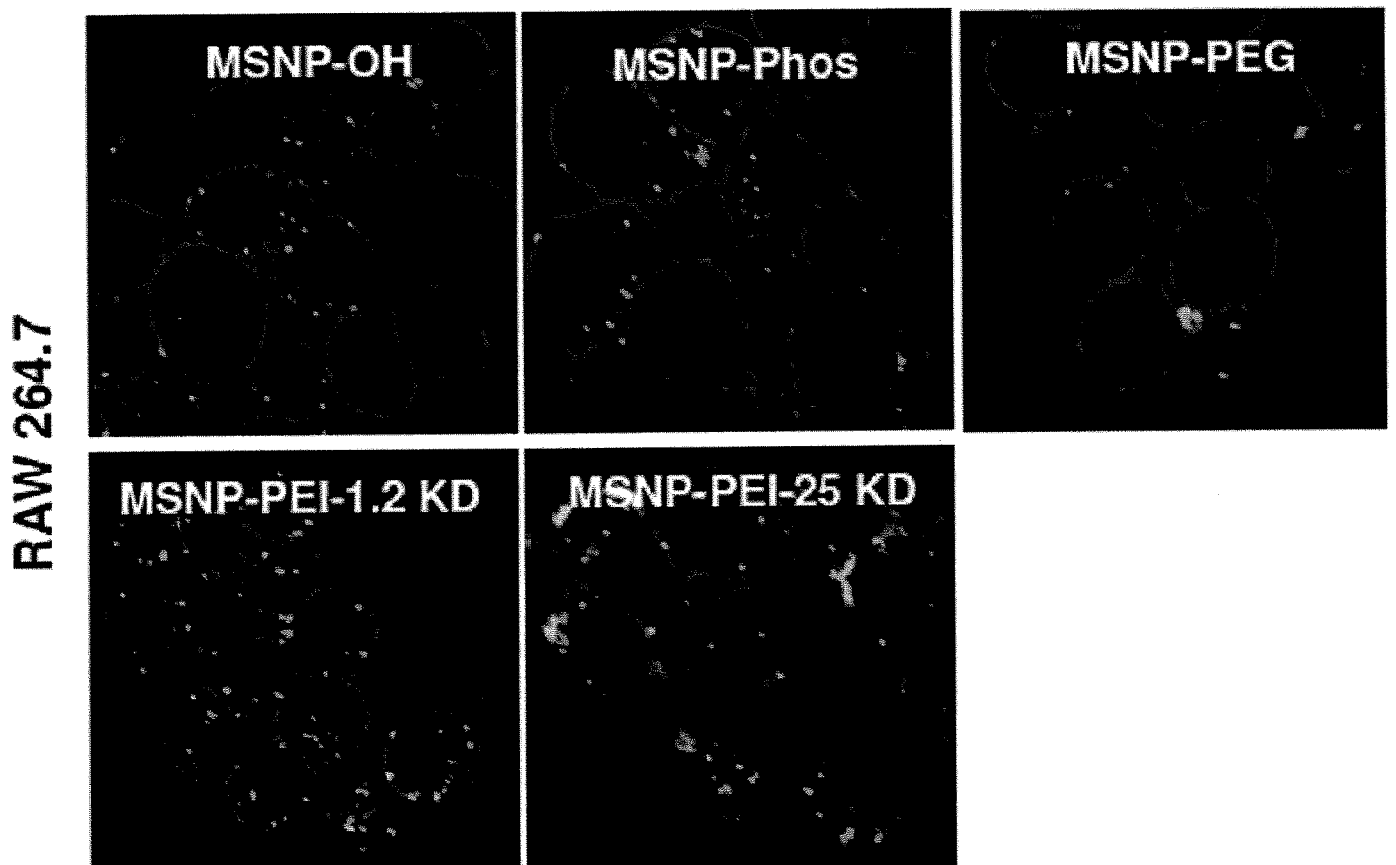


Figure 13

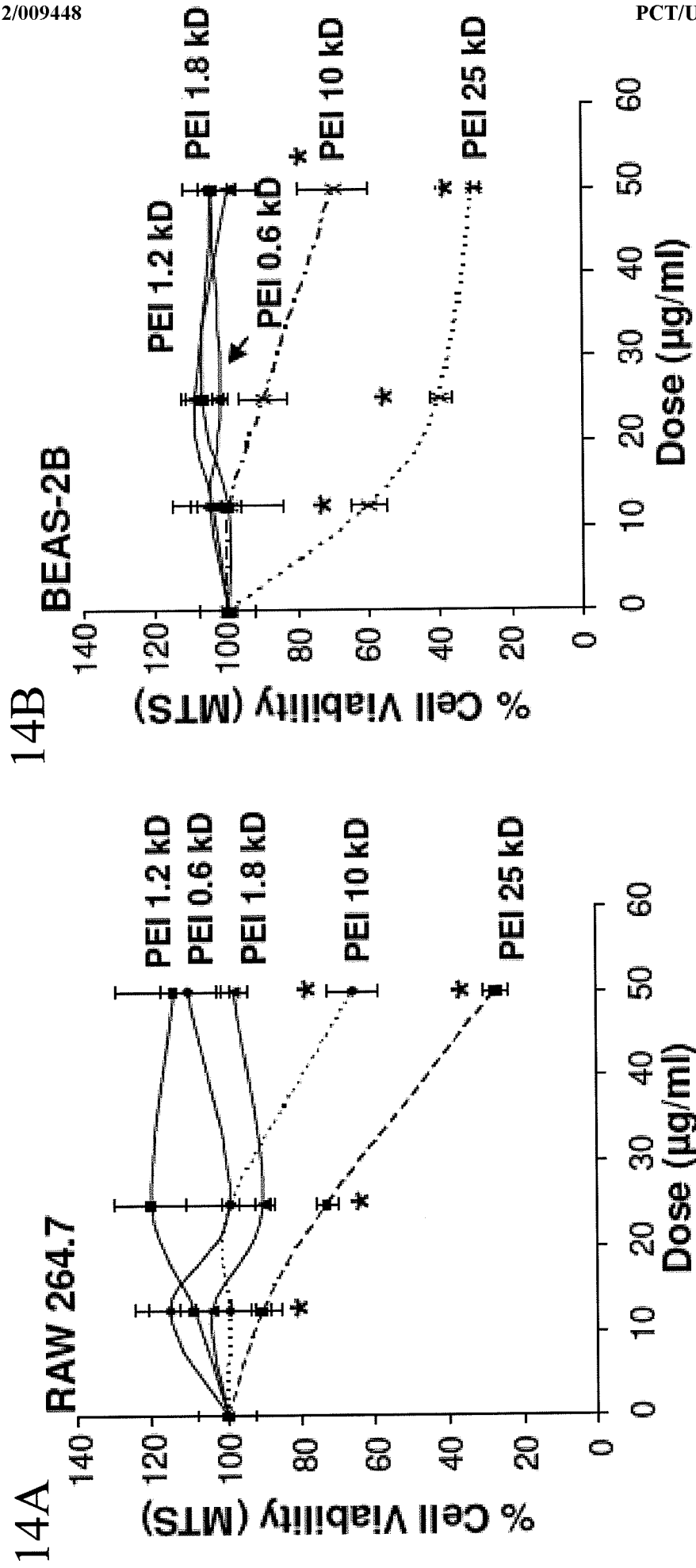
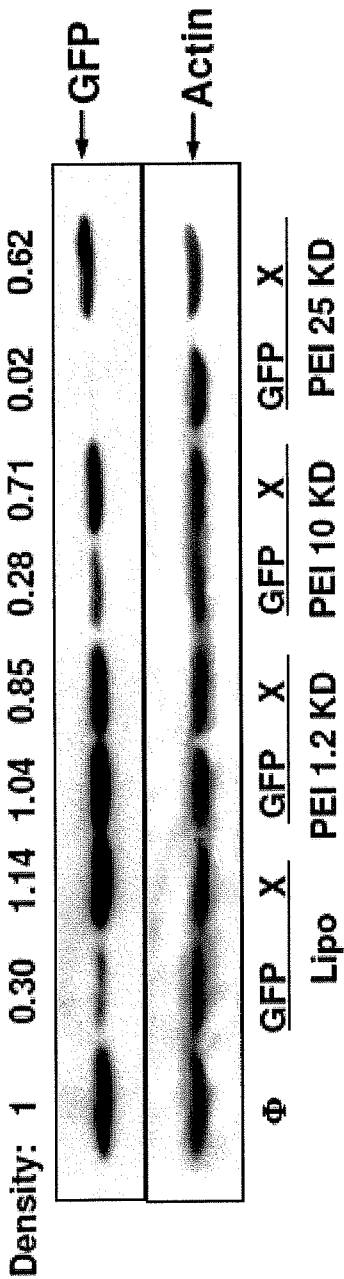


