

(51) International Patent Classification:
A61B 5/05 (2006.01)(21) International Application Number:
PCT/US2016/018417(22) International Filing Date:
18 February 2016 (18.02.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/118,817 20 February 2015 (20.02.2015) US
62/157,623 6 May 2015 (06.05.2015) US(71) Applicant: TRUSTEES OF BOSTON UNIVERSITY
[US/US]; One Silber Way, Boston, Massachusetts 02215
(US).(72) Inventors: WONG, Joyce Y.; 40 Princeton Rd., Chestnut
Hill, Massachusetts 02467 (US). WHITAKER, Ragnhild
D.; c/o One Silber Way, Boston, Massachusetts 02215
(US). RUIZ-OPAZO, Nelson; 175 Briar Lane, Westwood,
Massachusetts 02090 (US). HERRERA, Victoria L.M.;
175 Briar Lane, Westwood, Massachusetts 02090 (US).(74) Agents: EISENSTEIN, Ronald I. et al.; Nixon Peabody
LLP, 100 Summer St., Boston, Massachusetts 02110-2131
(US).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

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))

Published:

- with international search report (Art. 21(3))

[Continued on next page]

(54) Title: THERANOSTIC COMPOSITIONS AND USES THEREOF

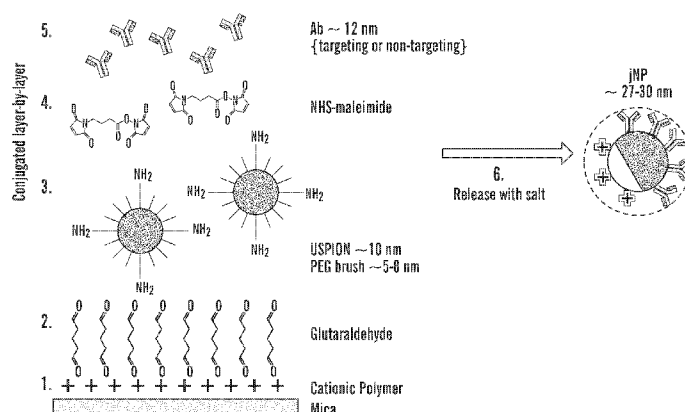


FIG. 1A

(57) Abstract: Provided herein are theranostic compositions comprising a Janus nanoparticle-coated microbubble that are useful for imaging (e.g., MRI, or ultrasound) and for delivering a therapeutic or bioactive agent (e.g., nucleic acid(s), drugs, etc), among other uses.



-
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

THERANOSTIC COMPOSITIONS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This Application claims benefit under 35 U.S.C. § 119(e) of the U.S. Provisional Application No. 62/118,817 filed February 20, 2015, and of the U.S. Provisional Application No. 62/157,623 filed May 6, 2015, the contents of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

[0002] The field of the invention relates to theranostic compositions useful in both imaging and treatment of diseases, such as cancer.

BACKGROUND

[0003] A high unmet need persists for successful delivery of nucleic acid therapy (*e.g.*, DNA, RNA) to address genetic and epigenetic heterogeneity, which underlie current cancer therapy failures. Multifunctional nanotechnologies are recognized as promising delivery platforms; however, key limitations in effective delivery of functionally intact nucleic acid payloads and/or in safety impede clinical translation of current nucleic acid nano-delivery systems.¹⁻⁴ Assuming tumor-specific targeting is achieved, efficacy of lipid and likely other nanoplateforms are dependent on endocytosis or macropinocytosis for intracellular delivery and remain inefficient due to limited (< 2%) escape of nucleic acid-cargo from endosomes into the cytosol^{2,5} and/or due to majority (>70%) of internalized RNA-cargo being exocytosed back to the extracellular space.⁶ Moreover, even if delivered intact, DNA/RNA-payloads need to integrate into the cellular machinery for functional efficacy.² Moreover, efficient *in vitro* nano-delivery systems, such as self-assembled cationic PEI-polyplexes, manifest toxic effects arising from excess cationic material remaining after self-assembly and/or from release of high MW or branched-PEI_{25kDa} as a result of disassembly *in vivo*.⁴ Alternatively, sonoporation-induced cytosolic-delivery of nucleic acid therapies has been tested to bypass inefficiencies of endosomal uptake, but remains inefficient with current microbubble technologies and modifications.^{7,60} To overcome key limitations, rational nanoparticle designs must consider not only charge, hydrophilicity, shape, and size⁸⁻¹³, but also the need to eliminate excess unincorporated materials, improve *in vivo* structural stability and prevent aggregation. Ideally, designs must also consider alternative endocytosis-independent mechanisms to deliver [DNA/RNA]-payloads,² modular multifunctionalities,¹⁴ and inherent versatility to accommodate multiple targeting and payload moieties to address genetic/ epigenetic heterogeneity in cancers, which to date, underlie current cancer therapy failures.

[0004] By virtue of their spatial separation of multifunctional components, thus eliminating confounding multi-component steric or chemical interactions, Janus nanoparticle designs have a unique potential to meet the foregoing criteria^{14,15} However, this potential has yet to be realized with

Janus NPs reported to date, which are typically larger than 100 nm, and were not designed to deliver nucleic acid therapies.¹⁴⁻¹⁹ Alternatively, nano/micro-hybrids bring unexplored potential, however, while dual MRI-ultrasound imaging flexibility has been attained using “nano-in-micro” composites of iron oxide nanoparticles encapsulated within microbubbles^{2,14} or within the microbubble shell,²⁰⁻²² these composite particles lack delivery functionality.

SUMMARY

[0005] Provided herein are methods and compositions that comprise dual functionality as a diagnostic reagent and a drug delivery agent (*e.g.*, act as theranostic agents). The compositions described herein are particularly useful in delivering nucleic acid agents to address genetic/ epigenetic heterogeneity in diseases, such as cancer, that underlie failure to respond to conventional treatment.

[0006] In one aspect, provided herein is a composition comprising: a microbubble with a Janus nanoparticle associated with the surface, wherein the Janus nanoparticle comprises an ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct (*e.g.*, spatially segregated) from one another.

[0007] In one embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is less than 75 nm in size (*e.g.*, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15 nm or smaller).

[0008] In another embodiment of this aspect and all other aspects described herein, the superparamagnetic iron oxide nanoparticle is less than 20 nm in size (*e.g.*, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 nm or smaller).

[0009] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is asymmetrically shaped.

[0010] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is avocado shaped.

[0011] In another embodiment of this aspect and all other aspects described herein, the targeting face is situated at the upper part of the avocado shaped nanoparticle, and the carrier face is situated at the round, bottom part of the avocado shaped nanoparticle.

[0012] In another embodiment of this aspect and all other aspects described herein, the targeting face comprises a targeting moiety selected from the group consisting of: a targeting peptide, a polyclonal antibody, a monoclonal antibody, an antibody binding fragment, a composite antibody, a recombinant antibody, a cell penetrating peptide and a matrix interacting peptide with *e.g.*, multiple units attached to the mixed PEG brush to impart efficiency of binding of the jNP through multivalent targeting moiety. In another embodiment of this aspect and all other aspects described herein, the composition comprises a long half-life in the circulation due to the properties of the targeting moiety components, for example, IgG antibodies that have a long half-life in the circulation.

[0013] In another embodiment of this aspect and all other aspects described herein, the targeting moiety binds to a cell surface or cell matrix protein. In some embodiments, the targeting moiety will permit part or all of the composition to be internalized within the cell.

[0014] In another embodiment of this aspect and all other aspects described herein, the carrier face comprises a polymer.

[0015] In another embodiment of this aspect and all other aspects described herein, the polymer comprises a polyelectrolyte.

[0016] In another embodiment of this aspect and all other aspects described herein, the polyelectrolyte is a cationic polyelectrolyte or an anionic polyelectrolyte.

[0017] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle comprises an iron oxide core with a PEG brush.

[0018] In another embodiment of this aspect and all other aspects described herein, the PEG brush is a mixed 3:1 short-long PEG brush.

[0019] In another embodiment of this aspect and all other aspects described herein, the moieties in the targeting and carrier faces are covalently coupled or tethered to the long PEG in the mixed PEG-brush.

[0020] In another embodiment of this aspect and all other aspects described herein, the iron oxide core is encapsulated within a polymer.

[0021] In another embodiment of this aspect and all other aspects described herein, the polymer-encapsulated iron oxide core further comprises a bioactive agent. In another embodiment, the polymer-encapsulated iron oxide core further encapsulates one or more bioactive agents.

[0022] In another embodiment of this aspect and all other aspects described herein, the composition further comprises a bioactive agent.

[0023] In another embodiment of this aspect and all other aspects described herein, the bioactive agent comprises a nucleic acid molecule.

[0024] In another embodiment of this aspect and all other aspects described herein, the nucleic acid molecule is sandwiched between the microbubble and the carrier face of the Janus nanoparticle.

[0025] In another embodiment of this aspect and all other aspects described herein, the composition is formulated for use as a magnetic resonance imaging (MRI) contrast agent.

[0026] In another embodiment of this aspect and all other aspects described herein, the MRI contrast agent comprises or exhibits a shorter magnetic resonance T2*-relaxivity.

[0027] Another aspect provided herein relates to a Janus nanoparticle comprising a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.

[0028] In one embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is less than 75 nm in size (*e.g.*, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15 nm or smaller).

[0029] In another embodiment of this aspect and all other aspects described herein, the superparamagnetic iron oxide nanoparticle is less than 20 nm in size (*e.g.*, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 nm or smaller). In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is about 10-40 nm or about 20-30 nm in size as measured using cryo-electron microscopy or about 10-60 nm or about 40-60 nm (*e.g.*, about 50 nm) in size as measured by dynamic light scattering (DLS).

[0030] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is asymmetrically shaped.

[0031] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is avocado shaped.

[0032] In another embodiment of this aspect and all other aspects described herein, the targeting face is situated at the upper part of the avocado shaped nanoparticle, and the carrier face is situated at the round, bottom part of the avocado shaped nanoparticle.

[0033] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle is (i) suitable for *in vivo* administration for use *e.g.*, in non-invasive *in vivo* contrast-enhanced magnetic resonance imaging (MRI), and/or (ii) suitable for *in vitro* self-assembly of a heteroplex comprised of the Janus nanoparticle with nucleic acid therapies (*e.g.*, miRNA, DNA, and/or siRNA) and amphoteric microbubbles for *in vivo* contrast-enhanced ultrasound molecular imaging and/or (iii) suitable for *in vivo* antibody-targeted delivery of nucleic acid therapy by sonoporation.

[0034] In another embodiment of this aspect and all other aspects described herein, the targeting face comprises a targeting moiety selected from the group consisting of: a targeting peptide, a polyclonal antibody, a monoclonal antibody, an antibody binding fragment, a composite antibody, a recombinant antibody, a cell penetrating peptide and a matrix interacting peptide.

[0035] In another embodiment of this aspect and all other aspects described herein, the targeting moiety binds to a cell surface or cell matrix protein. In some embodiments, the targeting moiety will permit part or all of the composition to be internalized within the cell.

[0036] In another embodiment of this aspect and all other aspects described herein, the targeting moiety is tethered directly to an amine group on the PEG brush, or via a linear or branched linker or cleavable spacer that can be linked by an enzyme.

[0037] In another embodiment of this aspect and all other aspects described herein, targeting is enhanced due to polyvalency of targeting moieties and non-interference by payloads due to the spatial segregation of the targeting face and the carrier face.

[0038] In another embodiment of this aspect and all other aspects described herein, the carrier face comprises a polymer.

[0039] In another embodiment of this aspect and all other aspects described herein, the polymer comprises a polyelectrolyte. In another embodiment of this aspect and all other aspects described herein, the polyelectrolyte comprises polyethyleneimine (PEI).

[0040] In another embodiment of this aspect and all other aspects described herein, the polyelectrolyte is a cationic polyelectrolyte or peptide or an anionic polyelectrolyte or peptide.

[0041] In another embodiment of this aspect and all other aspects described herein, the carrier face comprises a carrier moiety tethered directly to an amine group on the PEG brush, or via a linear or branched linker or cleavable spacer that can be linked by an enzyme.

[0042] In another embodiment of this aspect and all other aspects described herein, the carrier face forms a polyelectrostatic bridge with cognate opposing-charged payloads, such as cationic polyelectrolyte to negatively charged nucleic acids (*e.g.*, DNAs, oligonucleotides, miRNAs, miRNA-mimics, anti-miRNAs, siRNAs etc.), peptides, or drugs (*e.g.*, small molecule drugs).

[0043] In another embodiment of this aspect and all other aspects described herein, the carrying and delivery efficiency are enhanced by the polyvalency and spatial segregation of the polyelectrostatic bridge of the carrier face.

[0044] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle comprises an iron oxide core with a PEG brush.

[0045] In another embodiment of this aspect and all other aspects described herein, the PEG brush is a mixed 3:1 short-long PEG brush.