Figure 14

15A



15B

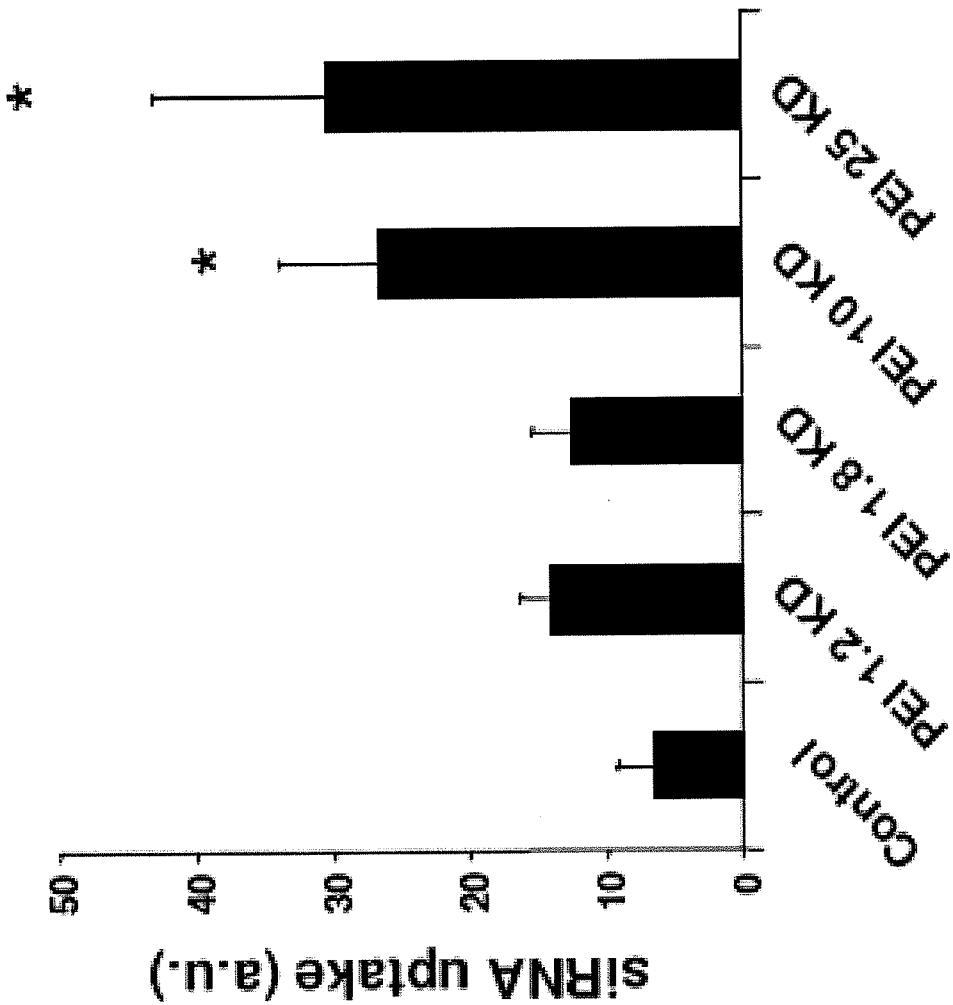


Figure 15

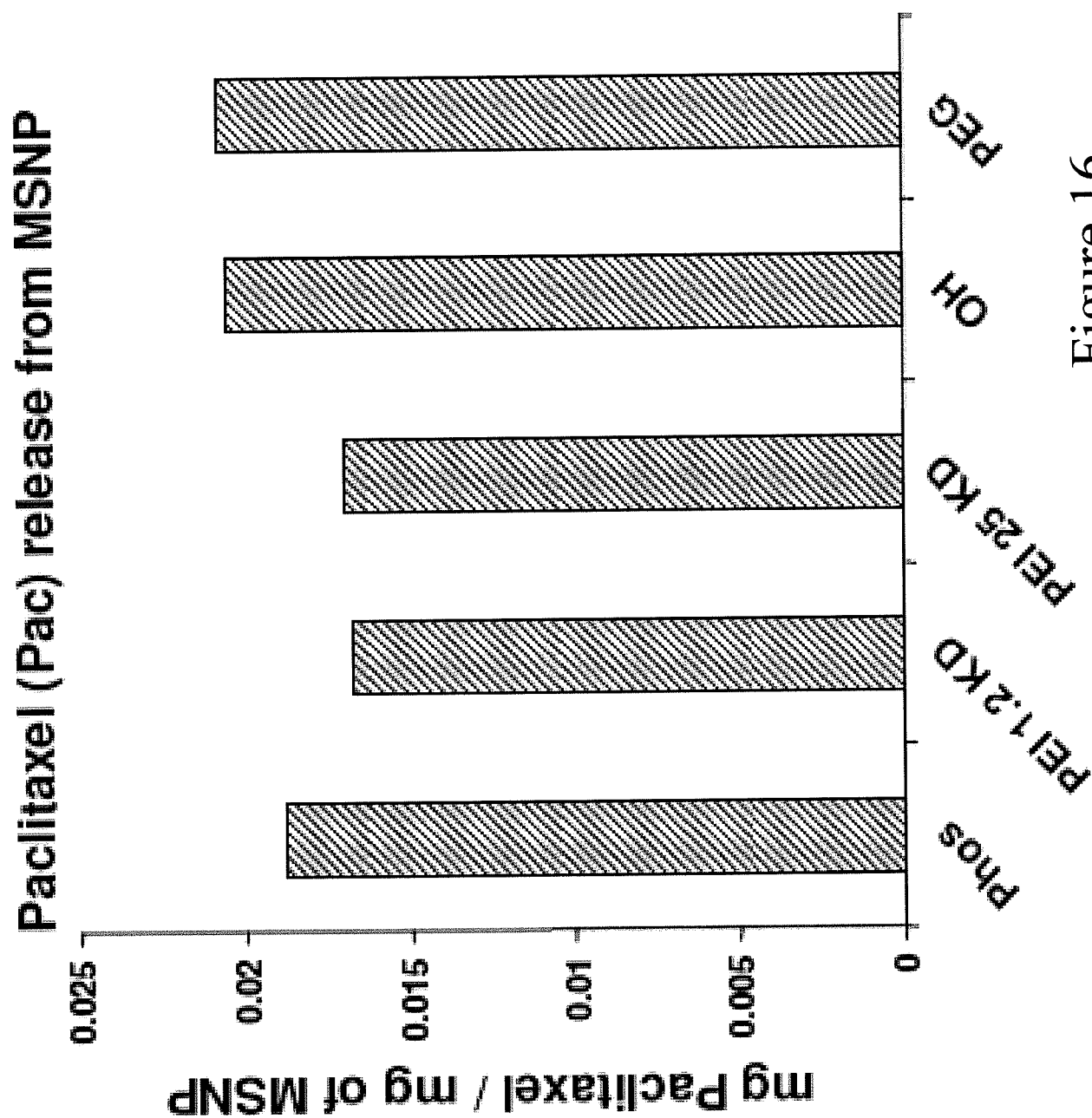
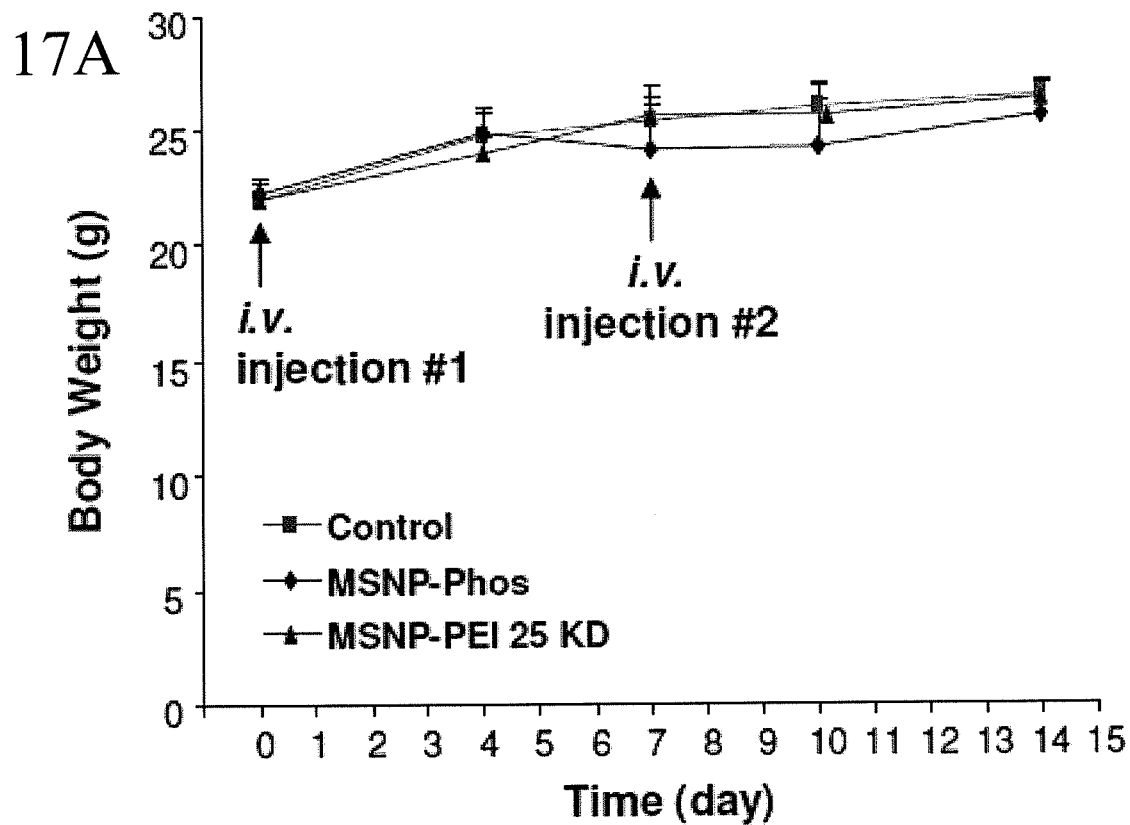


Figure 16



17B

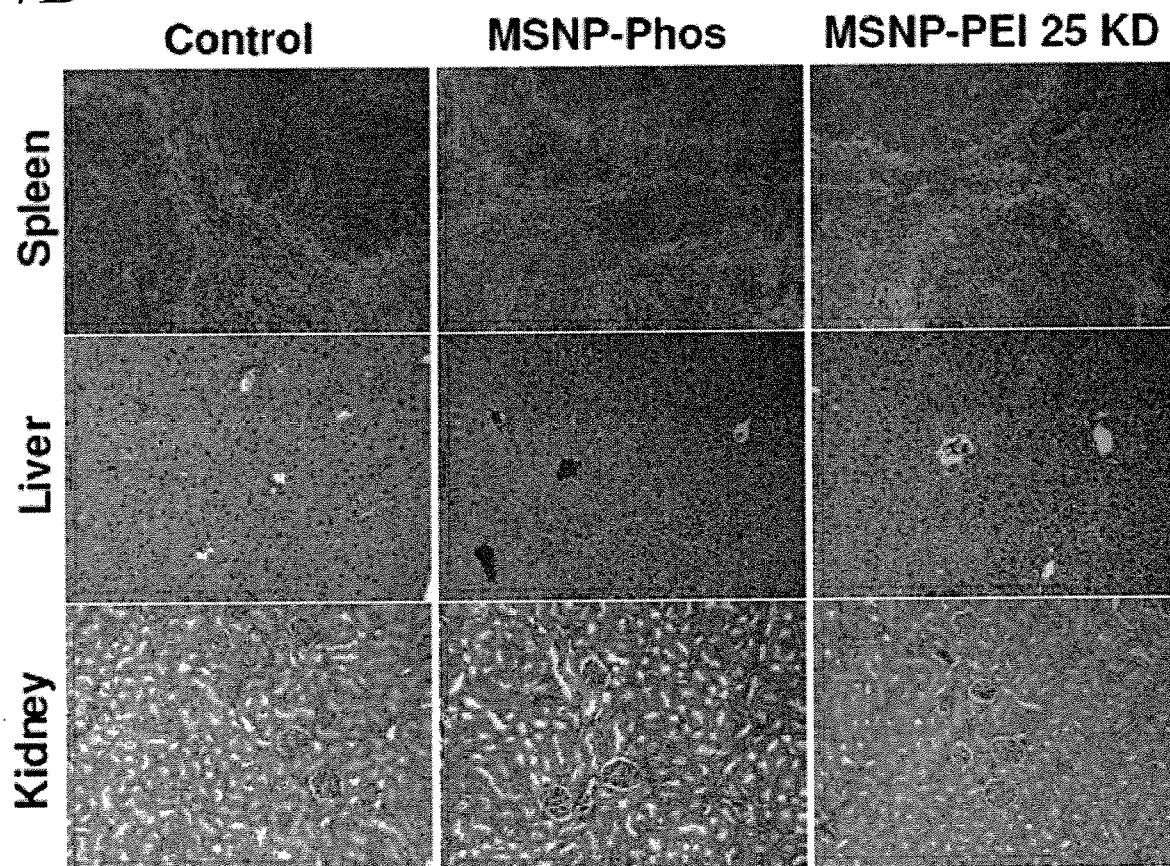
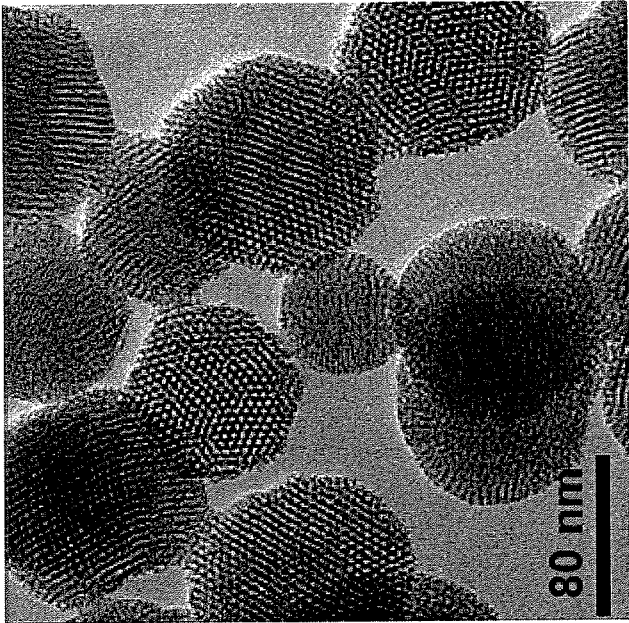


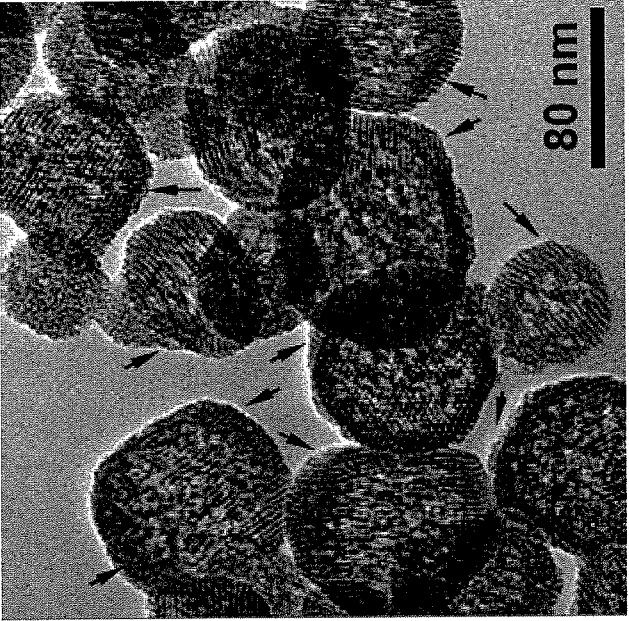
Figure 17

18A



Phos-MSNPs

18B

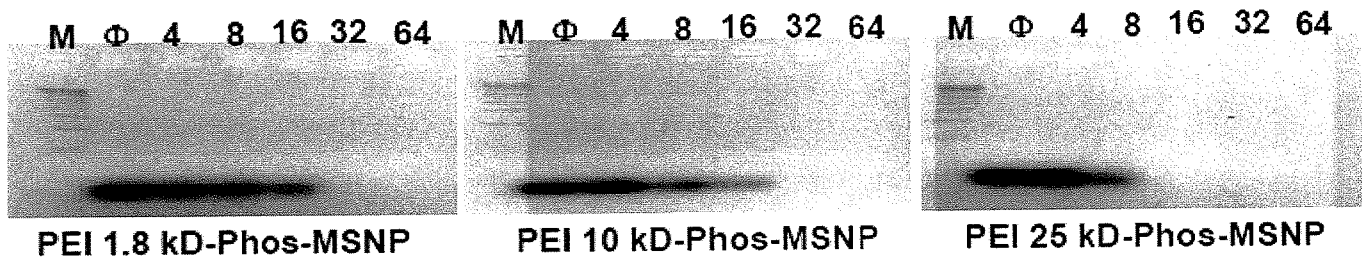


PEI-Phos-MSNPs

MSNPs Exterior	Size in H ₂ O (nm)	Size in BSA (nm)	Size in CDMEM (nm)	Zeta Potential in H ₂ O / CDMEM (mV)
PEI 1.8 kD-MSNPs	1201	360	294	+29.5/-4.6
PEI 10 kD-MSNPs	758	241	261	+34.1/-3.8
PEI 25 kD-MSNPs	1152	247	252	+31.7/-3.7