[0046] In another embodiment of this aspect and all other aspects described herein, the moieties in the targeting and carrier faces are covalently coupled or tethered to the long PEG in the mixed PEG-brush.

[0047] In another embodiment of this aspect and all other aspects described herein, the iron oxide core is encapsulated within a polymer.

[0048] In another embodiment of this aspect and all other aspects described herein, the polymer-encapsulated iron oxide core further comprises a bioactive agent, such as a small molecule drug or a chemotherapeutic. In another embodiment of this aspect and all other aspects described herein, the bioactive agent comprises a non-deliverable drug, for example, a drug that is hydrophobic, cytotoxic, or otherwise incompatible for delivery.

[0049] In another embodiment of this aspect and all other aspects described herein, the jNP with a polymer encapsulated iron oxide core and further comprising a bioactive agent, such as a small molecule drug or chemotherapeutic, facilitates the delivery of the bioactive agent (*e.g.*, a pharmacologically incompatible bioactive agent).

[0050] In another embodiment of this aspect and all other aspects described herein, wherein the iron oxide core is about 5-15 nm and covalently coupled to the targeting moiety(ies) on the targeting face and covalently coupled to the polyelectrolyte moieties on the carrier face.

[0051] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle enhances magnetic resonance (MR) contrast enhancement compared with non-Janus nanoparticle iron oxide nanoparticles due to changes in MR properties, such as relaxivity.

[0052] In another embodiment of this aspect and all other aspects described herein, MR-imaging is performed on a tumor and the MR-imaging of such tumors exhibits enhanced detection of tumor angiogenesis, extracellular matrix changes, edema, ischemia, inflammation, abnormal vasculature, invasiveness, metastasis and microtumors.

[0053] In another embodiment of this aspect and all other aspects described herein, the MR-imaging exhibits enhanced detection of features that distinguishes abnormal from normal, such as abnormal organ vascular patterns, normal and abnormal vascular integrity, normal and abnormal organ structures, angiogenic response, vascular leakiness, perivascular fibrosis, organ parenchyma, cellularity, extracellular matrix composition, fibrosis, among others.

[0054] In another embodiment of this aspect and all other aspects described herein, the Janus nanoparticle (jNP) can bind to DNA-coated or nucleic acid-coated microbubbles forming a heteroplex such as, a nNP[nucleic acid]microbubble heteroplex that performs better in ultrasound mediated molecular imaging and delivery of nucleic acid by sonoporation compared to all current non-[20-50nm]jNP-mediated methods of sonoporation.

[0055] Another aspect described herein relates to a composition for use as an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.

[0056] In one embodiment of this aspect and all other aspects provided herein, the MRI contrast agent is a negative contrast agent.

[0057] In another embodiment of this aspect and all other aspects provided herein, the composition is for use in detection and/or monitoring of electron paramagnetic resonance (EPR) imaging.

[0058] Another aspect provided herein relates to a method for imaging, the method comprising: (a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another, and (b) performing an MRI on the subject.

[0059] In another embodiment of this aspect and all other aspects provided herein, the MRI comprises electron paramagnetic resonance (EPR) imaging.

[0060] Another aspect provided herein relates to a composition for use as an MRI contrast agent for target-specific imaging, the composition comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged.

[0061] In one embodiment of this aspect and all other aspects provided herein, the target to be imaged is a tumor.

[0062] Another aspect provided herein relates to a method for target-specific imaging, the method comprising: (a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged, and (b) performing an MRI on the subject.

BRIEF DESCRIPTION OF THE FIGURES

[0063] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0064] **FIGs. 1A-1C** Conjugated layer-by-layer preparation and cryo-TEM/AFM imaging of Janus nanoparticles (jNPs). **FIG. 1A**, Schematic diagram of directional layer-by-layer method to prepare jNPs. Layers are: 1: cationic polymer, PEI, adsorbed onto a mica sheet; 2: glutaraldehyde, conjugated to amines in the PEI layer; 3: Fe_3O_4 ~10 nm USPION with mixed partially amine-terminated PEG2K/3.4K brush (~5-8 nm) conjugated to glutaraldehyde layer; 4: maleimide layer from conversion of free amines on USPION core by N-hydroxy-succinimide maleimide (NHS-maleimide); 5: targeting antibodies (Ab) conjugated to layers-4; 6: asymmetrically functionalized jNPs released from mica sheet with salt. **FIG. 1B**, Representative cryo-TEM images of jNPs from two independent jNP preparations showing an asymmetric, avocado-like ~22-32 nm particle with an electro-dense USPION core closer to the PEI cationic carrier-face, and targeting antibodies (~12 nm) comprising the opposing targeting-face. Scale bar = 20 nm. **FIG. 1C**, Representative atomic force microscopy (AFM) multi-parameter images (amplitude, topography, and phase) of individual jNPs. Scale bar = 50 nm.

[0065] **FIGs. 2A-2F** *In vitro* characterization of jNPs. **FIG. 2A**, Differential zeta-potential levels of partial jNP layered-composition stages with ultrasmall 10-nm SPION cores: SPION-PEG, SPION-PEG-NH₂, SPION-PEG-NH₂-PEI, and jNPs (n=3). ANOVA * $p < 0.05$; ** $p < 0.01$. **FIG. 2B**, Fluorescence intensity levels, documenting conjugation of AF594-labeled antibodies (Ab) as final layer (jNP) and targeting-face of jNPs compared to ultrasmall SPION-PEG-NH₂-PEI and water control (n=3). ANOVA * $p < 0.01$. **FIG. 2C**, Representative frequency plot of hydrodynamic diameters (nm) obtained via dynamic light scattering (DLS) at time-0. **FIG. 2D**, Representative serum-stability time-course plot of hydrodynamic diameters (nm) of jNPs from 0-120 hours (hr) in 85% serum. **FIG. 2E**, Representative AFM amplitude and topography images taken of jNPs at t-1 and

t-24 hr in 85% serum. Scale bar = 50 nm. **FIG. 2F**, Ethidium bromide (EtBr) dye exclusion assay for different amounts of DNA (300, 100, and 50 ng RFP-minigene plasmid DNA) exposed to increasing amounts of jNPs (# of jNPs $\times 10^{10}$) for 10 min incubation. Level of free DNA, unbound or unprotected by jNPs, is indicated by level of EtBr fluorescence intensity (arbitrary units, AU) emitted from EtBr upon intercalation into free DNA. Fluorescence [Ex. 260/Em.590 nm] at 10 min done in duplicate; highest jNP point in triplicate. Agarose gel analysis of EtBr fluorescence after intercalation into 'free' RFP plasmid 4.7 kb DNA. Lanes 1-4, increasing [0, 0.2, 0.6, 1.8×10^{10}] jNPs added to 100ng RFP-DNA for 10 min. MW, bands from 10, 8, 6, 5, 4, 3, and 2 kb DNA markers.

[0066] FIGs. 3A-3E *In vitro* analysis of Janus nanoparticle (jNP) targeting and carrier functions. **FIG. 3A**, Schematic diagram of self-assembly of targeted and non-targeted jNP-MBs. Multiple types of nucleic acids can be added to the MB as the payload. Coverage of jNPs on MB can be tuned. **FIG. 3B**, Representative FACS analysis of non-targeted jNPs with AF568-labeled IgG and AF488-labeled single strand 50-nt oligoDNA. Left panel shows control non-fluorescent MBs (Q4, MB). When compared to assembled jNP-MBs (right panel), double fluorescent jNP-MBs with projected density of $\sim 0.05\%$ jNP –coverage of MBs (Q2) are distinguished from single-fluorescent MB-DNA (Q3), and non-fluorescent MBs (Q4, MB). **FIG. 3C**, Representative FACS analysis of jNP-MB assembly. Panels 1-3: Control, non-fluorescent microbubbles (MB), compared with fluorescent targeted jNP-MBs with 1% and 50% coverage of MB surface by AF594-labeled monoclonal antibody against DEspR. Stacked bar graph of dose-response effects of increasing % jNP-coverage of MBs on % - fluorescent and % non-fluorescent (black) MBs, *** $p < 0.0001$ chi-square analysis. **FIG. 3D**, Representative baseline FACS analysis of controls 1-3: microbubbles, targeted AF594-labeled jNPs, and Panc1 tumor cells, respectively (Panels 1,2,3). Comparative FACS analysis of Panc1 tumors cells targeted by, and bound with jNP-MBs at 1:1 and 1:5 cell:jNP-MB ratio using jNP-MBs with 10% coverage. The percent (%) fluorescent units of total fluorescent counts are indicated in respective regions of interest. Free jNP-MBs are demarcated (53.2% and 75.2%) and not included in the $\pm$ fluorescent cell counts. Stacked bar graph of FACS data shows % fluorescent and % non-fluorescent (black) Panc1 cells from total cell counts at different cell:jNP-MB ratios, *** $p < 0.0001$ chi-square analysis. **FIG. 3E**, Epi-fluorescence microscopy analysis of Panc1 tumor cells with attached fluorescently-labeled targeted jNP-MBs ($\sim 2-3 \mu\text{m}$ diameter MBs) compared with control non-targeted jNP-MBs. bar, $10 \mu\text{m}$. Black/white stacked bar graph depicts % MB-positive cells when exposed to targeted jNP-mBs compared with control non-targeted jNP-MBs. ■, MB+ cells; □ MB(-) cells; chi-square analysis $p < 0.0001$; $n = 80$ (targeted jNP-MBs); $n = 30$ (non-targeted jNP-MBs). Bar graph depicts number of jNP-MBs per cell among MB+ cells comparing targeted jNP-MBs and non-targeted jNP-MBs. ***Mann Whitney test: $p = 0.0006$; targeted jNP-MBs $n = 14$; non-targeted jNP-MBs (white) $n = 6$ cells.

[0067] FIGs. 4A-4H Representative photomicroscopy images using identical exposures comparing *in vitro* fluorescence resulting from delivery of functionally intact reporter minigene-DNA (red fluorescent protein, RFP) to Panc1 cells 48-hours after sonoporation. **FIG. 4A**, Panc1 cells sonoporated with DEspR-targeted jNP[DNA]MB-heteroplexes exhibit RFP-positive expression. **FIG. 4B**, Less RFP-positive expression in Panc1 cells sonoporated with control DEspR-targeted non-jNP[DNA]MBs. Anti-DEspR mAb linked to MBs by biotin-streptavidin. **FIG. 4C**, Control non-sonoporated Panc1 cells with DEspR-targeted jNP[DNA]MBs. **FIG. 4D**, Control non-sonoporated non-jNP[DNA]MBs. **FIG. 4E**, Panc1 cells sonoporated with control non-targeted (isotype)-jNP[DNA]MB, and **FIG. 4F**, control sonoporated non-targeted (isotype) non-jNP[DNA]MBs. Bar = 100 μ m; identical experimental conditions: $\sim$ 1:5 cell:MB ratio, DNA-MB ratio (30 μ gDNA/ 10^8 MBs); 10% jNP-coverage used for DEspR-targeted and isotype-non-targeted jNP[DNA]MB-heteroplexes. **FIG. 4G**, high magnification image of RFP+ fluorescent Panc1 tumor cells ascertaining cell-specific expression. **FIG. 4H**, Bar graph of % RFP-positive cells in randomly selected sections (n = 3-19) from three independent experiments (4 sonoporation sites, 1 control site per experiment) of study groups represented in panels a-f. Kruskal Wallis non-parametric ANOVA $p < 0.0001$; panel a: #n=19; b: n=12; c: n=3, d: n=4; e: n=12; f: n=3. Post-hoc test: Dunn's multiple comparisons test, *, $p < 0.05$; ***, $P < 0.0001$; a vs. c: also $p < 0.0001$.