Figure 18

19A



19B

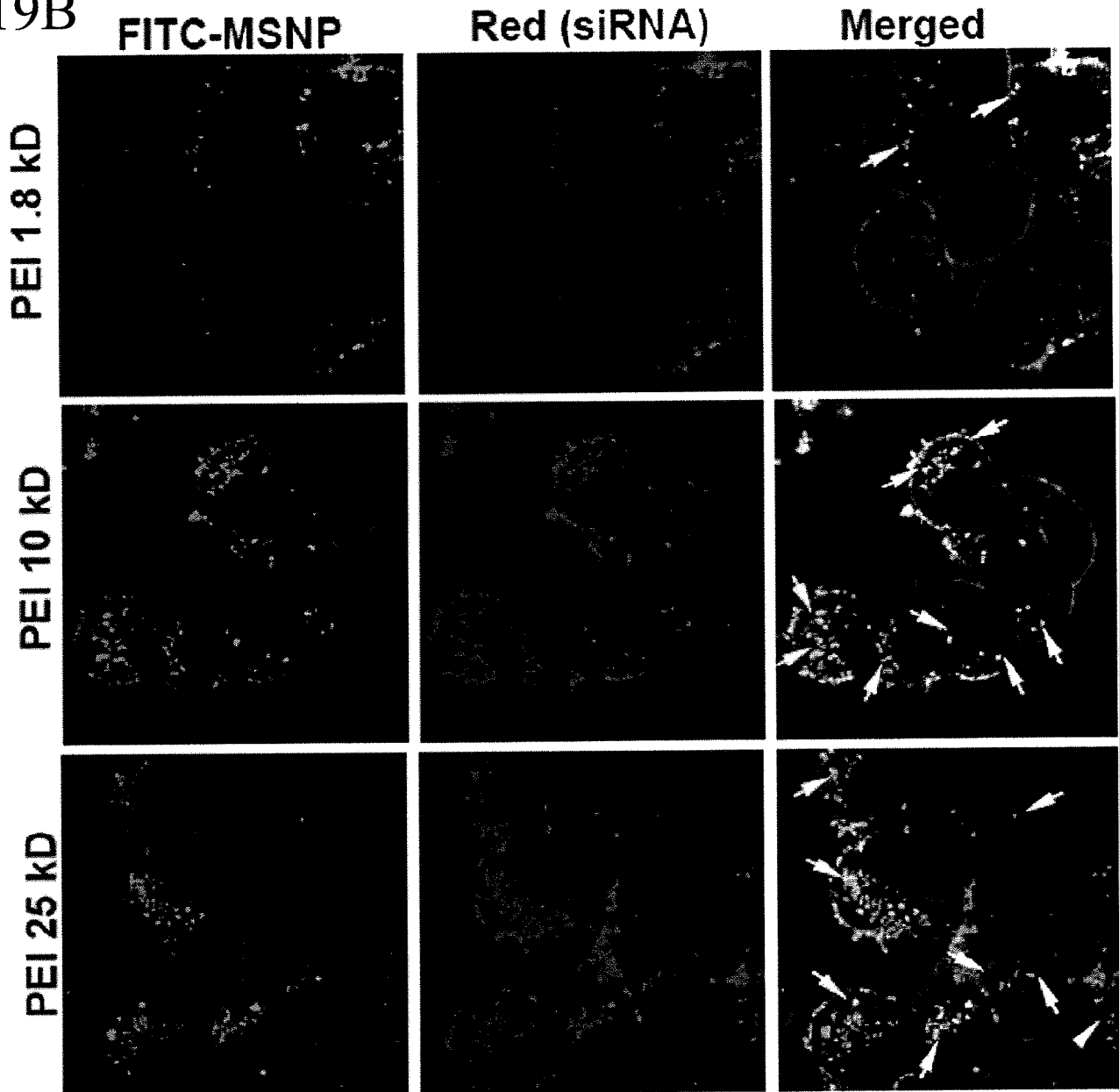


Figure 19

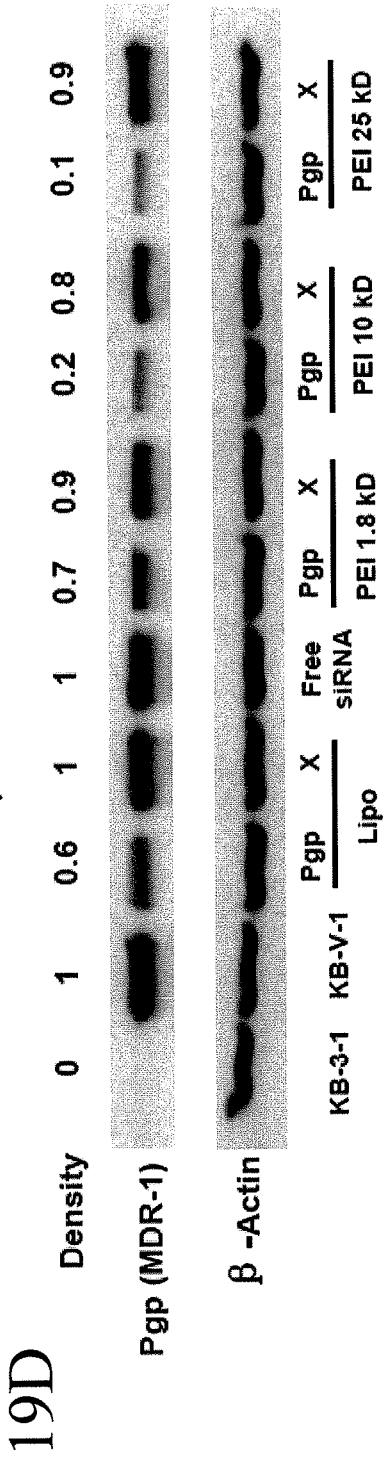
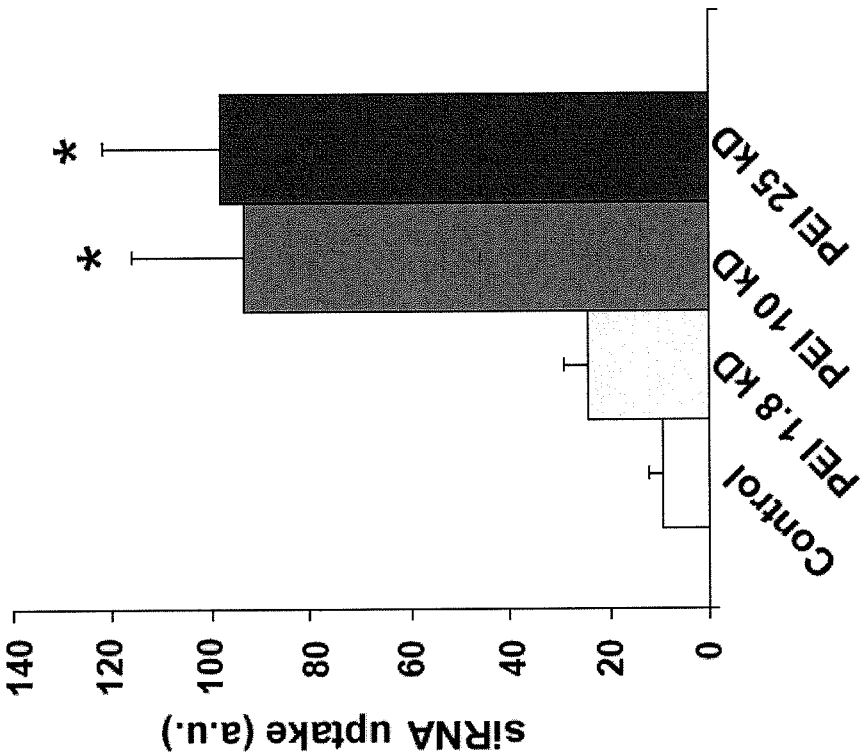


Figure 19 – cont.

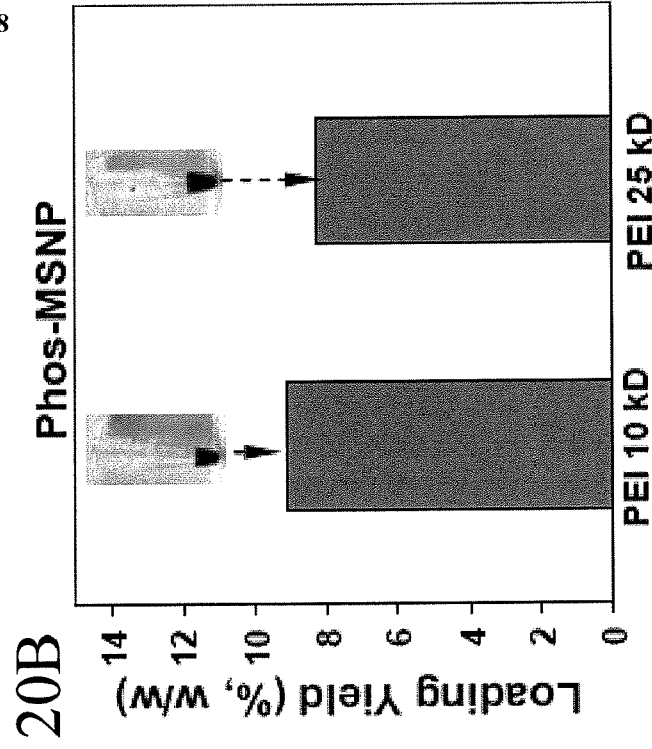
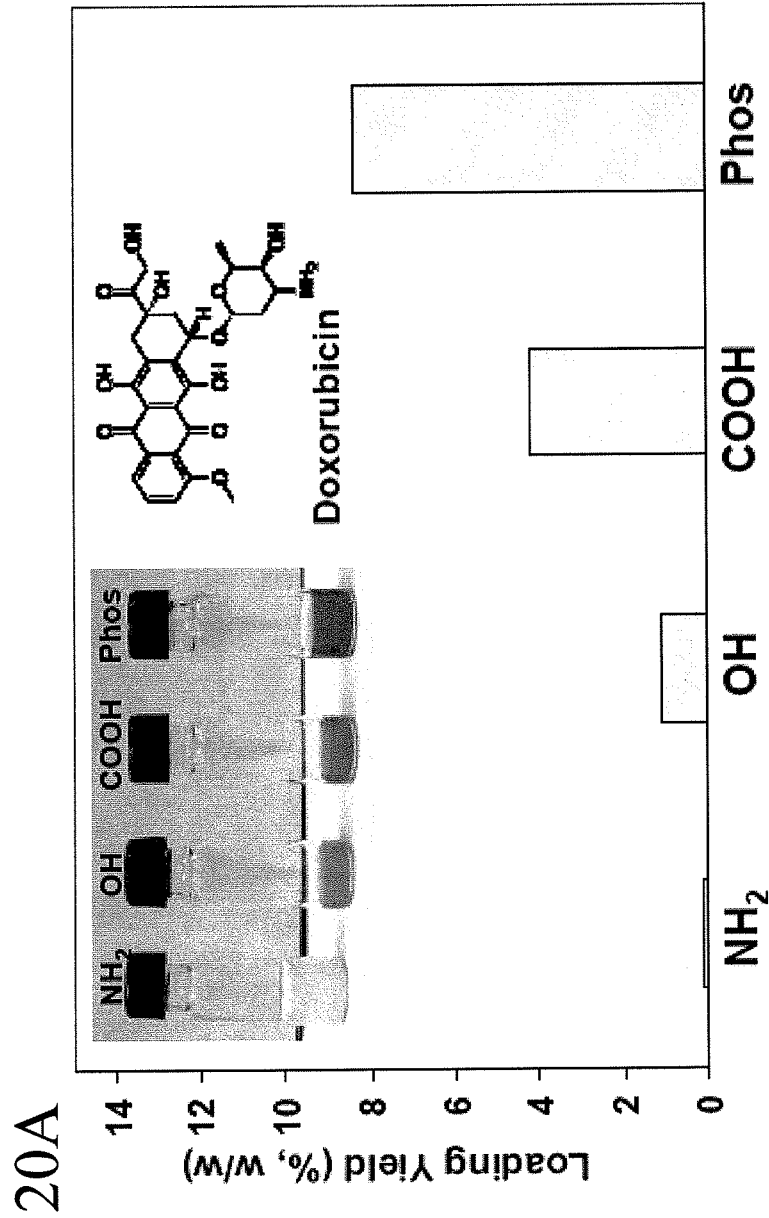