[0068] FIGs. 5A-5E Analysis of jNP diagnostic functionality for contrast-enhanced MR- and ultrasound imaging. **FIG. 5A**, Gradient-echo signal intensity versus echo time (TE, ms), from 10-100 ms, for jNP and precursor-USPION phantoms, both at 5×10^{10} /ml in 1% agar, and control blank 1% agar phantom. Each curve was normalized so that peak signal at TE = 4 ms is equal to 1. jNPs exhibited shorter T2*-relaxivity values (mean $\pm$ sd: 35.7 ± 1.2 ms) compared with precursor-USPIONs (57.9 ± 2.2 ms) and control blanks (82.0 ± 5.1 ms) $p < 0.001$, two-way ANOVA repeated measures. **FIG. 5B**, Representative susceptibility weighted intensity (SWI) magnetic resonance (MR)-images of Panc1 xenograft tumors in nude rats. Negative control: tumor with no jNPs; Positive control: tumor injected intratumorally with 50 μ l of 10^{12} /ml non-targeted isotype-jNPs; Right panel: tumor with intravenous (IV)-infused non-targeted jNPs imaged at t-24 hr consistent with tumor-selective EPR effects. Arrows indicate jNP localization of low-intensity regions, indicating increased iron content on SWI sequence; bar = 1cm. **FIG. 5C**, Contrast-enhanced ultrasound images: overlay of B-mode (grey to black) and contrast-enhanced images (pseudo-colored) in spontaneous rat mammary tumors comparing control DEspR-targeted non-jNP/MBs (Targeted Control-MBs) and DEspR-targeted jNP[DNA]MB-heteroplexes (Targeted jNP[DNA]MBs) at pre-destruct time point (t20:Pre-destruct), and pre- and post-destruct (t21:Post-destruct) time points. Regions of interest (ROI) for quantitation: intratumoral microvessels, extra-tumoral 'feeder vessels' at tumor base. (See FIG. 10 for schematic diagram). **FIG. 5D**, Representative time-intensity curves of contrast intensity signals in designated ROI at pre-destruct (PRE) and post-destruct (POST) time points, comparing extra-tumoral feeder vessels and intratumoral microvessels, and comparing Targeted jNP[DNA]MBs and Targeted

Control-MBs. Timepoint of high-power ultrasound MB-destruct sequence (dashed line) demarcating pre- and post-destruct contrast intensity signals. **FIG. 5E**, Quantitative analysis of peak contrast intensity signals (CIS) comparing DEspR-targeted jNP[DNA]MB heteroplexes (jNP) and control (con) non-jNP DEspR-targeted microbubbles (con) in both ROIs: extratumoral feeder vessels (fv) and intratumoral microvessels (mv) at two timepoints: t20 and t30 min. One-way ANOVA $p < 0.0001$, ***, Tukey's multiple pairwise comparison $p < 0.0001$, $n = 8$ groups, representing 10 random measures of peak CIS values in each ROI (extratumoral and intratumoral) at two time points (t20, t30) taken from 3 independent rat experiments using spontaneous mammary tumor rat model.

[0069] FIGs. 6A-6F *In vivo* analysis of jNP-DNA-MB delivery functionality and safety profile. **FIG. 6A**, Side-by-side IVIS image of RFP-fluorescence in spontaneous mammary tumors from a control mock-sonoporated rat with negative fluorescence (rat on left) and from a rat sonoporated with targeted jNP-DNA-MBs obtained two days after sonoporation (rat on right); IVIS image of control targeted MB-DNA (FIG. 11A). Fluorescence scale bar is in 10^9 units, in contrast to scale bar in 10^3 units for current technology MBs-DNA (FIG. 11B). Stacked bar graph of fold-increase in RFP fluorescence 48-hours (t-48 hrs) after sonoporation from baseline (white) comparing control targeted MBs-DNA ($n = 2$) with test targeted jNP-DNA-MBs ($n = 2$). **FIG. 6B**, Plot of melting temperatures (T_m) derived from real-time qRT-PCR analyses of miRNA-126 levels in rat xenograft tumors 48-hrs after sonoporation with targeted jNP-MBs: at 10^8 MBs, 10^{12} jNPs, 27 μ g ds miRNA-126-mimic compared to negative control tumors (non-sonoporated, non-infused). (black, control-T, negative control tumors ($n = 4$); MB 231-T, MDA-MB-231 CSC-derived breast cancer subQ tumors ($n = 4$); Panc1-T, Panc1-CSC-derived subQ tumors ($n = 6$). T_m plots from different samples are identical and consistent with expected T_m for miRNA-126 $\sim 75.4^\circ\text{C}$. **FIG. 6C**, Real-time qRT-PCR cycle threshold (Ct) plots of miRNA-126 comparing sonoporated breast and pancreatic xenograft tumors and control tumors; low Ct indicate high miRNA-126 levels. **FIG. 6D**, Bar graph of Ct values, means $\pm$ sem; dashed line for threshold Ct value for negative expression, ie $\text{Ct} > 34$; **, $p < 0.008$ Kruskal Wallis ANOVA on ranks; *, $p < 0.05$, multiple pairwise comparison of control tumor (tmr) vs other tumors; ns, not significant Kruskal Wallis ANOVA on ranks analyzing normal liver ($n = 4$), kidney ($n = 4$), MB231-tmr, and Panc1-tmr. **FIG. 6E**, Representative Western blot analysis of miRNA-126 target KRAS protein level 48-hr after delivery of miRNA-126 by sonoporation; β -actin protein levels serve as internal control. **FIG. 6F**, ELISA levels of key cytokines/chemokines (IL-1 α , IL-6, IL-4, IL-10, IL-12, IL-13: interleukins; TNF α : tumor necrosis factor alpha, GM-CSF: granulocyte macrophage colony stimulating factor, IFN- γ : interferon gamma, RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted or CCL5) produced by cells which are exposed to non-targeted jNPs in the circulation such as ECs, endothelial cells, MNC, monocytes, T- and B-cell leukocytes, PMNs, neutrophils or polymorphonuclear cells; Mast, complement-activated mast cells. Statistics performed: two-way (jNP-dose x cytokine levels) ANOVA (ns, not significant); cytokine-specific one way

ANOVA (ns, not significant); n = triplicates/rat x 2 rats/group x 3 groups: rats infused with 1) 10^{12} non-targeted jNPs in stroke prone rats; 2) 10^{13} non-targeted jNPs in normal rats, and 3) negative control age-, sex-, genotype-matched rats infused with vehicle saline.

[0070] FIGs. 7A-7B Representative Atomic Force Microscopy (AFM) images showing (**FIG. 7A**) analysis of an individual jNP showing amplitude, topography, and phase images with respective trace plots and (**FIG. 7B**) jNP binding to deposited lambda phage DNA on a mica surface. **FIG. 7A**, AFM amplitude, topography and phase images show the asymmetric avocado-like morphology of the jNP. The shortest radius, as calculated from the center of the iron oxide core to the closest edge, is 11.24 nm. All scaling of AFM images must account for the 20 nm radius tip. **FIG. 7B**, AFM image of deposited lambda-phage DNA strand shows typical Y-shaped DNA-strand with jNPs attached to DNA-strand. The phase diagram (far right) highlights the difference of the iron oxide cores and the DNA strand from the mica surface.

[0071] FIGs. 8A-8C Additional *in vitro* characterization of jNP binding to DNA, and to [DNA]MBs. **FIG. 8A**, Ethidium bromide (EtBr) dye displacement assay. Increasing amount of jNPs can displace EtBr intercalated into DNA base pairs (bp). Because EtBr fluoresces only when intercalated into DNA, loss of fluorescence from baseline indicates displacement of EtBr from pre-mixed DNA-bp:EtBr at a 4:1 ratio. **FIG. 8B**, Representative FACS image of side scatter area (SSC-a) detects increased granularity imparted by jNPs to MBs upon assembly of jNP[DNA]MB-heteroplexes. Y-axis, side scatter-area (SSC-a), corresponds to granularity; X-axis, forward scatter area, corresponds to size. Increased granularity is detected in jNP[DNA]MB-complexes with increased jNP-coverage of MBs: 50% ($\sim 5 \times 10^4$ /MB) vs 1% (10^3 jNPs/MB) coverage of [DNA]MBs. **FIG. 8C**, FACS analysis of different MB stocks (<6 hrs from suspension) with “bead marders” (left); equidistant 15-micron size marked by dash line.

[0072] FIG. 9 Representative MR-imaging of liver and kidney comparing control rats (no jNPs infused) and test rats (10^{12} jNPs/rat or 5 ng Fe/rat) 24 hours after IV infusion. *Ex vivo* 11.7T MR-susceptibility weighted images (SWI) of liver and kidney from xenograft tumor rats show differential hypointensities in the liver and kidney consistent with defined anatomical structures rather than confluent areas of hypointensities seen in xenograft tumors (**FIG. 5B**). *Ex vivo* 9.4T gradient echo (GRE) MR-images of control and test normal rat kidneys confirms T2*-MR hypointensities in jNP-infused rat kidney but not in the renal pelvis. Comparison of liver and kidney MR-images indicate hepatic clearance but not kidney excretion. MR-images were obtained with organs placed in fomblin. Determination of histological correlates needs to be studied

[0073] FIG. 10 Schematic diagram of targeted ultrasound contrast-enhanced imaging. Infused ultrasound contrast agent microbubbles (MBs) are confirmed at approximately 1 minute from bolus infusion (t-1 min) with increased contrast intensity signals (CIS) filling the blood vessel lumen. After 5 min (t-5 min), clearance of unbound “free” MBs is documented with decrease in contrast intensity (dashed line). At t-20 min, only adherent receptor targeted-MBs remain and impart contrast-intensity

signals detected for targeted jNP[DNA]MBs, and for targeted non-jNP[DNA]MBs. To show that increased contrast intensity signals are indeed from attached 'endothelial receptor-targeted' MBs, a high-intensity ultrasound (US) destruct sequence is done at approximately t=21 min, disrupting microbubbles as detected by a drop in CIS from release of MB-gas. Based on blood vessel size, run-off of MB-gas in the circulation can present as either a steep drop in CIS levels (fast runoff), as seen in larger blood vessels, or a sloping decline in CIS levels, as seen in lower flow microvessels, or with slower degradation of MBs. The sequence of imaging-destruction-imaging can be repeated multiple times in different tumor areas (e.g., at t=10, 20, 25, 30-min) depending on stability of the antibody-receptor interaction. jNP, Janus nanoparticle; MB, microbubbles; min, minutes; std, standard; t, timepoint; ⦿, microbubbles; ⦿, disrupted MBs after ultrasound destruct sequence; US, ultrasound.

[0074] FIGs. 11A-11F Representative ultrasound images of spontaneous mammary tumors in post-menopausal rats and corresponding contrast intensity signal (CIS) plots of bolus infusion and subsequent contrast-enhanced imaging using DEspR-targeted non-jNP MBs. **FIG. 11A**, pre-infusion baseline with zero pseudocolor-green contrast intensity signals (CIS). **FIG. 11B**, bolus infusion associated with enhanced CIS; **FIG. 11C**, plot of CIS along time (x-axis), and contrast intensity signals (y-axis) with peak CIS at around 60-65 units. **FIG. 11D**, Contrast-enhanced imaging detects positive DEspR-targeted molecular imaging at the base of the mammary tumor corresponding to tumor feeder vessels, prior to operator-triggered destruction of microbubbles, or pre-destruct. **FIG. 11E**, Image at identical settings after sequence-destruction of microbubbles, or post-destruct, indicating contrast derived from attached MBs present at specified time point. **FIG. 11F**, Scatter plot of CIS showing peak CIS from attached MBs in the region of interest (ROI) for feeder vessels (encircled), destruction sequence marked by the red line/arrow, followed by post-destruction analysis showing a drop in CIS, which indicates destruction (bursting) of attached receptor-targeted microbubbles with subsequent elimination of contrast-enhancement by blood flow.

[0075] FIG. 12 Baseline imaging of zero-background *in vivo* fluorescence using IVIS. Zero-fluorescence shown at baseline prior to delivery of red fluorescent protein (RFP) minigene-DNA by sonoporation using jNP[DNA]MB-heterplexes. Spontaneous mammary tumor in post-menopausal rat encircled in red. Any fluorescence after sonoporation indicates presence of functional red fluorescent protein, which implies that minigene-DNA was delivered intracellularly, transcribed into RNA, and translated into a functional protein that exhibits red-fluorescence.

[0076] FIG. 13 Analysis of the amount of double strand (ds) plasmid DNA payload (4707 bp-long plasmid dsDNA) assembled into MBs-DNA compared with payload amount assembled into jNP-DNA-MBs. At 1hr, 6-hrs, and 24-hrs, jNP-DNA-MBs had significantly more dsDNA payload (up to 9-fold, 6-fold and 2-fold greater at 1-, 6- and 24-hours) than the amount incorporated into MBs-DNA, $P < 0.0013$, two-way ANOVA (time x delivery system), showing that significant variation is due to the delivery system MBs-DNA vs jNP-DNA-MBs, and not due to time. Identical dsDNA and MB (MicroMarker Visual Sonics Inc) stocks were used. It is noted that at 1 and 6 hours after assembly of

jNP-DNA-MBs, the DNA payload contains $\geq 10^4$ dsDNA molecules per MB in jNP-DNA-MBs. This suggests that jNPs have an increased DNA payload by 9-fold at 1 hour compared to MBs-DNA. However, we also note that although MBs-DNA have less DNA bound than jNP-DNA-MBs, we detect $\geq 10^{11}$ DNA molecules in 10^8 MBs, or 10^3 DNA molecules per MB in MBs-DNA

[0077] FIGs. 14A-14B Comparative analysis of *in vivo* fluorescence imaging by IVIS. **FIG. 14A**, Representative *in vivo* fluorescence images by IVIS at baseline and 48-hours after sonoporation. Zero-fluorescence shown at baseline prior to delivery of red fluorescent protein (RFP) minigene-DNA by sonoporation using targeted jNP-DNA-MBs and targeted MBs-DNA. Spontaneous mammary tumor in post-menopausal rat is designated by red arrows. Color bar scale: 5.42×10^8 to 1.7×10^9 **FIG. 14B**, Comparative *in vivo* fluorescence images obtained by IVIS-imaging 48-hours after sonoporation. Sonoporation was done using MBs-DNA immediately after infusion while MBs filled the intravascular space (< 5 min) using a SoniGene sonoprotector. Identical number of MBs and amount of RFP-DNA, and sonoporation settings were used. Color bar scale: 1176 to 3930.