Figure 20

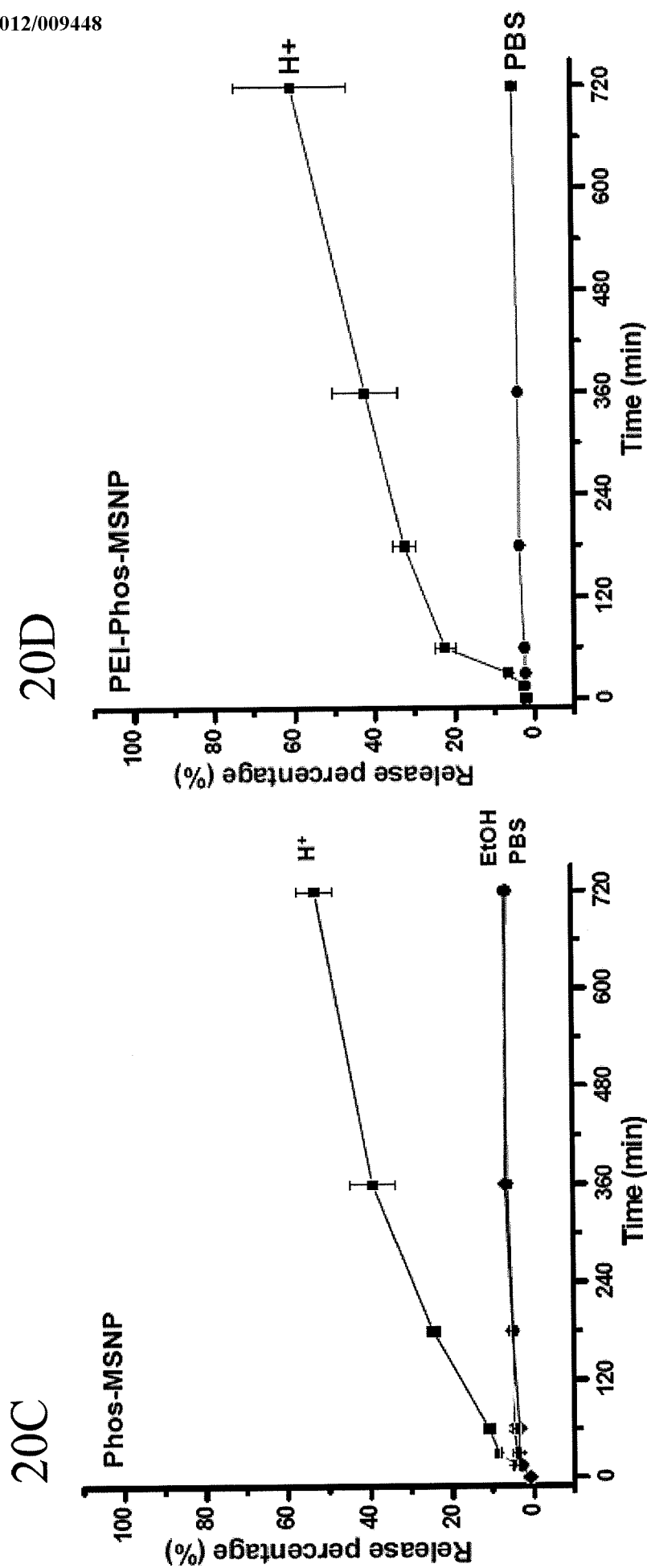


Figure 20 – cont.

20E

FITC-MSN

LAMP-1

Merged

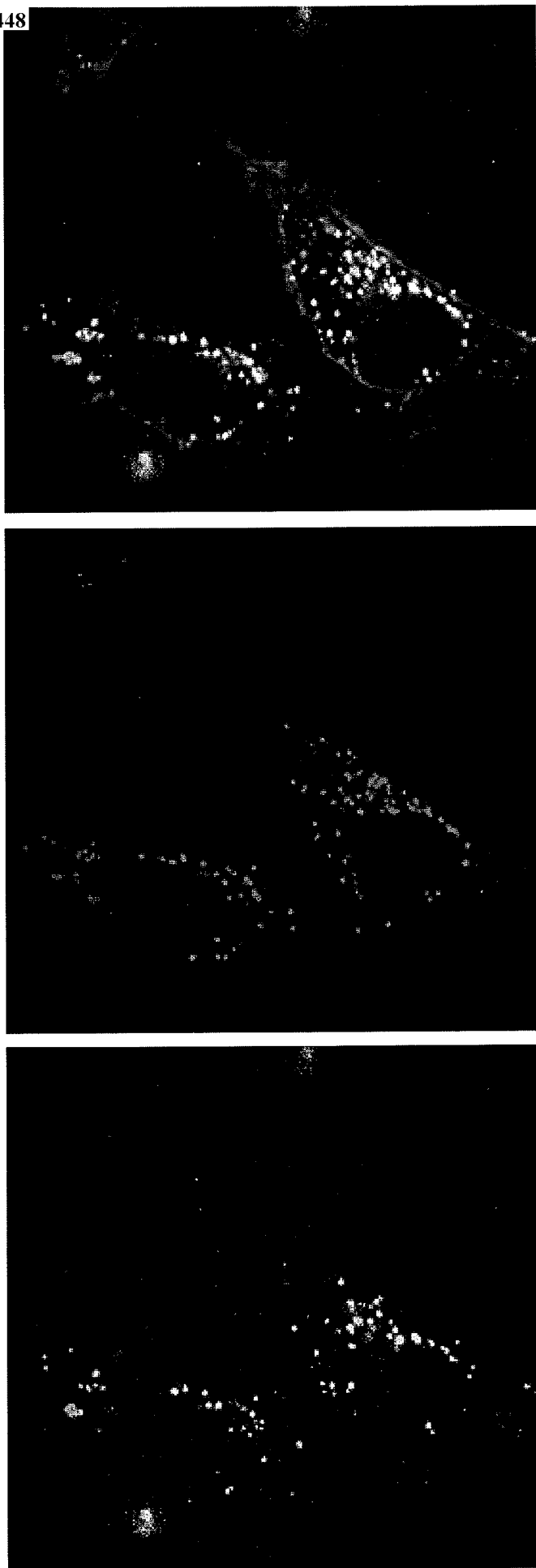


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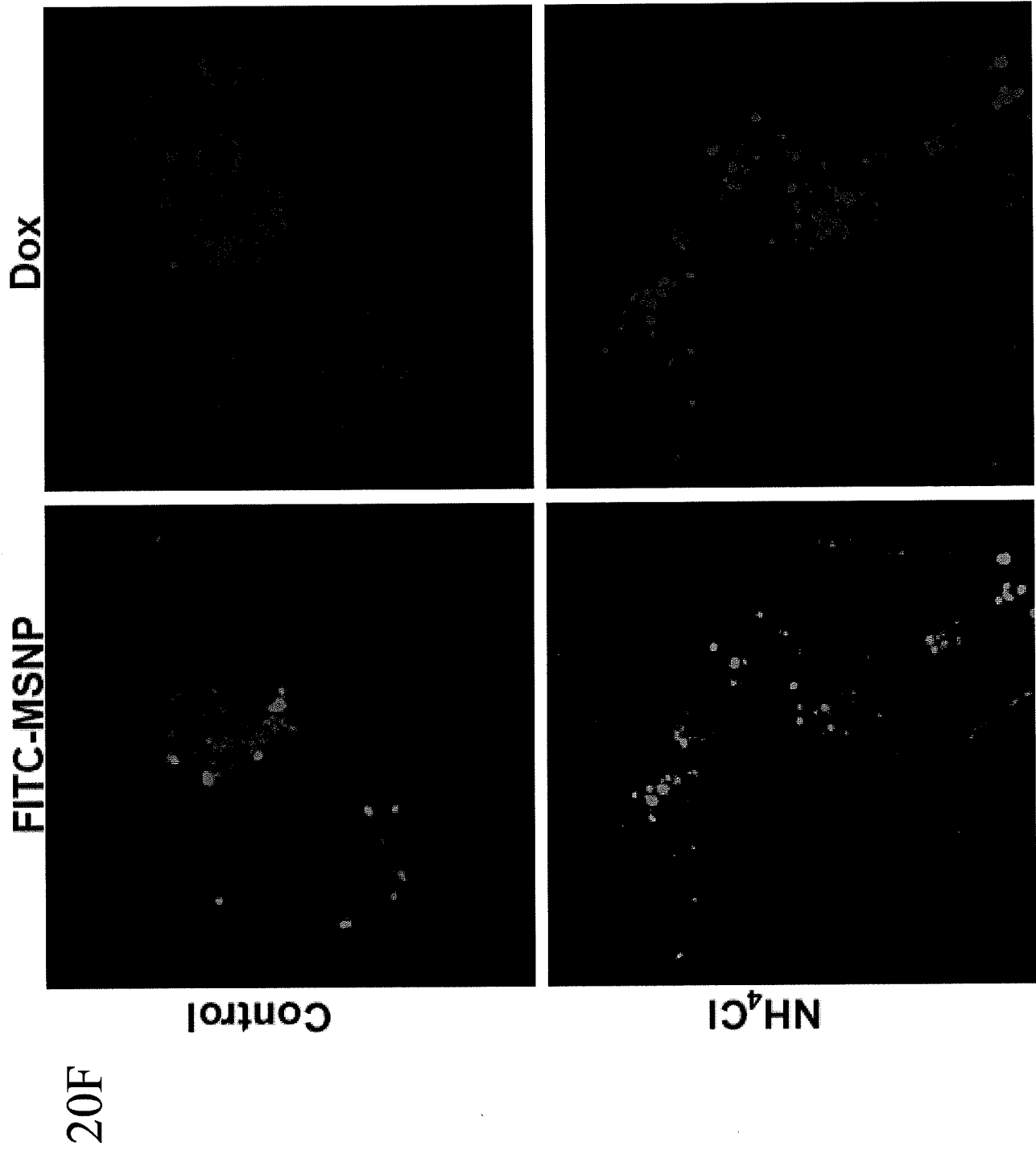


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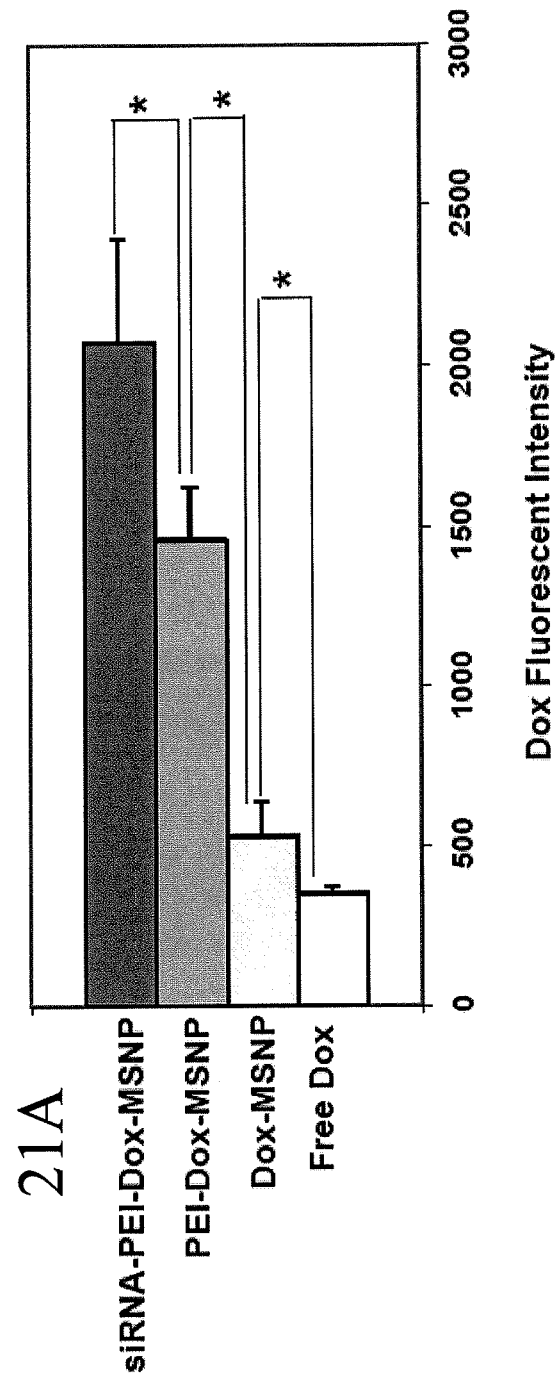
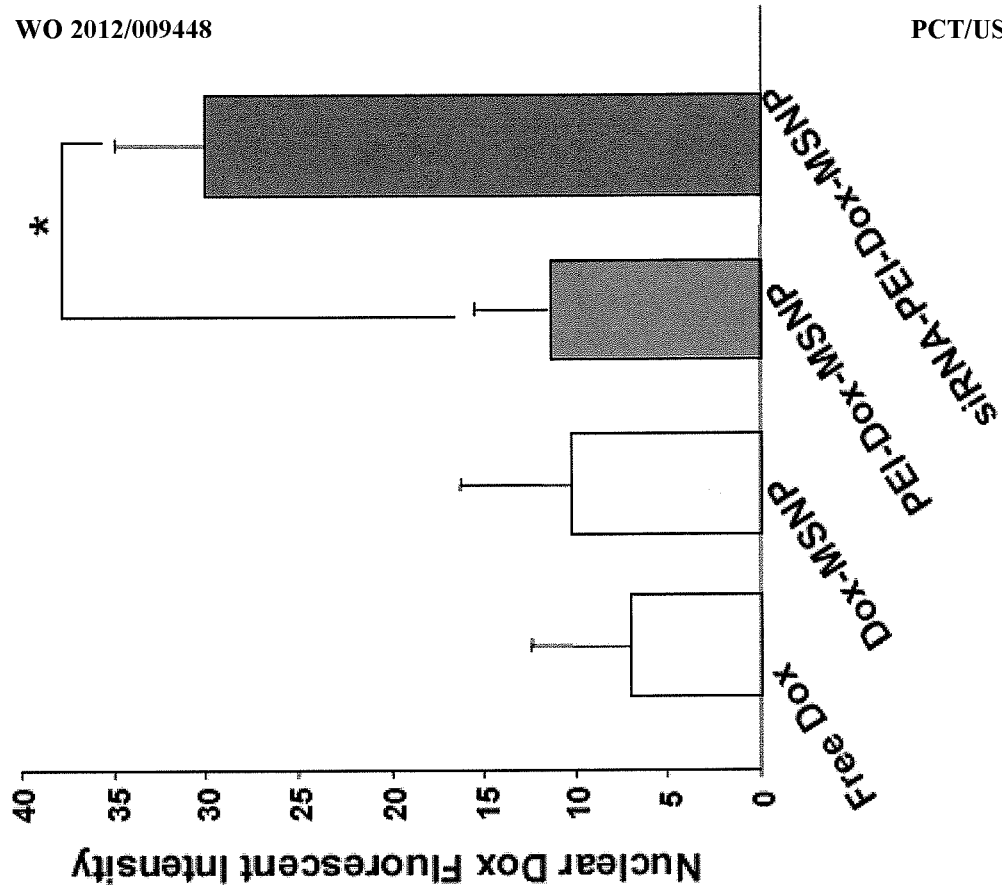


Figure 21

21C



21B

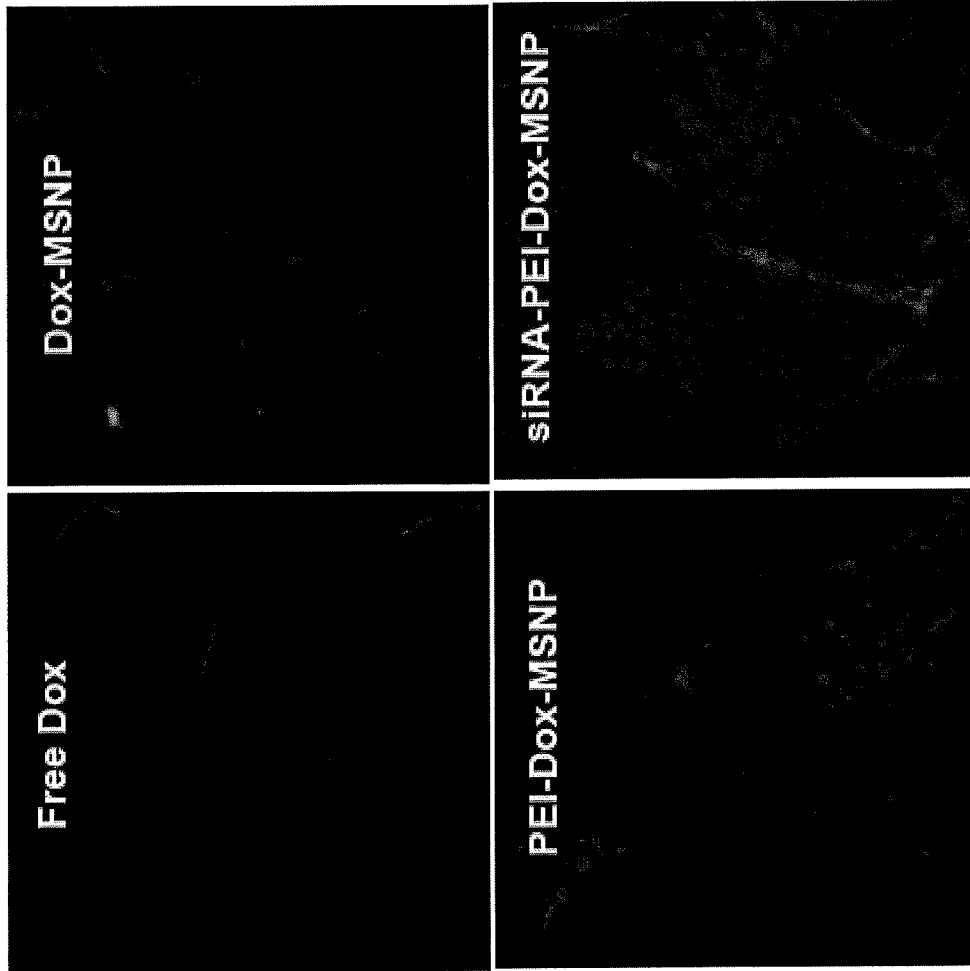


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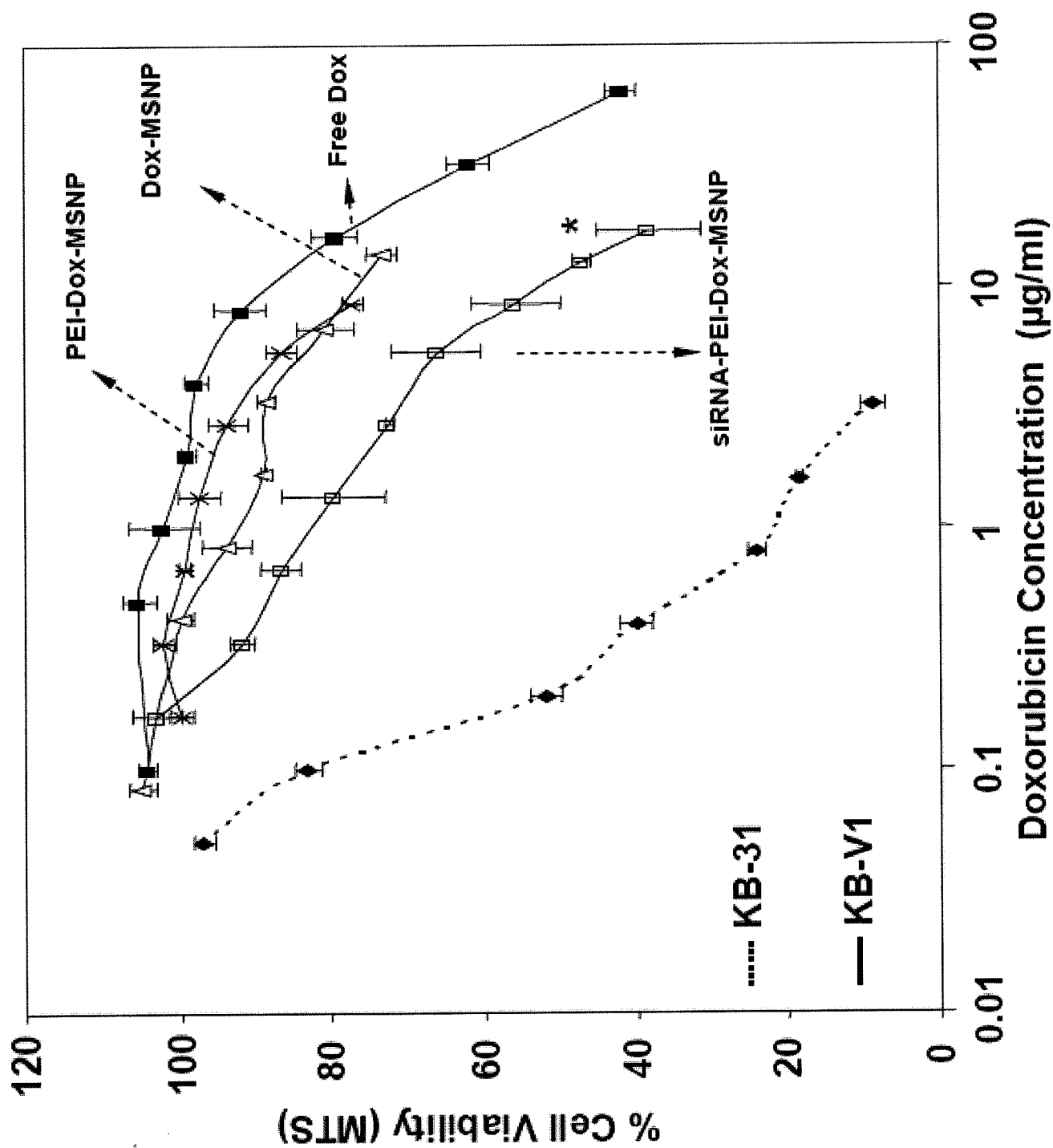


Figure 22

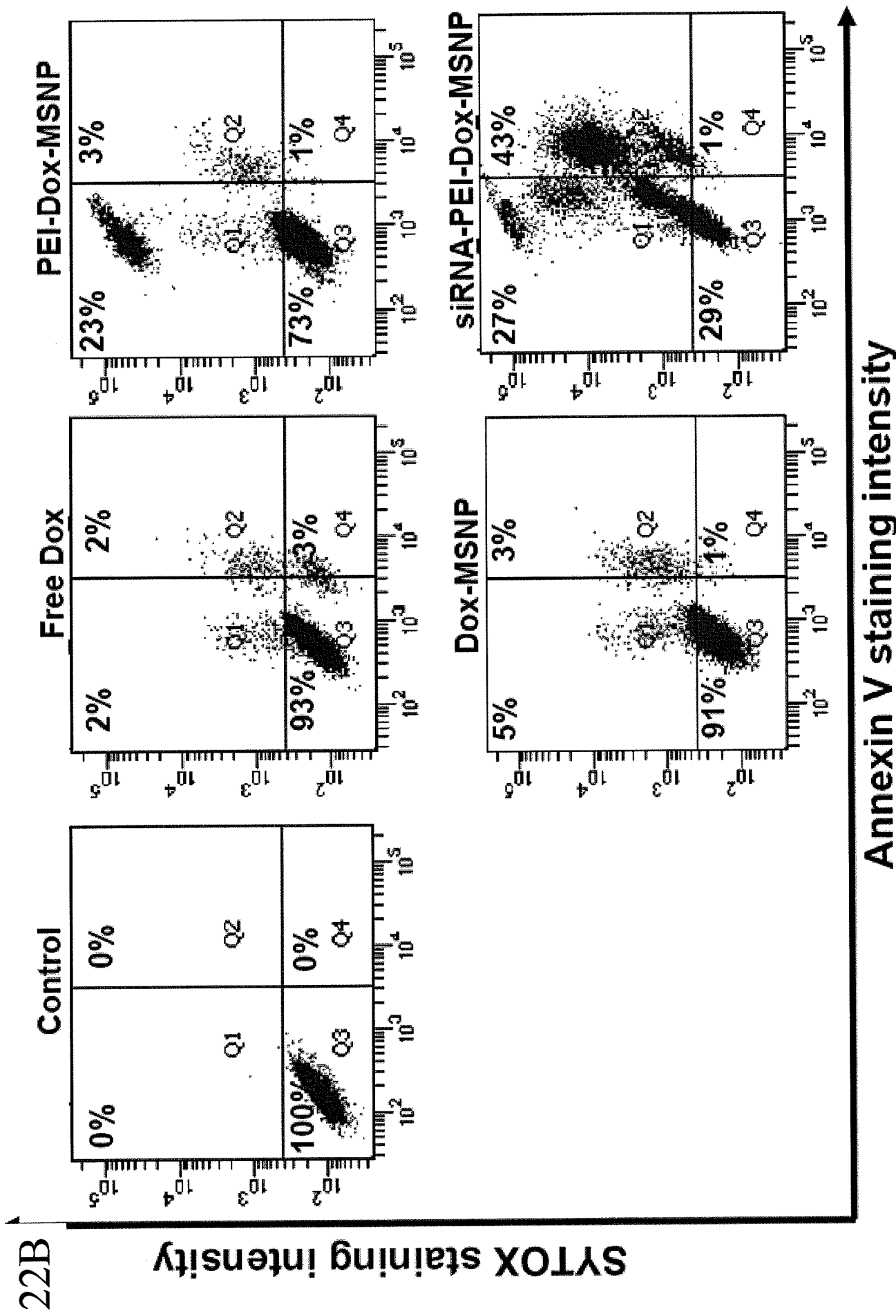


Figure 22 – cont.