[0078] FIGs. 15A-15B *In vitro* analysis of Terminal Complement Complex (Sc5b-9) levels in plasma with and without jNPs. **FIG. 15A**, Comparative analysis of the terminal complement complex, Sc5b-9 levels (ng/ml) shows non-elevation, hence no complement activation, when using 10^{10} to 10^{13} jNPs/ml plasma compared with normal human plasma. Differences in means ($n = 6$ from 2 independent jNP preparations) are not significant, Kruskal-Wallis ANOVA, followed by Dunns all pairs multiple comparisons test. Testing spans the range of jNPs/ml ($\leftrightarrow$) used *in vivo* for DNA and miRNA-126 delivery, MRI and safety studies: 10^{12} jNPs/rat dose $\sim 5 \times 10^{11}$ jNPs/ml plasma; 10^{13} jNPs/rat dose $\sim 5 \times 10^{12}$ jNPs/plasma. Normal plasma (0 jNPs/ml) level = 428.1 ± 86 . **FIG. 15B**, Standard curve for Sc5b-9 complex as per manufacturer's specifications (Quidel™, MicroVue SC5b 9 Plus Enzyme Immunoassay). All measurements were done in appropriate dilutions to obtain values within the linear range of the standard curve.

[0079] FIGs. 16A-16D Demonstration that jNPs can be targeted with different antibody targeting moieties using the same covalent-layering methodology and function to deliver reporter function DNA (RFP-minigene construct) by sonoporation *in vitro* to Panc1 tumor cells. **FIG. 16A**, Expression levels of red fluorescent protein (RFP) (red fluorescent-positive cells) in Panc1 tumor cells sonoporated using DEspr-targeted jNP[DNA]MBs. **FIG. 16B**, Expression levels of RFP-positive expression sonoporated using non-jNP control DNA-MBs wherein the antibody is coupled by avidin-biotin. **FIG. 16C**, RFP expression levels in Panc1 tumor cells of CD44-targeted jNPs. **FIG. 16D**, quantitative analysis reveals statistically significant enhancement of DNA-delivery compared with non-jNP DNA-MBs. ***, $P < 0.0001$, One way ANOVA with Tukey's multiple comparison testing. Identical numbers of jNPs (10^{12} jNPs/ 10^8 MBs), amounts of DNA ($30 \mu\text{g}/10 \text{ ml}$), MBs ($10^8 / 10 \text{ ml}$), and % confluence of Panc1 tumor cells were used. Sonoporation was performed using 0.153 MPa mechanical index after 45 minutes of incubation with constant rotation, and all unbound MBs

removed by media change. These data indicate that covalent layering methodology facilitates modular targeting: jNPs can be targeted using different antibodies to target different cell surface receptors, for example, DEspR (dual endothelin1/VEGFsp receptor), CD44 (receptor for hyaluronic acid), both present on cancer cells.

[0080] FIGs. 17A-17L Representative epi-fluorescent microscopy images showing best image with *in vitro* fluorescence from expression of delivered reporter minigene-DNA (red fluorescent protein, RFP) to Panc1 cells 48-hours after sonoporation. **FIG. 17A**, Panc1 cells sonoporated with test-construct: targeted jNP-DNA-MBs exhibit RFP-positive expression. **FIG. 17B**, Control-1: Panc1 cells sonoporated with targeted MBs-DNA. **FIG. 17C**, Control-2: Panc1 cells sonoporated with DEspR targeted jNP-DNA (no MBs). **FIG. 17D**, Control-3: non-sonoporated Panc1 cells with DEspR-targeted jNP-DNA-MBs. **FIG. 17E**, Control-4: non-sonoporated Panc1 cells with DEspR-targeted MB-DNA (no jNP). **FIG. 17F**, Control-5: non-sonoporated Panc1 cells with DEspR-targeted jNP-DNA (no MBs). **FIG. 17G**, Control-6: Panc1 cells sonoporated with non-targeted (isotype IgG) jNP-DNA-MBs. **FIG. 17H**, Control-7: Panc1 cells sonoporated with non-targeted (isotype) MBs-DNA. **FIG. 17I**, Control-8: Panc1 cells sonoporated with non-targeted (isotype) jNP-DNA. **FIG. 17J**, Kruskall Wallis non-parametric ANOVA $P < 0.0001$ of 9 study groups represented in panel a: n=16, b: n=12, c: n=6, d: n=3, e: n=4, f: n=4, g: n=12, h: n=3, i: n=3, photomicroscopy images (0.54 mm^2) of fluorescent areas, if any. Post-hoc test: Dunn's multiple comparisons test for all pair-wise comparison to panel-a, $P < 0.05$; bar = 100 μm , panels 17A-17I. Taken together, these data indicate that jNP-DNA can deliver reporter function DNA via internalization of jNP-DNA as a function of the targeting moiety through receptor internalization. In this example, DEspR is internalized upon engagement with its antibody. **FIG. 17K**, Relative efficacy of *in vitro* DNA delivery by jNP-MBs compared with current technology cationic microbubbles (CMBs) reported. Parameters for comparison: peak % cell transfection, and peak % cell viability. **FIG. 17L**, Comparison of DNA and MB doses used per ml media in *in vitro* transfection experiments.

[0081] FIG. 18 Non-targeted jNPs were infused intravenously and MRI was performed 24 h later. A gradient echo with flow compensation (GEFC) was performed *ex vivo* on xenograft pancreatic peritoneal metastatic tumors in nude rats. Two echo times (TE) of 6.5 ms and 13 ms were used to analyse the tumors. Images show negative (darker) contrast enhancement of tumors in the presence of non-targeted jNPs in contrast to negative control. This negative contrast enhancement is increased with longer TE times from 6.5 ms to 13 ms consistent with the presence of ultrasmall superparamagnetic iron-oxide nanoparticles (USPIONS) in jNPs. Bar = 0.5 cm; t, tumor; gut, small intestine.

[0082] FIG. 19 Comparison with Resovist™ and Ferridex™ reveals efficiency of jNPs in that dose measured as Fe g/kg is much smaller in jNPs, assuming similar sensitivity/specificity for MRI-detection of tumors. Notably, Ferridex™ is approved for liver cancer only, indicating Ferridex™ is

taken up by the normal reticuloendothelial system in the liver, which is absent or abnormal in liver tumors.

[0083] FIG. 20 Comparative analysis of *in vitro* DNA delivery. Comparison of amount of DNA loaded onto each microbubble (MB) measured as DNA in pictograms (pg)/MB and mechanical index (MI) used to sonoporated in megapascals (Mpa). Data for CMB-1 and CMB-2 compiled from Phillips *et al.* (2012) *IEEE Transaction on Ultrasonics, Ferroelectrocs and Frequency Control* 59:1596-1601, and Jin *et al.* (2013) *PLoS One* 8:e76544, respectively. Data for CMB-3 compiled from Zhou *et al.* (2015) *Theranostics* 4(4):399-417.

[0084] FIG. 21 Comparative analysis of *in vivo* ultrasound contrast-enhanced imaging or molecular imaging. Comparison *in vivo* molecule imaging efficacy of jNP-MB heteroplex with cationic microbubbles (CMBs) by ultrasound-contrast enhanced imaging. Efficacy is measured as peak contrast intensity signals (CIS). *Inset*: Comparison of microbubble size range (MB) and time when *in vivo* molecular imaging was performed. Data for CMB-3 and CMB-4 compiled from Zhou *et al.* (2015) *Theranostics* 4(4):399-417 and Xie *et al.* (2012) *JACC Cardiovasc Imaging* 5:1253-1262, respectively.

[0085] FIGS. 22A-22C Comparative analysis of *in vivo* ultrasound-mediated delivery of reporter function-DNA. **FIG. 22A**, Average photons per second per area cm^2 per solid angle of one steradian (sr) in jNP-MBs (n = 4 tumors, with 2 measured at 24- and 48 hrs after sonoporation) compared with published CMB reports of reported CMBs*: targeted: CMB-3, Zhou *et al.* (2015) *Theranostics* 4(4):399-417; CMB-4, Xie *et al.* (2012) *JACC Cardiovasc Imaging* 5:1253-1262; non-targeted: CMB-5: Panje *et al.* (2012) *Theranostics* 2:1078-1091; CMB-6: Sun *et al.* (2014) *J Control Release* 182:111-120; CMB-7: Wang *et al.* (2012) *Radiology* 264:721-732. All used IVIS imaging of fluorescence or bioluminescence. **FIG. 22B**, Bar graph of amount of DNA loaded onto MBs in picogram (pg), and the mechanical index (MI, in megapascals (MPa) used for *in vivo* sonoporation or ultrasound-mediated DNA delivery. **FIG. 22C**, Bar graph of dose of DNA and microbubbles (MB) used per gram body weight. jNP-MBs were tested in rat tumor model; CMBs were tested in mouse models.

DETAILED DESCRIPTION

[0086] Provided herein are theranostic compositions comprising a Janus nanoparticle-coated microbubble that are useful for imaging (*e.g.*, MRI, or ultrasound) and for delivering a therapeutic or bioactive agent (*e.g.*, nucleic acid(s), drugs, etc).

Definitions

[0087] As used herein, the terms “functionalization”, or “functionalized” are used to describe modifications to an iron oxide particle and can encompass, for example attaching a polymer, a linker, a

particle, a targeting moiety, a chemical side group, a ligand, or any combination of these.

Functionalization also encompasses attaching, for example a particle to a linker molecule on the surface of the cell

[0088] As used herein, “a” or “an” means at least one, unless clearly indicated otherwise. As used herein, to “prevent” or “protect against” a condition or disease means to hinder, reduce or delay the onset or progression of the condition or disease.

[0089] The term “statistically significant” or “significantly” refers to statistical significance and generally means two standard deviations (2SD) or more above or below normal or a reference. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[0090] As used herein, the term “comprising” means that other elements can also be present in addition to the defined elements presented. The use of “comprising” indicates inclusion rather than limitation.

[0091] As used herein the term “consisting essentially of” refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[0092] The term “consisting of” refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0093] Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0094] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used in connection with percentages can mean $\pm 1\%$.

[0095] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc.,

1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), and Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005 (ISBN 0471142735), the contents of which are all incorporated by reference herein in their entireties.

Imaging using Contrast Agents

[0096] The use of contrast agents in medical diagnostic techniques to enhance tissue contrast or to facilitate the study of body processes is well established. The manner in which contrast enhancement occurs varies from imaging modality to imaging modality but in magnetic resonance imaging most of the conventional contrast agents derive their contrast enhancing power from their effects on the tissue selection times.

[0097] MRI, the most representative tomography technique, is a method used to obtain 3-dimensional images non-invasively and that has been widely used for the diagnosis of disease owing to its excellent contrast and spatial resolution. One of the great advantages of MR imaging is the high degree of intrinsic tissue contrast present from tissue relaxations times.

[0098] MRI contrast agents are used to improve the visibility of internal body structures by increasing contrast between normal tissues and abnormal tissues in MRI. MRI contrast agents alter the T1 and T2 relaxation times of tissues and body cavities where they are present. Depending on the image weighting, this can give a higher or lower signal. Most MRI contrast agents work through shortening the relaxation time of protons located nearby. Generally, MRI contrast agents are divided into two groups: paramagnetic contrast agents and superparamagnetic contrast agents.

[0099] Superparamagnetic nanoparticles comprising superparamagnetic iron oxide (SPIO) as magnetite (Fe_3O_4) and maghemite (Fe_2O_3) are used clinically. Characteristics of superparamagnetic iron oxide particles include (i) hydrophobicity, (ii) a high ratio of volume to surface area, (iii) can form clusters, (iv) has low stability indicating that both structural and magnetic characteristics can be easily changed; (v) biodegradable; and (4) toxicity. Surface modifications of the particle can be used to overcome toxicity when administered to subjects.

[0100] The application of superparamagnetic iron oxide in MR-imaging derives from their ideal combination of a large effect on tissue signal intensity which results in powerful contrast enhancement, and their highly specific targetability. The potential targets of particulate agents are many, depending on the administration route and the physicochemical properties of the particulate

material, in particular the particle size and surface characteristics. Their two main applications are by enteral administration for gastrointestinal investigations, and by parenteral administration for investigations of the blood pool compartment and/or the reticuloendothelial system and regions of its anatomical distribution, e.g. the liver, spleen, bone marrow, and lymph nodes. Ultrasmall iron oxide particles with a diameter of less than approximately 30 nm have a relatively long intravascular half-life when compared to larger conventional iron oxide particles. In addition to the T2 shortening typically associated with the iron oxide particles, ultrasmall particles also produce T1 shortening thereby increasing signal within the vessels. Recent advances in particulate agents have also made targeting with receptor ligands or antibodies/antibody fragments possible. A brief summary of described applications of different superparamagnetic agents is given in Fahlvik et al., JMRI 3:187-194 (1993).