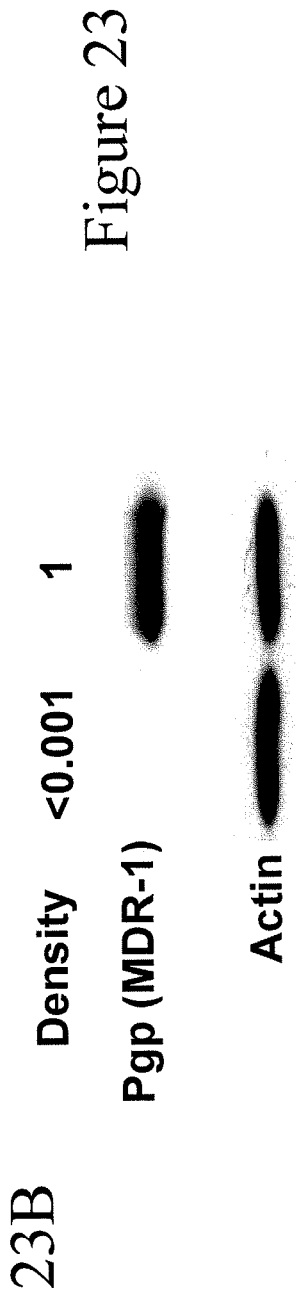
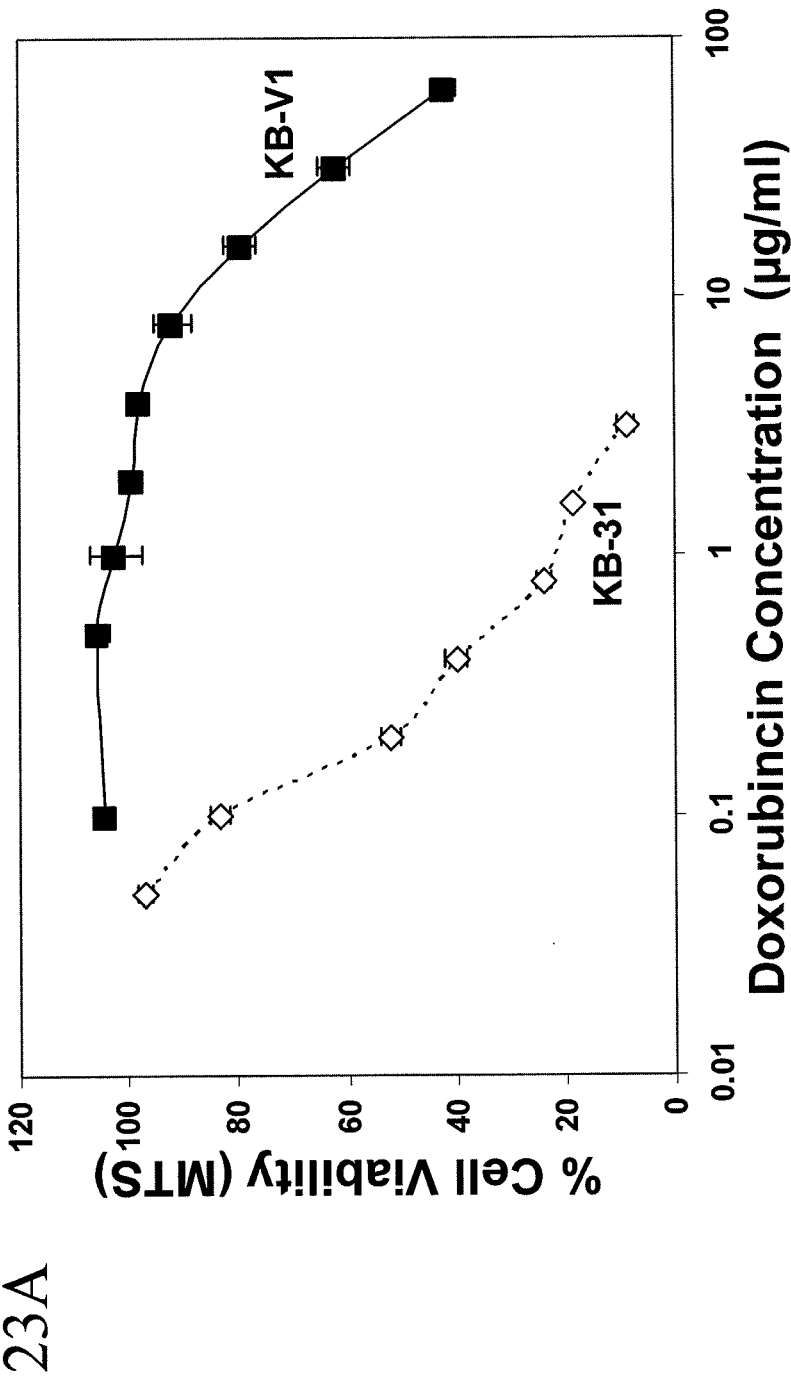