Superparamagnetic Iron Oxide particles

[0101] Iron oxide based magnetic nanoparticles have been widely used in a variety of biomedical applications such as magnetic separation, magnetic resonance imaging, hyperthermia, magnetically-guided drug delivery, tissue repair, and molecular diagnostics. For most applications, a polymeric coating is needed to improve the nanoparticles' aqueous stability, biocompatibility and conjugation properties. Typically, dextran-coated iron oxide nanoparticles have been successfully used as magnetic resonance imaging (MRI) contrast agent, due to their strong ability to dephase water protons in surrounding tissue, which results in a decrease in the MRI signal. In addition, the dextran coating can be cross-linked and functionalized with amino groups to facilitate the conjugation of targeting ligands for MRI and in vitro diagnostics applications.

[0102] Superparamagnetic iron oxide crystals can be prepared using any method known to those of skill in the art. For example, superparamagnetic iron oxide crystals can be precipitated from an aqueous solution of a mixture of iron salts by rapid addition of a base to pH above 10 while stirring vigorously or during sonication. A wide range of iron salts can be used such as $\text{FeCl}_2 \cdot n\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot n\text{H}_2\text{O}$, Fe(III) citrate, Fe(II) gluconate, $\text{FeSO}_4 \cdot n\text{H}_2\text{O}$, $\text{Fe}_2(\text{SO}_4)_3$, Fe(II)oxalate, $\text{Fe}(\text{NO}_3)_3$, Fe(II) acetylacetonate, Fe(II) ethylenediammonium sulfate, Fe(II) fumarate, Fe(III) phosphate, Fe(III) pyrophosphate, ammonium Fe(III)citrate, ammonium Fe(II)sulfate, ammonium Fe(III) sulfate and ammonium Fe(II) oxalate. The ratio between ferrous and ferric iron can be within the range of 1:5 to 5:1. Such precipitated iron oxide crystals can be represented by the following formula: $(\text{FeO})_x \cdot \text{Fe}_2\text{O}_3$ where x can be a number in the range of $0 \leq x \leq 1$. Maghemite, $\gamma\text{-Fe}_2\text{O}_3$, represents the lower x-value while magnetite, Fe_3O_4 , represents the upper value for x. Bases for precipitating superparamagnetic iron oxide can include a wide range of strong inorganic or organic bases such as NaOH, NH_4OH , LiOH, KOH, triethylamine and guanidine. In general, the counter ions for the metal and the base should be physiologically tolerable ions so as to minimize the need for the precipitated crystals to be cleansed of potentially toxic by-products.

[0103] The precipitation of iron oxide or, alternatively, the co-precipitation of iron oxide and at least one polymer, can take place in water, a mixture of water and organic solvent(s), or in a viscous medium. As an example, organic solvents like methanol, ethanol, acetone, ethers, and hexane may be used. The viscous matrix can consist of hydrogels of polysaccharides or polyamines, tri-iodinated aromatics, glycerol or polyethylene- and polypropylene-glycols. Precipitation from aqueous solution free from non-physiologically tolerable co-solvents is of course preferred as again the need for post-production purification is reduced. Nanoparticles of a desired size can be isolated and purified by differential column chromatography, centrifugation, and dialysis. Electron microscopy, X-ray diffraction and laser light scattering which may be used to measure median diameter of the resulting nanoparticles.

[0104] In one embodiment, the Janus nanoparticle is less than 75 nm in size (*e.g.*, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15 nm or smaller).

[0105] In another embodiment, the superparamagnetic iron oxide nanoparticle is less than 20 nm in size (*e.g.*, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 nm or smaller).

Carrier Face

[0106] In some embodiments, the carrier face of the iron oxide preparation has a polyelectrolyte structure. Without wishing to be bound by theory, polyelectrolytes have the added advantage of improving stability and minimizing toxicity of iron oxide preparations. Polyelectrolytes can include polyanionic and polycationic compounds or a mixture of these that bind strongly to the iron oxide surface through *e.g.*, multiple attachment points.

[0107] Coating materials can be grouped depending on their charge and functional groups, such as negatively charged polymers with functional groups containing phosphorus or sulphur atoms or carboxy groups, and positively charged polymers with functional groups containing nitrogen atoms. Examples of negatively charged polymers are certain modifications of carboxycellulose, alginates, carrageenans, polygalacturonate, heparins and heparinoid compounds such as chondroitin-4-sulphate, dermatansulphate, keratin sulphate and hyaluronate, synthetic polymers such as poly-styrenesulphonate and amino acids such as polyglutamate and polyaspartate. Examples of positively charged polymers include chitosan and polylysine. In one embodiment, the polymer(s) must comprise multiple (more than one) functional groups to secure multiple attachment points to the metal oxide crystals and give the particles a charged surface.

Targeting Face

[0108] The targeting face of Janus nanoparticle comprises at least one targeting moiety, *e.g.*, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25 or more targeting moieties.

[0109] The targeting moiety can be a small molecular weight compound (e.g. mono or disaccharide) or a macromolecule (e.g. protein, polysaccharide) and can be associated with the nanoparticle by covalent or non-covalent interactions.

[0110] In certain embodiments, the targeting moiety can target the theranostic composition to a binding pair, antibodies, monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')₂ fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent binding reagents including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ((scFv)₂ fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (i.e., leucine zipper or helix stabilized) scFv fragments; and other targeting moieties include for example, aptamers, receptors, ligands, and fusion proteins.

[0111] In general, antibodies can be raised to any desired antigen, using the many conventional techniques now well known in the art.

[0112] When targeting a cancer cell, a specific antigen can be chosen from cell surface markers such as, for example, members of the MUC-type mucin family, an epithelial growth factor (EGFR) receptor, a carcinoembryonic antigen (CEA), a human carcinoma antigen, a vascular endothelial growth factor (VEGF) antigen, a melanoma antigen (MAGE) gene, family antigen, a T/Tn antigen, a hormone receptor, growth factor receptors, a cluster designation/differentiation (CD) antigen, a tumor suppressor gene, a cell cycle regulator, an oncogene, an oncogene receptor, a proliferation marker, an adhesion molecule, a proteinase involved in degradation of extracellular matrix, a malignant transformation related factor, an apoptosis related factor, a human carcinoma antigen, glycoprotein antigens, DF3, 4F2, MGFM antigens, breast tumor antigen CA 15-3, calponin, cathepsin, CD 31 antigen, proliferating cell nuclear antigen 10 (PC 10), and pS2. For other forms of cancer and their metastases, a specific marker or markers may be selected from cell surface markers such as, for example, vascular endothelial growth factor receptor (VEGFR) family, a member of carcinoembryonic antigen (CEA) family, a type of anti-idiotypic mAB, a type of ganglioside mimic, a member of cluster designation differentiation antigens, a member of epidermal growth factor receptor (EGFR) family, a type of a cellular adhesion molecule, a member of MUC-type mucin family, a type of cancer antigen (CA), a type of a matrix metalloproteinase, a type of glycoprotein antigen, a type of melanoma associated antigen (MAA), a proteolytic enzyme, a calmodulin, a member of tumor necrosis factor (TNF) receptor family, a type of angiogenesis marker, a melanoma antigen recognized by T cells (MART) antigen, a member of melanoma antigen encoding gene (MAGE) family, a prostate membrane specific antigen (PMSA), a small cell lung carcinoma antigen (SCLCA), a T/Tn antigen, a hormone receptor, a tumor suppressor gene antigen, a cell cycle regulator antigen, an oncogene antigen, an oncogene receptor antigen, a proliferation marker, a proteinase involved in

degradation of extracellular matrix, a malignant transformation related factor, an apoptosis-related factor, and a type of human carcinoma antigen.

Encapsulation of jNP

[0113] The Janus nanoparticles (jNP) described herein can be coated with a polymeric shell comprising *e.g.*, non-biodegradable or biodegradable polymers, however, biodegradable polymers are preferred. The polymer can be natural or synthetic, with synthetic polymers being preferred due to the better characterization of degradation and, where appropriate, release profile of an incorporated agent. The polymer can be selected based on the period over which degradation or release of an agent is desired, generally in the range of at several weeks to several months, although shorter or longer periods may be desirable.

[0114] Representative, non-limiting examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(hydroxybutiric acid), poly(valeric acid), and poly (lactide-co-caprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin, and other hydrophilic proteins. The compositions described herein can also comprise bioerodible hydrogels which are prepared from materials and combinations of materials such as polyhyaluronic acids, casein, gelatin, gluten, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly (isobutyl methacrylate), poly (hexylmethacrylate), poly (isodecyl methacrylate), poly (lauryl methacrylate), poly (phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate). Preferred biodegradable polymers are polyglycolic acid, polylactic acid, copolymers of glycolic acid and L- or D,L-lactic acid, and copolymers of glycolide and L- or D,L-lactide. Those of skill in the art will appreciate that the molecular weight of the polymer can be varied to tailor the properties of the particle.

[0115] The foregoing exemplary natural and synthetic polymers are, of course, either readily available commercially or are obtainable by condensation polymerization reactions from the suitable monomers, comonomers, or oligomers. For instance, homopolymers and copolymers of glycolic and lactic acids can be prepared by direct poly-condensation or by reacting glycolide and lactide monomers (Gilding, D. K., et al., *Polymer*, 20:1459 (1979)).

[0116] The compositions described herein can also include a conjugate of a lipid and a hydrophilic polymer, referred to as a 'lipopolymer.' Lipopolymers can be obtained commercially or can be synthesized using known procedures. For example, lipopolymers comprised of methoxy(polyethylene glycol) (mPEG) and a phosphatidylethanolamine (*e.g.*, dimyristoyl phosphatidylethanolamine, dipalmitoyl phosphatidylethanolamine, 1,2-distearoyl-3-sn-glycerophosphoethanolamine (distearoyl

phosphatidylethanolamine (DSPE)), or dioleoyl phosphatidylethanolamine) can be obtained from Avanti Polar Lipids, Inc. (Alabaster, Ala.) at various mPEG molecular weights (350, 550, 750, 1000, 2000, 3000, 5000 Daltons). Lipopolymers of mPEG-ceramide can also be purchased from Avanti Polar Lipids, Inc. Preparation of lipid-polymer conjugates are known in the art and are not described in detail herein.

[0117] The hydrophobic component of the lipopolymer can be virtually any hydrophobic compound having or modified to have a chemical group suitable for covalent attachment of a hydrophilic polymer chain. Exemplary chemical groups are, for example, an amine group, a hydroxyl group, an aldehyde group, and a carboxylic acid group. Preferred hydrophobic components are lipids, such as cholesterol, cholesterol derivatives, sphingomyelin, and phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), where the two hydrocarbon chains are typically between about 8-24 carbon atoms in length, and have varying degrees of unsaturation. These lipids are exemplary and are not intended to be limiting, as those of skill can readily identify other lipids that can be covalently modified with a hydrophilic polymer and incorporated into the particles described herein. In some embodiments, the lipopolymer is formed of polyethylene-glycol and a lipid, such as distearoyl phosphatidylethanolamine (DSPE), PEG-DSPE. PEG-DSPE has some degree of biodegradability *in vivo*, by virtue of the hydrolysable bonds between the fatty acids and the glycerol moiety.

[0118] It is also contemplated herein that the iron oxide nanoparticles are encapsulated in a polymer, for example, PLGA such that the PLGA forms a shell around the iron oxide nanoparticle. Encapsulation of the compositions described herein can be used *e.g.*, to deliver poorly soluble drugs. The polymer-coated nanoparticles remain magnetic and can be prepared using the techniques described herein, for example, a surface layer-by-layer covalent coupling method. Other polymer shell compositions are also contemplated herein.

Bioactive Agents

[0119] In some embodiments, the compositions described herein further comprise a biologically active agent. A variety of different pharmaceutical/therapeutic agents can be used in conjunction with the methods and compositions described herein and include, but are not limited to, small molecules, proteins, antibodies, peptides and nucleic acids. In one embodiment, the bioactive agent comprises a nucleic acid, such as DNA, RNA, siRNA, miRNA, oligonucleotide, shRNA, etc.

[0120] In general, bioactive agents which can be administered via the invention include, without limitation: anti-infectives such as antibiotics and antiviral agents; chemotherapeutic agents (i.e. anticancer agents); anti-rejection agents; analgesics and analgesic combinations; anti-inflammatory agents; hormones (e.g., steroids); growth factors (e.g., bone morphogenic proteins (i.e. BMP's 1-7), epidermal growth factor (EGF), fibroblast growth factor (i.e. FGF 1-9), platelet derived growth factor

(PDGF), insulin like growth factor (IGF-I and IGF-II), transforming growth factors (i.e. TGF- β -III), vascular endothelial growth factor (VEGF)); anti-angiogenic proteins such as endostatin, and other naturally derived or genetically engineered proteins, polysaccharides, glycoproteins, or lipoproteins. Additionally, the particles described herein can be used to deliver any type of molecular compound, such as for example, pharmacological agents, vitamins, sedatives, steroids, hypnotics, antibiotics, chemotherapeutic agents, prostaglandins, and radiopharmaceuticals. The compositions described herein are suitable for delivery of the above materials and others including, but not limited to, proteins, peptides, nucleotides, carbohydrates, simple sugars, anti-thrombotics, anti-metabolites, growth factor inhibitors, growth promoters, anticoagulants, antimitotics, fibrinolytics, anti-inflammatory steroids, drugs, and monoclonal antibodies, among others.

[0121] In one embodiment, the bioactive agent is a chemotherapeutic agent. Chemotherapeutic agents are typically cytotoxic antineoplastic drugs that find use as part of a standardized regimen of cancer treatment. Chemotherapy can be given with a curative intent or it may aim to prolong life or to palliate symptoms. It is often used in conjunction with other cancer treatments, such as radiation therapy or surgery. Certain chemotherapeutic agents also have a role in the treatment of other conditions, including ankylosing spondylitis, multiple sclerosis, Crohn's disease, psoriasis, psoriatic arthritis, rheumatoid arthritis, and scleroderma.

Linker Groups

[0122] In some embodiments, the compositions provided herein further comprise a linker group, for example, between the polymer and a targeting moiety or between the polymer and the iron oxide core. In some embodiments the linker moiety is cleavable by an enzyme e.g., an esterase present in a target tissue or cell. In some embodiments, the linker moiety is cleavable by one type of esterase (e.g., a first type of an esterase present in a first cell type, e.g., a kidney cell) and is not cleavable by another type of esterase (e.g., a second type of esterase present in a second cell type, e.g., a cardiac cell). Thus, cleavage of the linker and activation of the drug and detectable moieties can be specific for a targeted cell type.

[0123] In one embodiment, the linker group comprises a functionalized phospholipid, such as a thiol-functionalized phospholipids, an amine functionalized phospholipids, or any combination thereof. In another embodiment, the amine-functionalized phospholipids can comprise DSPE-PEG(2000)Carboxylic Acid, DSPE-PEG(2000)Maleimide, DSPE-PEG(2000)PDP, DSPE-PEG(2000)Amine, DSPE-PEG(2000)Biotin, or any combination thereof. In another embodiment, the thiol-functionalized phospholipids can comprise phosphatidylthioethanol (PTE).

[0124] The present invention may be as defined in any one of the following numbered paragraphs:

[0125] 1. A composition comprising: a microbubble with a Janus nanoparticle associated with the surface, wherein the Janus nanoparticle comprises a superparamagnetic iron oxide nanoparticle

having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.

- [0126] 2. The composition of paragraph 1, wherein the Janus nanoparticle is less than 75 nm in size.
- [0127] 3. The composition of paragraph 1 or 2, wherein the superparamagnetic iron oxide nanoparticle is less than 20 nm in size.
- [0128] 4. The composition of paragraph 1, 2, or 3, wherein the Janus nanoparticle is asymmetrically shaped.
- [0129] 5. The composition of paragraph 4, wherein the Janus nanoparticle is avocado shaped.
- [0130] 6. The composition of paragraph 5, wherein the targeting face is situated at the upper part of the avocado shaped nanoparticle, and the carrier face is situated at the round, bottom part of the avocado shaped nanoparticle.
- [0131] 7. The composition of any one of paragraphs 1-5, wherein the targeting face comprises a targeting moiety selected from the group consisting of: a targeting peptide, a polyclonal antibody, a monoclonal antibody, an antibody binding fragment, a composite antibody, a recombinant antibody, a cell penetrating peptide and a matrix interacting peptide.
- [0132] 8. The composition of paragraph 7, wherein the targeting moiety binds to a cell surface or cell matrix protein.
- [0133] 9. The composition of any one of paragraphs 1-8, wherein the carrier face comprises a polymer.
- [0134] 10. The composition of paragraph 9, wherein the polymer comprises a polyelectrolyte.
- [0135] 11. The composition of paragraph 10, wherein the polyelectrolyte is a cationic polyelectrolyte or an anionic polyelectrolyte.
- [0136] 12. The composition of any one of paragraphs 1-11, wherein the Janus nanoparticle comprises an iron oxide core with a PEG brush.
- [0137] 13. The composition of any one of paragraphs 1-12, wherein the PEG brush is a mixed 3:1 short-long PEG brush.
- [0138] 14. The composition of any one of paragraphs 1-13, wherein the moieties in the targeting and carrier faces are covalently coupled or tethered to the long PEG in the mixed PEG-brush.
- [0139] 15. The composition of any one of paragraph 1-14, wherein the iron oxide core is encapsulated within a polymer.
- [0140] 16. The composition of paragraph 15, wherein the polymer-encapsulated iron oxide core further comprises a bioactive agent.
- [0141] 17. The composition of any one of paragraphs 1-16, wherein the composition further comprises a bioactive agent.
- [0142] 18. The composition of any one of paragraphs 1-17, wherein the bioactive agent comprises a nucleic acid molecule.

- [0143] 19. The composition of paragraph 18, wherein the nucleic acid molecule is sandwiched between the microbubble and the carrier face of the Janus nanoparticle.
- [0144] 20. The composition of any one of paragraphs 1-18, wherein the composition is formulated for use as a magnetic resonance imaging (MRI) contrast agent.
- [0145] 21. The composition of any one of paragraphs 1-20, wherein the MRI contrast agent comprises a shorter T2*-relaxivity.
- [0146] 22. The composition of paragraph 20, wherein the MRI contrast agent is a negative contrast agent.
- [0147] 23. A composition for use as an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.
- [0148] 24. The composition of paragraph 23, wherein the MRI contrast agent is a negative contrast agent.
- [0149] 25. The composition of paragraph 23 or 24 for use in detection and/or monitoring of electron paramagnetic resonance (EPR) imaging.
- [0150] 26. A composition for use as an MRI contrast agent for target-specific imaging, the composition comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged.
- [0151] 27. The composition of paragraph 26, wherein the target to be imaged is a tumor.
- [0152] 28. A method for imaging, the method comprising: (a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another, and (b) performing an MRI on the subject.
- [0153] 29. The method of paragraph 28, wherein the MRI comprises electron paramagnetic resonance (EPR) imaging.
- [0154] 30. A method for target-specific imaging, the method comprising: (a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged, and (b) performing an MRI on the subject.
- [0155] 31. Use of a composition of any one of paragraphs 1-27 as an MRI contrast agent.
- [0156] 32. Use of a composition of any one of paragraphs 1-27 as a drug delivery device.

[0157] 33. Use of a composition of any one of paragraphs 1-27 as a nucleic acid delivery device.

EXAMPLES

[0158] Nanotechnologies present great untapped potential to solve much needed minimally invasive cancer monitoring and effective delivery of nucleic acid, DNA and RNAi (siRNA/miRNA) therapies that directly address genetic and epigenetic changes associated with non-curable cancers. However, to date multifunctional nanotechnologies have shown key limitations in efficacy and/or safety that impede clinical translation.¹⁻³ Even with tumor-specific targeting, nanoplateforms dependent on endocytosis/macropinocytosis for intracellular delivery remain inefficient due to limited (< 2%) escape of nucleic acid-cargo from endosomes into the cytosol^{1,4} and the likelihood that the majority (>70%) of internalized nucleic acid-cargo will be exocytosed.⁵ Even if delivered intact, DNA/RNAi-payloads must integrate into cellular machinery for functional efficacy.¹ Moreover, many of the efficient *in vitro* nano-delivery systems, *e.g.* self-assembled PEI-polyplexes, display toxic effects arising from excess cationic material and/or from release of high MW or branched-PEI_{25kDa} resulting from disassembly *in vivo*.² While sonoporation-induced cytosolic-delivery of nucleic acids carried by microbubbles (MBs) may bypass some drawbacks associated with endosomal uptake, current MB technologies remain inefficient.^{6,7} New nanoparticle designs should thus explore more efficient endocytosis-independent mechanisms to deliver DNA/RNAi-payloads, and at the same time, eliminate excess unincorporated materials.¹ Ideally, such a particle would also be modular, multifunctional,¹² and inherently versatile to accommodate multiple targeting and payload moieties to address genetic/epigenetic heterogeneity in cancers. Other considerations include charge, hydrophilicity, shape, and size,⁸⁻¹¹ as well as non-aggregability.

[0159] Janus nanoparticle designs spatially separate multifunctional components to eliminate confounding multicomponent steric/chemical interactions,^{12,13} and thus have potential to meet criteria for versatility and multifunctionality. Although existing jNPs are typically >100nm,¹²⁻¹⁷ and hence too large for efficient cellular uptake and nucleic acid delivery, efficient delivery would be possible with ultrasmall jNPs capable of forming composites with MBs. Such composites offer the additional advantage of unexplored dual mode magnetic resonance (MR) and ultrasound diagnostic potential. This type of dual mode MR/ultrasound imaging has already been described for “nano-in-micro” composites of iron oxide nanoparticles encapsulated within microbubbles^{1,14} or within their shell,^{18,19} but these composites are not designed for nucleic acid delivery.^{1,14,18-19} Based on inherent advantages of Janus-designs and nano-in-micro composites, it was hypothesized that covalent-layered ultrasmall jNPs alone or composite^{16,17} could facilitate tumor-specific detection and targeted nucleic acid delivery with modular versatility in targeting and payload.

[0160] Presented herein, in part, is the novel design and preparation of ultrasmall, covalent-layered jNPs comprised of a 10-nanometer (nm) iron oxide core surrounded by opposing antibody-targeting and cationic-payload faces. These jNPs and self-assembled jNP-MBs, comprise a modular targetable

nanoplatfrom that enhances MR- and ultrasound-imaging of tumor-specific features, and delivers both DNA and miRNA to tumors more efficiently and safely than current technologies. These results demonstrate that jNPs and jNP-MBs provide a versatile and tunable theranostic platform to advance cancer detection and delivery of nucleic acid therapy.

[0161] Provided herein, in one embodiment, are Janus nanoparticles (jNPs; 20-30 nm by Cryo-TEM), consisting of an ultra-small (10 nm) superparamagnetic iron-oxide (USPIO) core with a mixed PEG-brush onto which a payload-face is covalently layered on one side and an antibody targeting-face layered on the opposing side. The jNPs are stable, bind DNA, exhibit shortened MR-T2* relaxivity for contrast-enhanced MRI-detection of tumors at 24-hours using 50ng Fe/kg, without inducing adverse events or inflammation. The jNPs self-assemble with echogenic [DNA/miRNA]-coated microbubbles (MBs) to form non-aggregating jNP[DNA/miRNA]MB-heteroplexes. jNP heteroplexes enhance ultrasound molecular-imaging of tumor microvessels and can deliver reporter-gene DNA and tumor suppressor miRNA-126 by sonoporation to breast and pancreatic tumor models, resulting in expected tumor-fluorescence and decreased oncogenic-KRAS protein levels, respectively. The jNPs can encapsulate pharmacologically incompatible drugs or small molecules in order to have favorable pharmacokinetic or pharmacodynamics profiles for efficacious delivery, for efficacious targeted delivery via the jNP targeting moieties, and for combinatorial delivery of nanocapsule encapsulated bioagents. Greater efficiency is noted in imaging and delivery compared to current technologies. Altogether, these data provide proof-of-principle that jNPs and jNP/MB-heteroplexes are a modular dual imaging and delivery platform for DNA/miRNA-therapy for cancer.

EXAMPLE 1: RESULTS

Covalently layered, ultrasmall Janus-nanoparticle (jNPs)

[0162] Janus-nanoparticles were sequentially covalent-layer-coupled via modified surface-initiated conjugation¹⁰ (FIG. 1). Layer-1 (“payload-face”), is comprised of IPEI_{25K} adsorbed onto mica to enable unidirectional sequential layering (FIG. 1A). Layer-2 (glutaraldehyde) covalently links amines in IPEI_{25K} (Layer-1) with amine-terminated PEG chains in Layer-3 (USPIONS pre-synthesized with a mixed polymer brush).²⁰ The mixed PEG_{2K/3.4K-NH₂} brush was selected to prevent USPION aggregation^{21,22} and to expose free amines for antibody targeting²³ (FIG. 1A). The 10-nm USPION-size was selected to optimize MR-imaging²⁴ and enhance MR-T2*-weighted contrast by PEI.²⁵ Layer-4 (N-hydroxysuccinimide-ester-maleimide (NHS-MAL))^{22,26} crosslinks free amines on Layer-3 to cysteine thiol groups of targeting-antibodies (Layer-5).