Figure 23

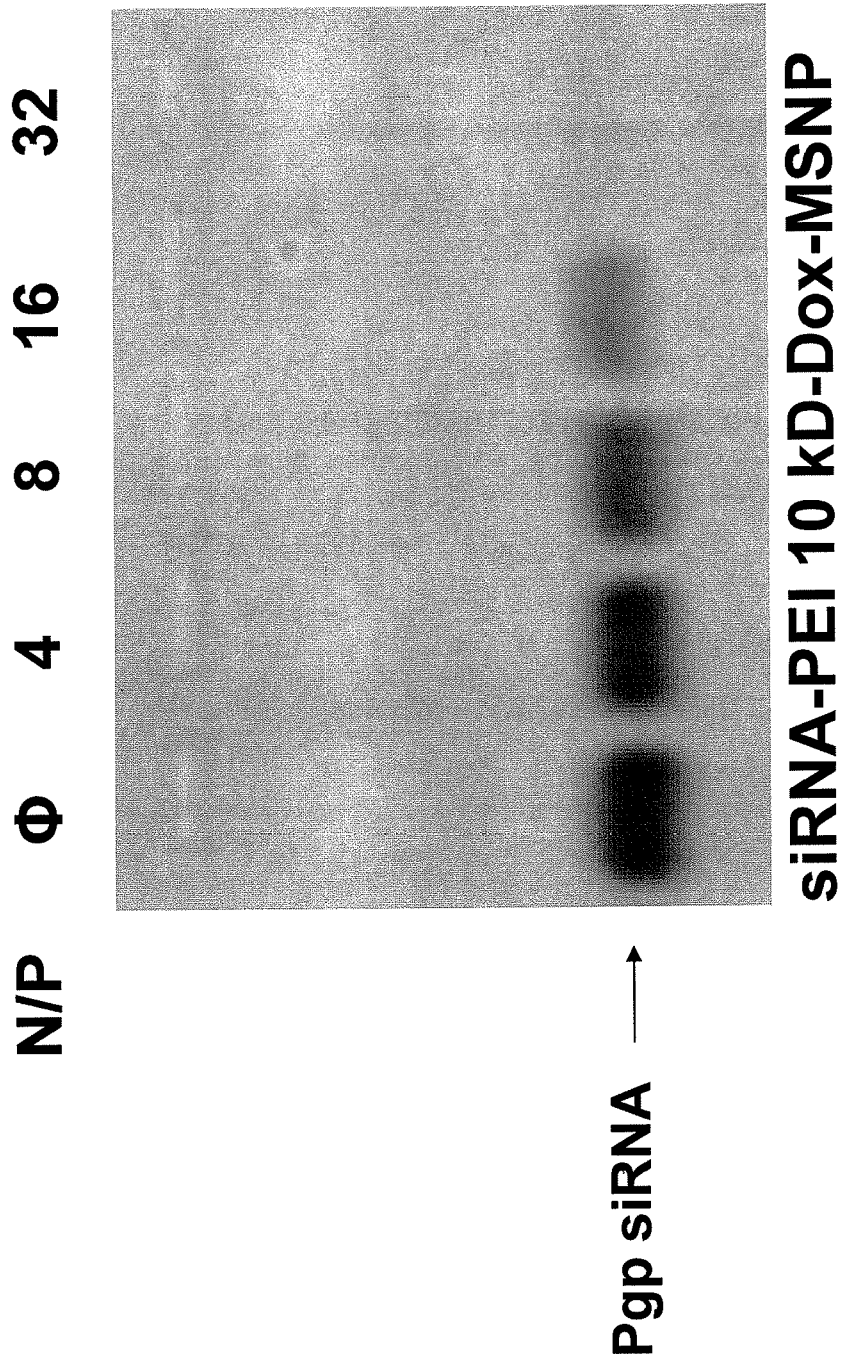


Figure 24

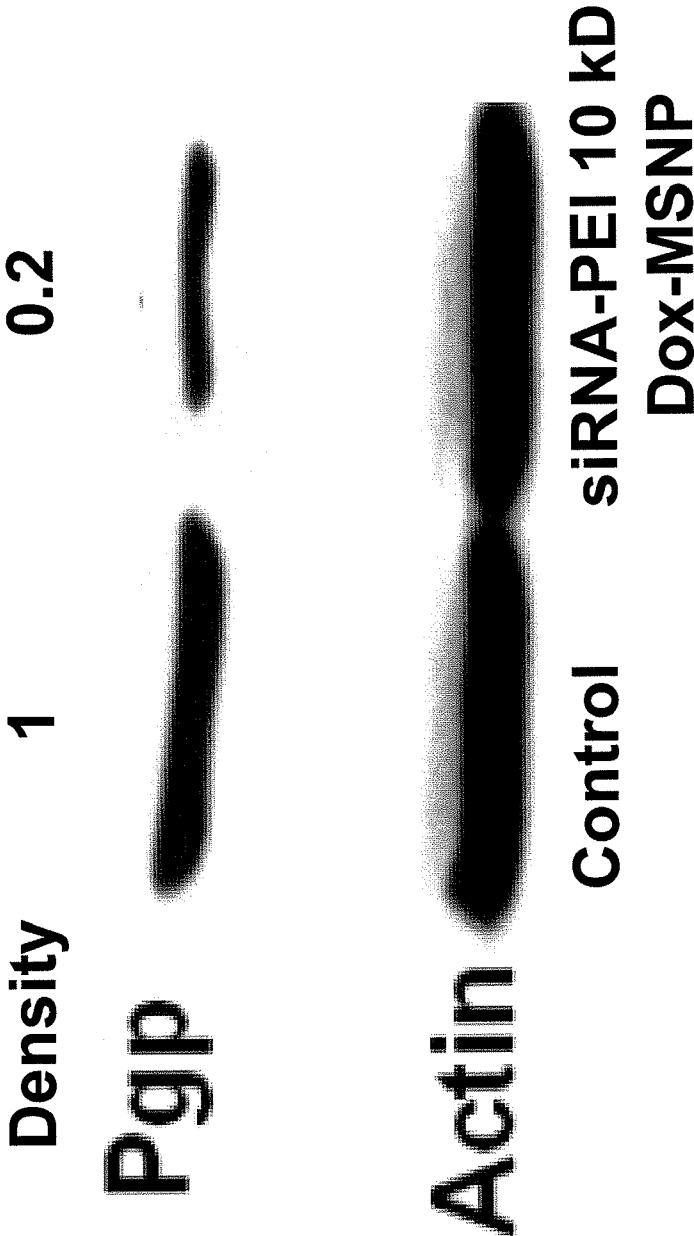


Figure 25

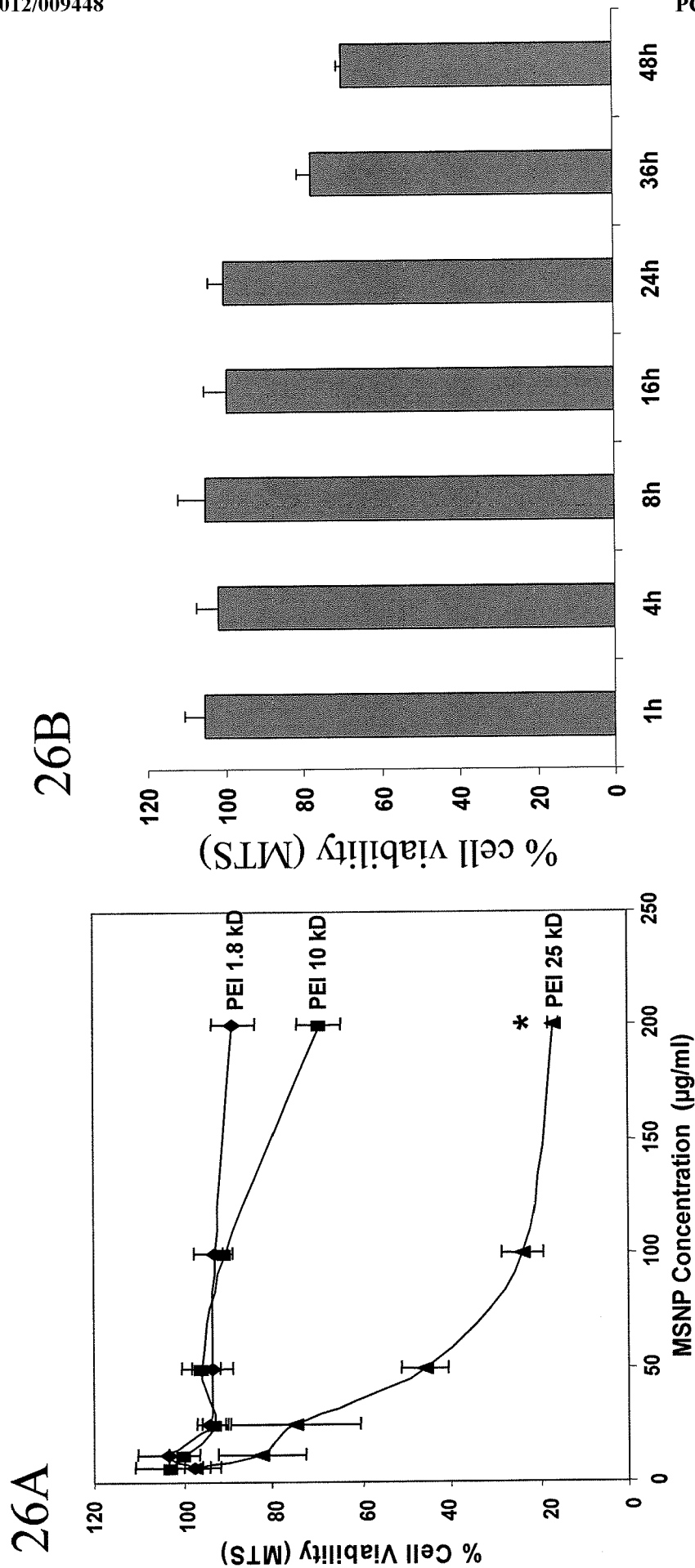


Figure 26

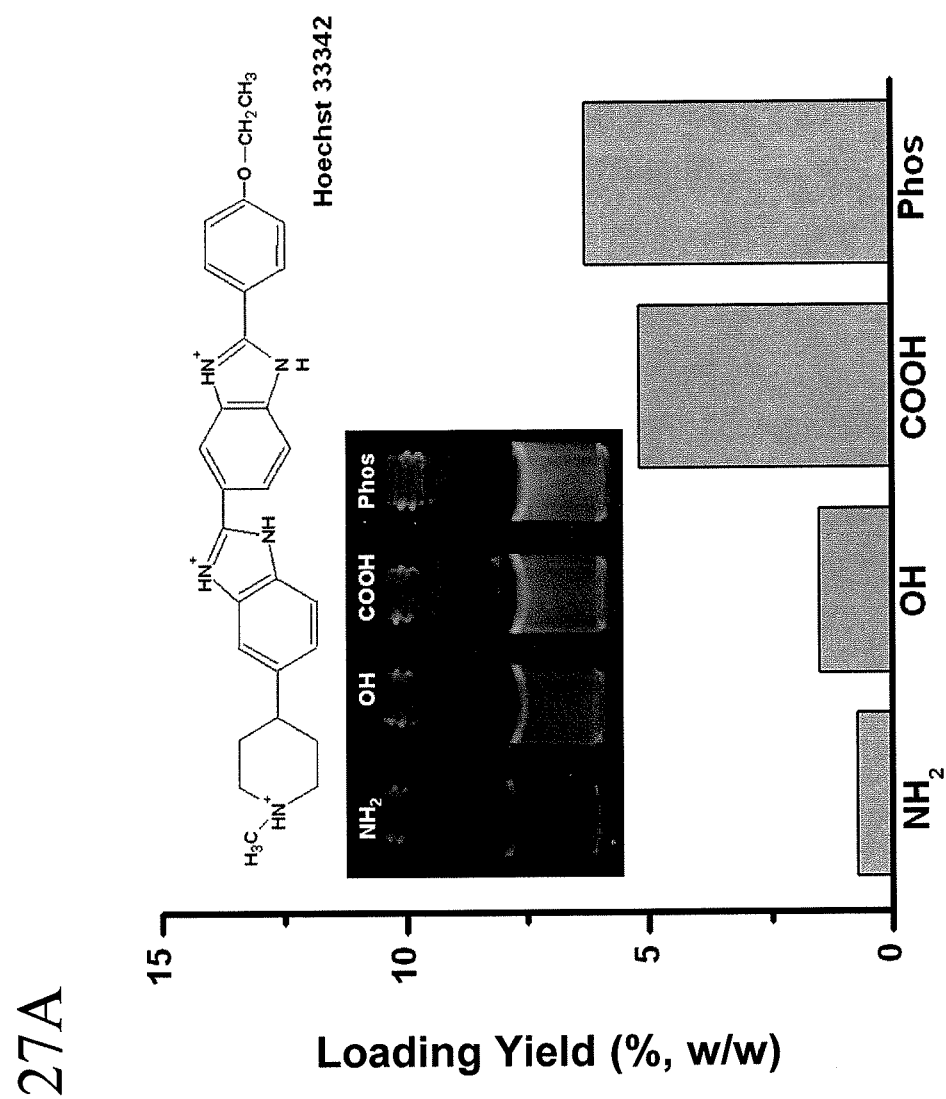
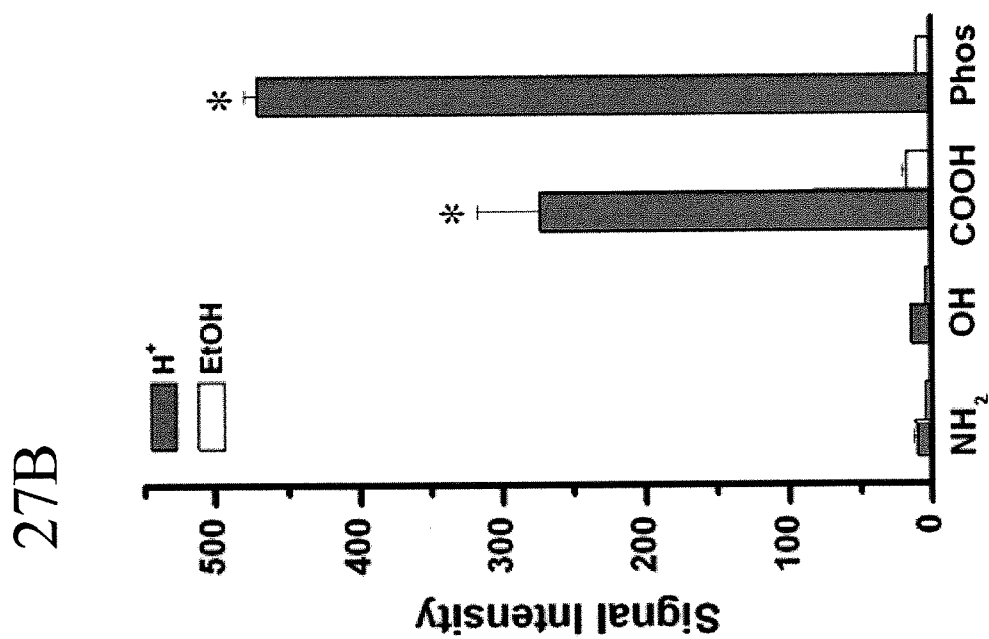