[0163] The jNP is projected to be ~27-30 nm based on a 10-nm USPION-core, and ~5–8-nm PEG mixed brush layer. Cryo-transmission electron microscopy (cryo-TEM) revealed asymmetric 22–30-nm particles with the USPION-core resembling an “avocado pit” and a ~12-nm opposing tapered face (FIG. 1B). Mostly single jNPs were observed, but free USPIONS, doublets, triplets, or clusters of jNPs (~35-70% in different cryo-TEM images) were also seen, permitting room for further

optimization. Atomic force microscopy (AFM) confirmed asymmetrically functionalized USPIO cores, distinguishing dense USPIOs from less dense material (FIG. 1C). AFM measurements (~55-nm avocado-shape) were slightly larger than cryo-TEM measurements, likely due to flattening of 3-dimensional jNPs during imaging (FIG. 1C).

[0164] Coupling of layer-specific components was confirmed by measuring changes in zeta-potential with each layer addition (FIG. 2A) and by gain of fluorescence upon fluorophore-labeled antibody attachment (FIG. 2B). Measurement of protein/jNP in 3 independent jNP batches detected a range of 3-12 antibody molecules/jNP (Table 1), providing multivalency for improved targeting.²⁷ This finding is consistent with the 9 antibodies/jNP predicted theoretically if antibodies are approximated as 12-nm diameter spheres.²⁷ Typically, 10^{12} – 10^{13} jNPs/mica²⁹ (10^{12} -jNPs ~ 5-ng Fe) were obtained. Dynamic light scattering (DLS) gave larger average hydrodynamic diameters ~30-47 nm (FIG. 2C), consistent with known surface charge effects.^{30,31}

Table 1: Determination of the number of antibodies per jNP

	DLS (nm)	# jNPs/ml	1:20 dilution μg protein/ml	1:1 μg protein/ml	μg protein/jNP	# of ab/jNP:
1	143	1.3×10^{13}	1.97	39.46	$3.0\text{E}+14$	12 abs/jNP
2	50.6	3.3×10^{13}	3.47	69.38	$2.3\text{E}+14$	9 abs/jNP
3	32	9.2×10^{13}	4.02	80.43	$8.9\text{E}+13$	3.5 abs/jNP
Legend: ab, antibody; DLS, dynamic light scattering; μg, microgram; nm, nanometer. Calculation of ab/jNP based on 2.5×10^{-13} μg/ab.						

[0165] The number of antibody particles to janus nanoparticle was estimated at 9 mAbs/jNP by using assumptions of 12-nm globular-shaped IgG antibody molecule arranged around a 10 nm-USPIO core while sitting on a flat surface (mica sheet in the layer-by-layer covalent conjugation process). To verify this estimate, 3 independent preparations of jNPs were analyzed— each with different measured diameters by dynamic light scattering (DLS), and specific number of jNPs per batch prepared. Protein amounts were determined by standard BCA assays following manufacturer's specifications, and within the ideal optimal sensitivity-range for assay detection.

[0166] ~3.5 to 12 antibody molecules were detected per jNP. The number of antibodies per jNP trended with DLS measurement of average diameter of jNPs. It is noted that the 50 nm diameter jNP has 9 antibody molecules as predicted.

jNP stability in serum and nucleic acid binding affinity

[0167] To test jNP stability for *in vivo* applications, the inventors assessed changes in jNP size and morphology over 24-hr in 75%-serum. A representative jNP batch showed size stability at ~ 85 nm for 12-hrs, increasing to 134.5-nm by 24-hr and 112-nm at 3 days (FIG. 2D). Although batch-to-batch variations occur, size increases were limited and non-progressive (FIG. 2D). Likewise, AFM

amplitude and topography images showed that jNPs retain characteristic avocado-shaped morphologies seen on cryo-TEM for at least 24-hr in 75%-serum (FIG. 2E).

[0168] To demonstrate jNP function as carriers for nucleic acids, the inventors visualized binding of lambda phage double-stranded DNA to jNPs by AFM (FIG. 7B) and confirmed jNP-DNA binding via an ethidium bromide (EtBr) dye exclusion assay.^{32,33} In the latter, jNPs bind DNA in a concentration-dependent manner and compete with EtBr-intercalation into DNA base-pairs, resulting in decreasing EtBr-fluorescence with increasing jNP concentrations (FIG. 2F). jNP-dependent binding to, and resultant protection of DNA from EtBr intercalation, was confirmed on agarose gel electrophoresis (FIG. 2F). Moreover, the reverse experiment showed that jNPs displace EtBr intercalated into DNA using a 4:1 DNA-EtBr ratio prior to addition of jNPs (FIG. 8A).

Self-Assembly of jNP-MBs

[0169] We next verified jNP-MB self-assembly (FIG. 3A) using differential-fluorophore fluorescence-activated cell-sorting (FACS) analysis (FIG. 3B). From 1 to 24-hours after addition of jNPs, the inventors detected DNA binding to ~60-90% of non-fluorescent MBs by single-fluorescent AF488-positive MBs (Q3, FIG. 3B), and detected jNP (0.05% MB-coverage) binding to 3-4% of MB-DNA, forming jNP-DNA-MBs as detected by double-fluorescence (Q2, FIG. 3B). Similar findings at 1-hr (data not shown) and 24-hr indicate relative stability of MB-DNA and of jNP-DNA-MBs in 150 mM NaCl.

[0170] To obtain a higher yield of jNP-DNA-MBs and test 'dose-response' specificity of jNP binding to MB-DNA, increasing amounts of jNPs were added to increase %-MB surface coverages: 1% (10^3 jNPs/MB), 10% (10^4 jNPs/MB) and 50% (5×10^4 jNPs/MB) (FIG. 3A) and studied results by FACS. In contrast to control non-fluorescent MBs (FIG. 3C-left panel), increasing %-MB surface coverage – 1%, 10% (data not shown) and 50% – led to increasing levels of fluorescent jNP-DNA-MBs (80%, 97%, 99%), respectively, $p < 0.0001$ (FIG. 3C). Concordantly, FACS side-scatter analysis showed dose-dependent increases in jNP-MB granularity (FIG. 8B). For all subsequent experiments, 10^4 jNPs/MB were used (10%-coverage), which would project to 97% of the MBs associated with jNPs.

jNP-MBs *in vitro* multifunctionality

[0171] FACS was then used to test targeting functionality of jNPs prepared with AF594-fluorescent-labeled monoclonal antibody against the dual endothelin1/VEGFsp receptor (DEspR), a cell-surface receptor increased on tumor vascular endothelium and tumor cells.³⁴ Fluorescently labeled targeted jNP-DNA-MBs were prepared, using 10^{12} jNPs, 30 μ g DNA, and 10^8 MBs (~10% jNP-coverage of MBs), and binding to DEspR-positive human pancreatic cancer (Panc1)-cells³⁴ was tested *in vitro*. Panc1-cells gained fluorescence upon binding of fluorescently labeled targeted jNP-MBs in a dose-dependent manner, in contrast to non-fluorescent MBs and cells alone (FIG. 3D). Notably, 1:1 jNP-MB-to-cell ratio resulted in ~40% binding of total Panc1 cells, whereas a 5:1 ratio resulted in ~90%

binding of total cells, $p < 0.0001$ (FIG. 3D). Microscopy confirmed fluorescent targeted jNP-DNA-MBs bind significantly more (80%) of Panc1 cells compared to non-targeted jNP-DNA-MBs (30%), $p < 0.0001$ (FIG. 3E). Similarly, significantly more targeted than non-targeted jNP-DNA-MBs bound to a given cell, $p = 0.006$ (FIG. 3E).

[0172] It was next investigated whether targeted jNP-DNA-MBs can deliver RFP-minigene DNA into DEspR-positive Panc1-cells by sonoporation more efficiently than multiple controls. Cells were incubated with test and pertinent control constructs (Table 2), unattached jNP-MBs or MBs were removed after 45 min through media change, and sonoporation was performed to test delivery of DNA-payloads.

Table 2: Table of test and corresponding control constructs. Additional controls comprised of: different % coverage of MBs by jNPs (0.05%, 1%, 10%, 50%), $\pm$ sonoporation.

Test construct	Symbol	Control	Symbol
Non-targeted jNP		USPION	
		USPION-PEG-NH2	
		USPION-PEG-NH2-PEI	
Non-targeted jNP-DNA		EtBr-DNA	
		EtBr	
		DNA	
Non-targeted jNP-ssDNA-MB		MB	
		ssDNA	
		Non-targeted jNP	
Targeted jNP-DNA-MB		Non-targeted jNP-DNA-MB	
		Targeted jNP	
		MB	
		Targeted MB-DNA	
		MB-DNA	

		Targeted MB	
		Non-targeted MB	
Targeted jNP-miRNA-MB		Targeted jNP-siRNA-MB	
Legend: • Non-Targeting Ab • Targeting Ab • miRNA • siRNA • MicroMarker MB • Target-ready MB      			

[0173] After 48-hours, fluorescence microscopy showed that Panc1-cells sonoporated with targeted jNP-DNA-MBs exhibit significantly more fluorescence (index of successful delivery of functionally intact red fluorescent protein (RFP)-DNA and subsequent RFP-expression) (FIG. 4A) than those sonoporated with non-targeted jNP-DNA-MBs (FIG. 4B), targeted MB-DNA (FIG. 4E), or non-targeted MB-DNA (FIG. 4F). Non-sonoporated cells exhibit little, if any, red fluorescence (FIG. 4C, 4D). High magnification confirms intracellular location of fluorescence (FIG. 4G). Quantitative analysis detects significant differences (FIG. 4H), confirming advantages of jNP-DNA-MBs as a DNA-delivery system via sonoporation. This is further supported by detection of 10- and 7-fold greater DNA payloads carried by jNP-DNA-MBs compared with the parent MBs at 1- and 6-hours of self-assembly respectively (FIG. 13).

jNP *in vivo* diagnostic multifunctionalities

[0174] Given the USPIO core and ability of jNPs to assemble with echogenic MBs, jNPs were tested for dual modality contrast-enhanced magnetic resonance (MR)-imaging and ultrasound molecular imaging. For MR-contrast-imaging, it was first determined that jNPs exhibit shorter T2*-relaxivity compared with precursor-USPIOs and control blanks across a range of 10-100 milliseconds echo times, $P < 0.001$ (FIG. 5A). An *in vivo* test was next conducted to determine whether jNPs localize to tumors by tumor-selective enhanced permeability and retention (EPR) effects and provide contrast-enhanced MR-imaging. Compared to control tumors receiving no jNPs, tumors injected intra-tumorally *ex vivo* with ~50 μ l of jNPs [10^{12} /ml] as a positive control exhibited significant MR-T2*-hypointensity. Similar results were seen in tumors from rats infused with 10^{12} jNPs; these tumors exhibited MR-T2*-hypointensity confluent 'regions-of interest' [(ROI) ~ 60 mm²] with scattered intratumoral hypointensities (FIG. 5B). It was also found that contrast-enhanced

imaging is better at 24-hr (FIG. 5B) compared with 1- and 4-hr after infusion (data not shown). Because non-targeted jNPs were used to test universal MR-imaging of tumors, the MR-T2*-hypointensity ROIs detected only in tumors and not in liver or kidney (FIG. 9) suggest jNP tumor-localization via EPR-effects, a known time-dependent and tumor-selective characteristic requiring exposure to a contrast reagent for more than 6-hrs at a high plasma concentration³⁵. In contrast, MR-T2*-hypointensities in the liver at 24-hrs exhibit structural patterns consistent with clearance via the hepatic Mononuclear Phagocyte System, as expected (FIG. 9). Likewise, detection of MR-T2*-hypointensities with anatomical structural features in the kidney cortex but not in the renal medulla or pelvis (FIG. 9) indicate jNP-presence in the microvascular space and non-clearance through the kidney. This is consistent with observations that kidney clearance occurs only for nanoparticle sizes < 6 nm³⁶.

[0175] As a second imaging modality, it was next tested whether targeted jNP-MBs enhance ultrasound molecular imaging of tumor vasculature. For receptor-targeted ultrasound molecular imaging, peak contrast intensity signals (CIS) were measured from successful receptor-targeted/adherent MBs persisting after clearance of freely circulating MBs typically by 5-min,³⁷ and confirmed that CIS derive from MBs by demonstrating subsequent loss of increased CIS-levels upon operator-triggered MB-destruction (FIG. 10). Compared to targeted MBs (FIG. 5C, left), targeted jNP-MBs exhibited greater contrast-enhanced ultrasound imaging of extratumoral feeder vessels and intratumoral microvessels (FIG. 5C, middle) – confirmed by decreased CIS upon operator-triggered ultrasound destruction of attached MBs (FIG. 5C, right). Analysis of contrast-intensity signal (CIS) time curves showed that targeted jNP-MBs attain 2X-higher peak CIS-levels in feeder vessels and 6X-higher CIS-levels in intratumoral microvessels at t-20 and t-30-min compared to targeted MBs (FIGs. 5D, 5E), and also compared to peak CIS levels after bolus infusion (FIGs. 11A-11F). While jNP-MBs exhibit a slower decline in CIS after ultrasound destruction, CIS levels still reach baseline levels equivalent to control targeted MBs after 15-seconds (FIG. 5D).

jNP *in vivo* delivery efficacy and safety profile

[0176] The inventors next investigated *in vivo* jNP-MB delivery functions for nucleic acid payloads to tumors via sonoporation (FIG. 6), which *a priori* requires successful target interaction and payload-binding, carrying, release and delivery. After establishing baseline zero-fluorescence (FIG. 14A), sonoporation of tumors with i) targeted RFP-loaded jNP-DNA-MBs [10^{12} jNPs/30 μ g DNA/ 10^8 MBs], ii) control targeted RFP-loaded MB-DNA and iii) mock control tumor was performed. Forty-eight hours later, *in vivo* fluorescence-imaging (IVIS) detected no fluorescence in mock control compared with marked RFP-fluorescence in rat tumors sonoporated with targeted jNP-DNA-MBs (FIG. 6A, FIG. 14a). *In vivo* RFP-fluorescence obtained with targeted jNP-DNA-MBs was also significantly greater than that attained with control targeted MBs (FIG. 6A bar-graph, FIG. 14a), and non-targeted MBs (FIG. 14B).