Figure 27

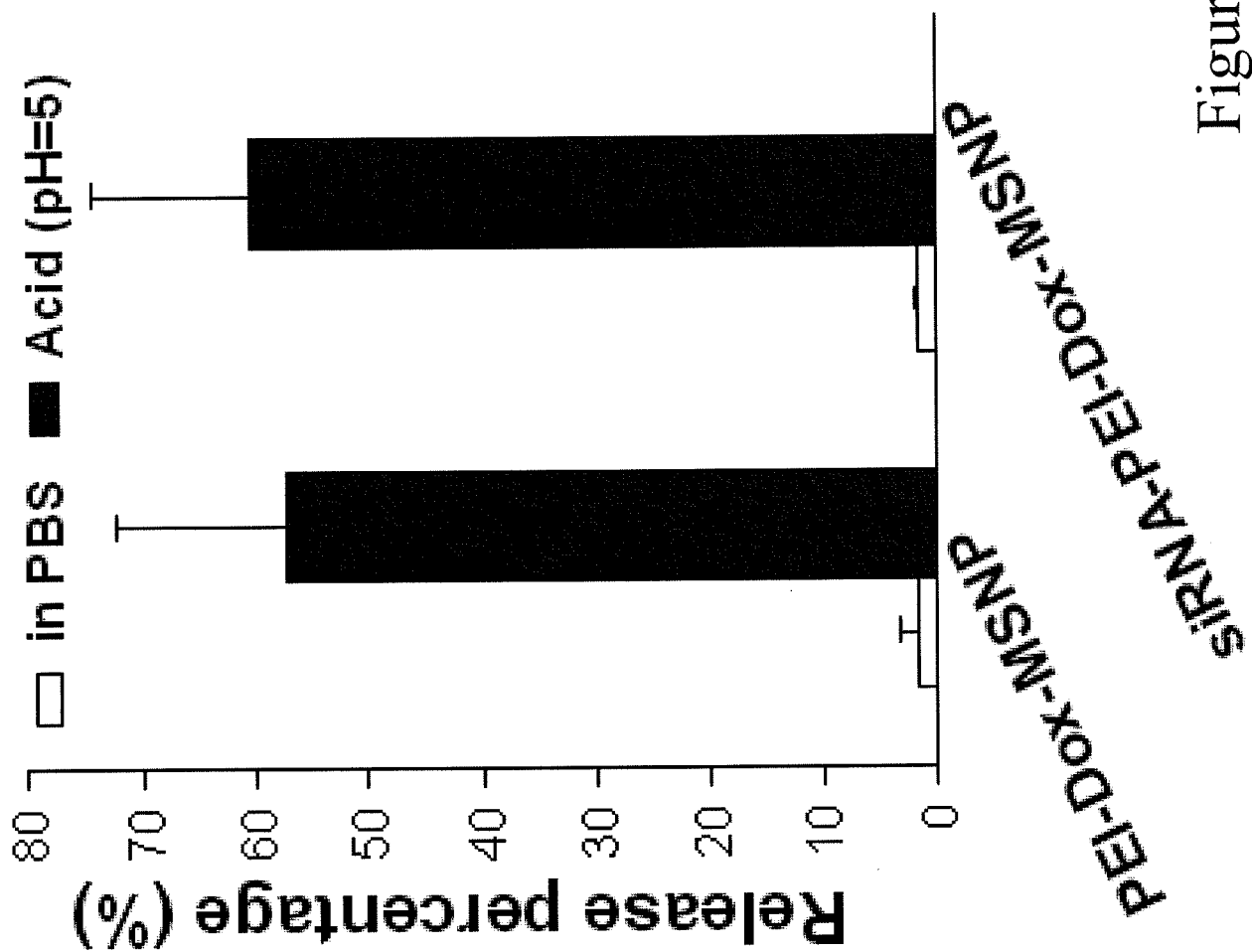


Figure 28

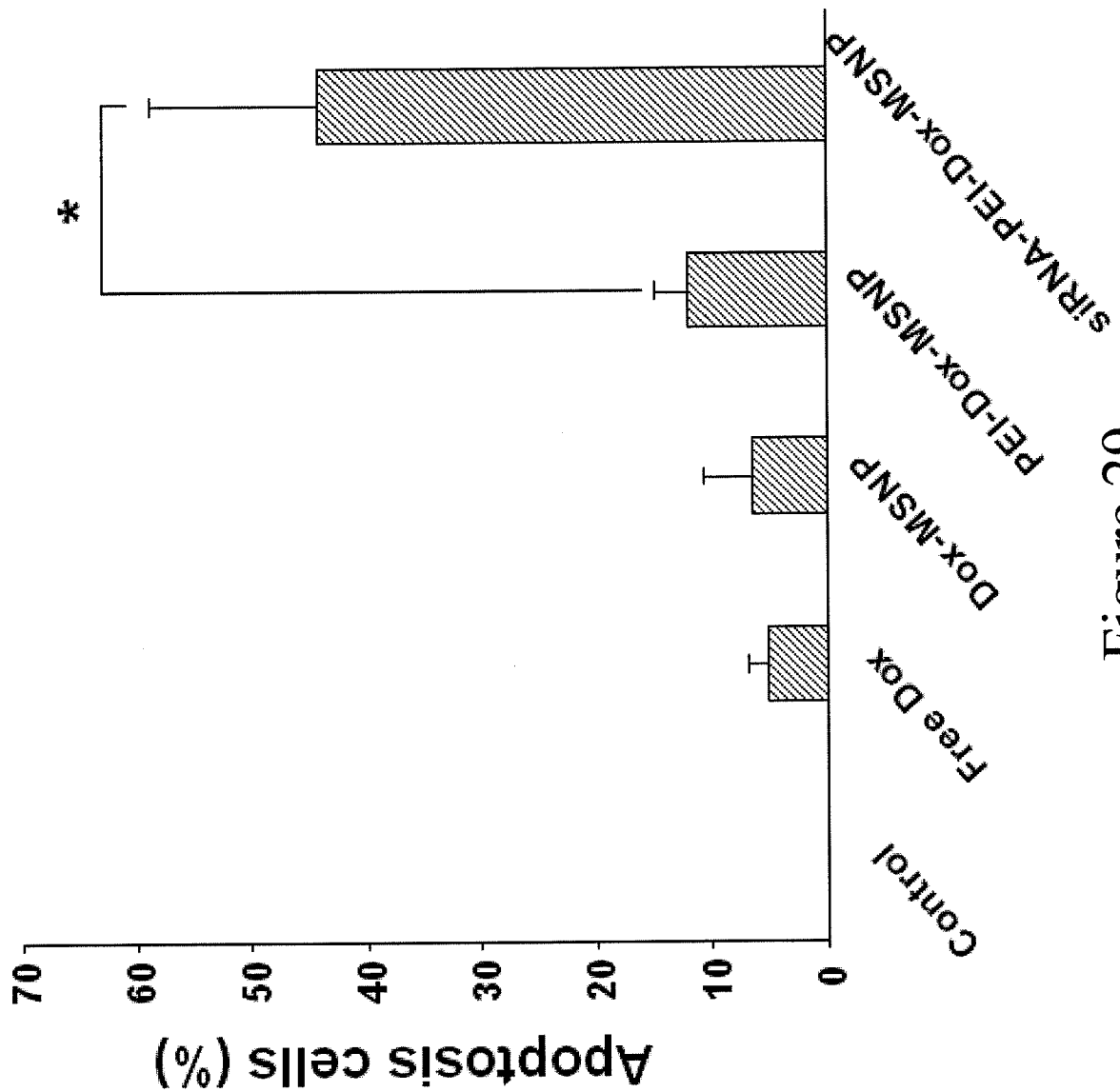


Figure 29

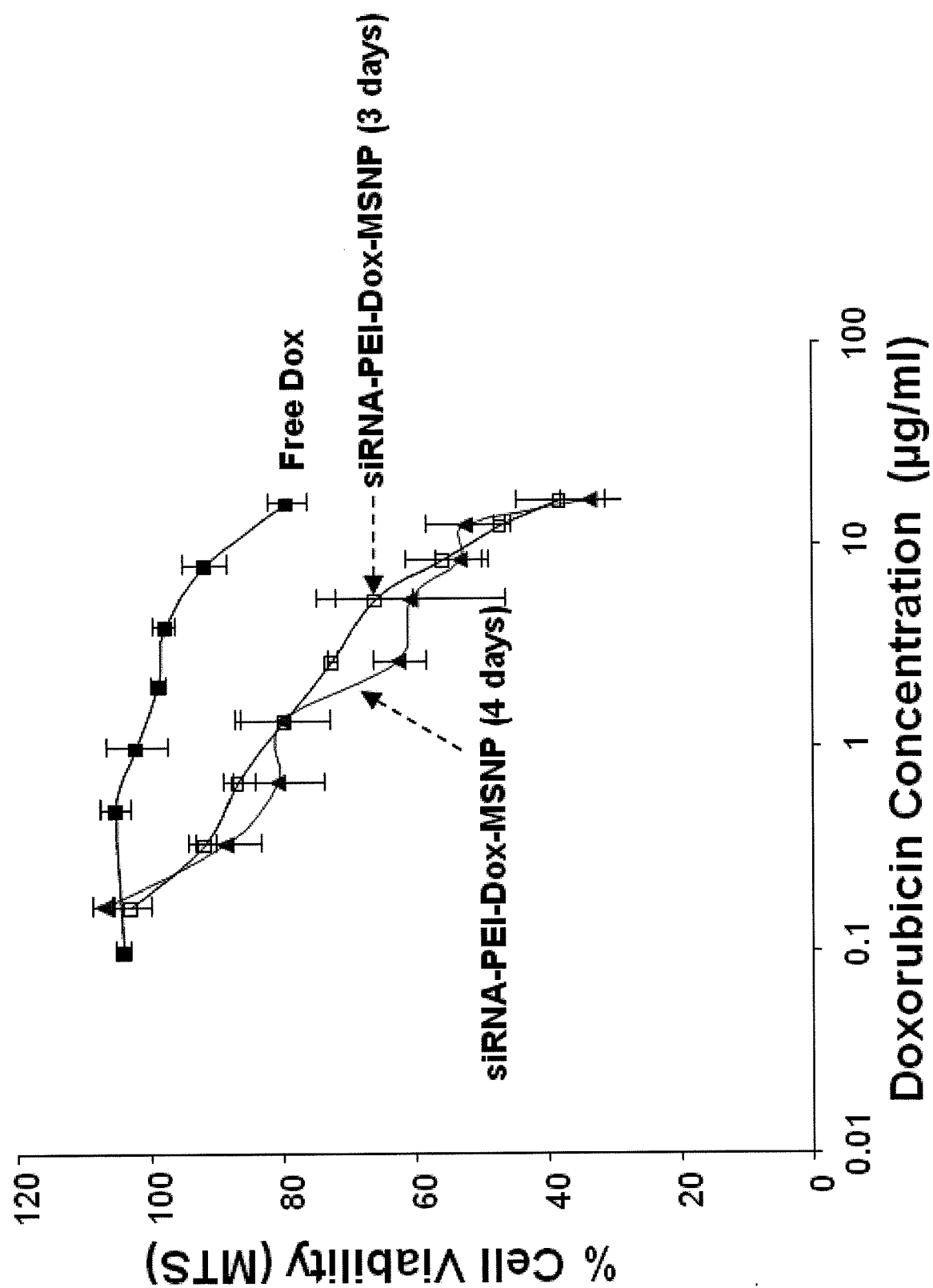


Figure 30

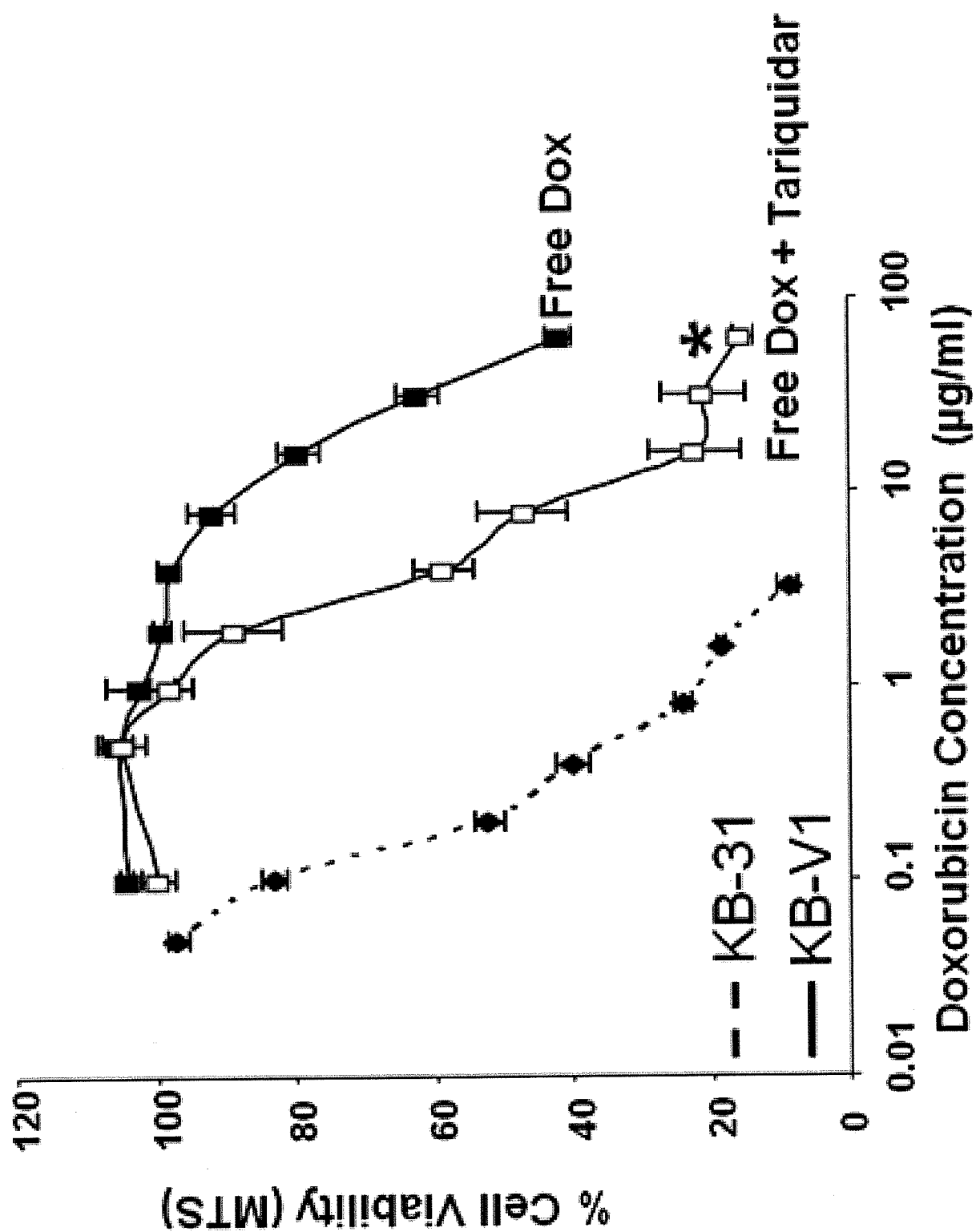


Figure 31

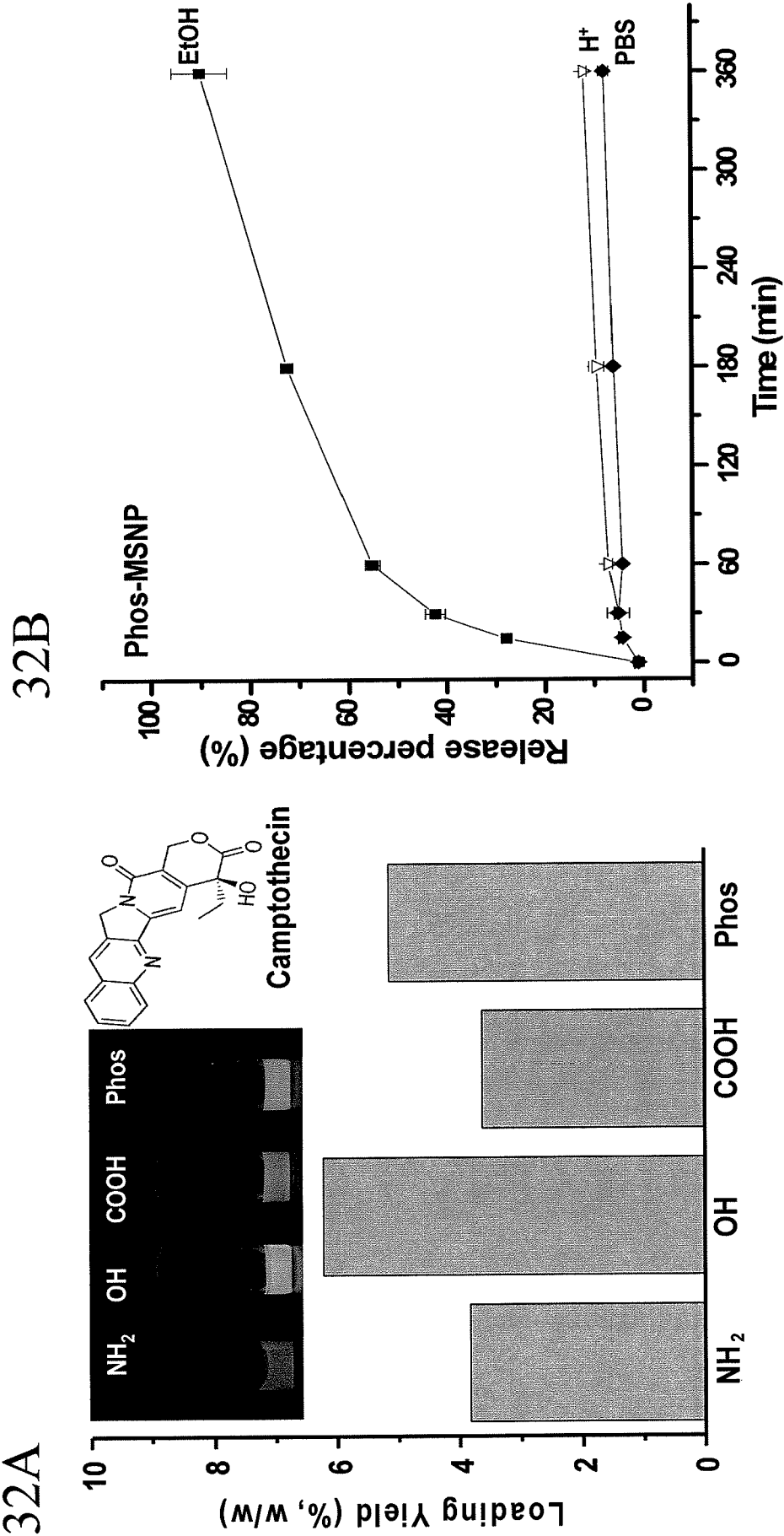


Figure 32