[0177] To test modular versatility in payload delivery, it was tested if targeted jNP-MBs could deliver miRNA-126, a tumor suppressor miRNA markedly decreased in human breast and pancreatic cancers. Decreases in miRNA-126 levels contribute to increased KRAS (Kirsten rat sarcoma viral oncogene homologue), which promotes cancer progression, metastasis and tumor-resistance,³⁸⁻⁴⁰ thus successful delivery of functionally active miRNA-126 would be expected to decrease KRAS protein levels. Following procedures used for successful RFP-DNA delivery, miRNA-126 delivery was tested by targeting jNP-MBs to two xenograft tumor models: breast cancer KRAS^{G13D}-mutant MDA-MB-231 cancer stem-like cells (CSCs), and pancreatic cancer KRAS^{G12D}-mutant Panc1-CSCs. Real-time quantitative reverse transcriptase PCR (real-time qRT-PCR) analysis confirmed specificity of miRNA-126 amplification products (expected ~74.8°C melting temperature for miRNA-126) (FIG. 6B) and showed > 1000-X increase in miRNA-126 levels ($P < 0.0001$) in xenograft breast and pancreatic tumors sonoporated with jNP-miRNA-126-MBs relative to the levels in non-manipulated tumors (FIGs. 6C, 6D). Moreover, delivery of miRNA-126 in these studies resulted in tumor levels similar to normal-high levels of miRNA-126 detected in normal liver and kidney tissues (FIG. 6D). In contrast, delivery of 4-fold lower dose of miRNA did not work in identical conditions (data not shown), indicating that dosing is important and delivery-specific.

[0178] To validate delivery of functionally intact miRNA-126, Western blot analyses of KRAS, a key target gene for miRNA-126, were performed. Notably, KRAS-levels were decreased up to 2.8-fold in xenograft breast tumors (FIG. 6E), indicating functional association of delivered miRNA-126 with RNA-induced silencing complex (RISC), a prerequisite for inhibition of target gene translation. Variation in extent of KRAS-decrease among tumors with delivered miRNA-126 observed in xenograft Panc1 tumors and breast tumors (data not shown) indicate need for further study, along with analysis of other downstream targets and off-target effects.

[0179] To assess *in vivo* safety of jNPs and jNP-MBs, daily monitoring was performed on all tumor model rats infused with 10^{12} jNPs or jNP-MBs, as well as hypertensive stroke-prone rats infused with 10^{12} -jNPs, and normal rats infused with 10^{13} -jNPs. No adverse events were observed acutely or up to 2-weeks. Additionally, measurements of plasma levels of cytokines/chemokines two-weeks after infusion of 10^{12} and 10^{13} jNPs, respectively, in stroke-prone and normal rats showed that jNPs do not induce inflammation, endothelial activation or complement activation compared with control rats (FIG. 6F). Concordantly, *in vitro* analysis for SC5b-9, the complement activation end-product regardless of activation mechanism, corroborated non-activation of complement system by jNPs *in vivo* (FIG. 15). These data contrast with toxicities reported in studies using larger lipopolyplexes⁴² and other SPIO MR-contrast agents for tumor imaging²⁴, but are similar to observed *in vivo* safety of LINEAR-PEI_{25K} polyplexes in mice.⁴²

[0180] In contrast to previously reported Janus nanoparticles, all larger than 100 nm,¹²⁻¹⁷ covalently layered ultrasmall jNPs reported here exhibit modularity and multifunctionality that offer key advantages: efficient and safe delivery of nucleic acid therapies and dual-mode imaging of tumor-specific features. Attaining 10-fold greater reporter-gene fluorescence compared to MBs, the jNP-MB nucleic acid delivery system uses 6-fold less payload (~30 µg/rat) and 10-fold fewer microbubbles (10⁸-MBs/rat) compared to recent mouse studies (200 µg DNA/mouse and 10⁹ MBs/mouse,⁴⁴ or 500 µg DNA/mouse and 10⁸ MBs/mouse)⁷ despite rats being ~5-10-fold heavier than mice. Thus, our system is hundreds-fold more efficient than systems used in published mouse studies. This efficiency is likely attributable at least in part to stable DNA-binding by jNPs and 10-fold greater payloads in jNP-MBs compared with MBs. The advantage of jNP-MBs is further demonstrated by observations that jNP-MBs attain DEspR-positive molecular imaging of intratumoral vessels compared to minimal detection by MBs despite their identical targeting moiety, and attain effective DEspR-targeted delivery of both DNA and miRNA-payloads at clinically safe ultrasound energies, 153 kPa. The low acoustic energy requirement is also a distinct advantage over polymer microbubbles that typically require higher energy.

[0181] *In vivo* RFP-fluorescence detection and decreased KRAS protein levels demonstrate delivery of functionally intact DNA and miRNA that are properly processed by the cell. These data validate cytosolic delivery by jNP-MB sonoporation as a highly efficient, endosomal-independent^{6,45} delivery paradigm, thereby avoiding cellular toxicity associated with intra-endosomal inflammatory pathways reported for PEI-DNA/siRNA nano-polyplexes.⁴⁶ Because targeted jNP-MB sonoporation can potentially overcome limitations of passive delivery systems relying on EPR^{16,49} and subsequent endocytic-uptake and escape,⁵⁰ this system has the potential to restore the reduced tumor suppressor miRNA levels often associated with cancer aggressiveness (e.g. miRNA-126).³⁸

[0182] Contrast-enhanced MR-imaging of tumor-specific EPR-sites indicates the hypothesis that hemispherical segregation of payload- from targeting-face in jNP-design is key. Notably, using 10¹²-jNPs/rat or 0.05 µg Fe/kg dose is 10³- to 10⁴-fold less than clinically approved human doses for Resovist™ (60 nm, 90 µg Fe/kg) and Ferridex™ (120-180 nm, 560 µg Fe/kg), respectively. Better contrast-enhanced MR-imaging at 24-hours compared with 4-hours indicate jNP circulation at high levels beyond 6-hours since a minimum 6-hours is required for MR-imaging of tumor-selective EPR-effects.³⁵

[0183] Although formal toxicity studies are needed prior to clinical translation, the initial safety profile show that jNPs do not trigger adverse events, inflammation or complement activation, which contrasts reported acute adverse events for PEI-DNA/siRNA nano-polyplexes,^{10,46-47} and 500-x higher cytokine levels for free linear[25-kDa]-PEI in culture.⁴⁸ Altogether, these data indicate that the nano-design is safe and effective for use of linear-PEI_{25kDa} at a maximum 10 µg PEI in 10¹² jNPs/dose. Without wishing to be bound by theory, it is hypothesized that the observed

‘inflammation/complement-stealth’ property is due to the antibody targeting-face design that allows for IgG-like long circulation times using targeting-antibody isotypes with insignificant ADCC and CDC for targeted jNPs, or non-specific antibodies that do not engage antigen for non-targeted jNPs.⁴¹

[0184] In summary, jNP theranostic multifunctionality, relative structural stability, sufficient circulation time, and initial safety profiles collectively are indicative of a balanced efficacy/safety ratio, thus providing proof-of-concept that jNPs and jNP-MBs comprise an ideal theranostic nanosystem for cancer.

EXAMPLE 2: EXEMPLARY METHODS & MATERIALS

Preparation of Janus nanoparticles (jNP).

[0185] Ultrasmall superparamagnetic iron oxide nanoparticles⁵² were assembled through covalent coupling of sequential layers. Monodisperse citric acid-coated Fe₃O₄ USPIOs (~ 10-nm in diameter) were functionalized to have a 4:1 PEG_{2K/3.4K-NH₂} mixed brush as previously described⁵³. A modified “layer-by-layer” technique^{54,55} was used to prepare asymmetrically functionalized jNPs – resulting in nanoparticles with cationic PEI on one face, and a targeting monoclonal antibody (mAb) moiety (anti-DEspR mAb³⁷) on the opposite face. Non-targeted jNPs are prepared using isotype IgG2 or non-specific IgG antibodies (Pierce Thermo Scientific™, Rockford, IL) for the antibody-face. Stepwise layering was documented by changes in zeta potential or gain of fluorescence. To prepare fluorescently labeled jNPs for heteroplex assembly studies, the inventors used antibodies labeled either with red-fluorescent AF594 (jNP^{AF594}) or with AF568 (jNP^{AF568}) for the targeting-face.

In vitro characterization of jNPs.

[0186] Cryo-transmission electron microscopy (cryo-TEM) was performed to visualize jNPs using a CM12 Transmission Electron Microscope (Philips Electron Optics™, Eindhoven, Netherlands). AFM was performed on jNPs. Determination of jNP diameters by DLS was performed per manufacturer’s specifications. Study of jNP binding to DNA-payload was done via ethidium bromide (EtBr) dye exclusion assay as described,³⁵ with jNPs and DNA mixed prior to addition of EtBr, and via EtBr dye displacement assay³⁶ with DNA and EtBr mixed first in a 4:1 ratio, followed by addition of jNPs. Dose-response was done with increasing number of jNPs using different DNA concentrations.

Assembly and analysis of jNP[DNA]MB-heteroplexes.

[0187] Stepwise assembly of the jNP[DNA]MB heteroplex is done in physiological saline at room temperature with mild rotation. For heteroplex assembly, the inventors used commercially available ~2-μm diameter microbubbles (MicroMarkers™, Visual Sonics-Fuji™, Canada) characterized for acoustic properties and successful delivery of DNA^{44,56}. The inventors used the following formulation: 10⁸ MBs, 30-μg RFP-minigene DNA as nucleic acid payload, and 10¹²-jNPs for projected 10% coverage of 10⁸ MBs, or 10⁴ jNPs/MB. Gain of fluorescence marks assembly and cell-targeting

endpoints as tracked by FACS analysis using fluorescently labeled DEspR-targeted jNP^{AF594}. FACS analysis was used to quantify dual-labeled heteroplex assembly to show binding of DNA to MBs, and jNP binding to [DNA]MBs after 1- and 24-hr, using differential fluorescent labeling of nucleic acid payload: oligoDNA^{AF468} (Integrated DNA Technologies™, Coralville, IA), and jNPs^{AF568}.

***In vitro* analysis of jNP[DNA]MB-heteroplex multifunctionality.**

[0188] Targeting and stable binding of DEspR-targeted jNP[DNA]MB heteroplexes to Panc1 cancer cells is documented *in vitro* by FACS analysis and confocal fluorescence microscopy fluorescently labeled jNPs^{AF594}. *In vitro* delivery of red fluorescent protein (RFP) mini-gene DNA was done by sonoporation and gene expression assessed by confocal microscopy *in vitro*. Quantitation was done on 12-16 high power fields from 8 independent sonoporation sites per study group (n= 8).

***In vivo* analysis of jNPs and jNP[DNA]MB-heteroplex multifunctionality.**

[0189] *In vivo* tests of jNP[DNA]MB-heteroplexes were done using identical component ratios validated *in vitro* for heteroplex assembly. Three rat tumor models were used: spontaneous mammary tumors in post-menopausal female rats for ultrasound contrast-enhanced molecular imaging and for DNA delivery; nude rat heterotopic, xenograft tumor models of human pancreatic cancer (Panc1) and breast cancer (MDA-MB-231) developed from Panc1- and MDA-MB-231 cancer stem-like cells (CSCs) isolated from each line as described³⁷.

[0190] *Ex vivo* 11.7-Tesla (T) MR-imaging of tumors was done 24-hr after intravenous infusion of mock/non-targeted jNPs (10¹² jNPs/ml) using susceptibility weighted imaging (SWI) analysis. Comparative 9.4Tesla MR-imaging of jNP, precursor-USPIONS and blank control phantoms in 1% agar was conducted using gradient-echo at varying echo times (TE). Targeted molecular imaging was performed using identical conditions for test and control rats via Vevo770 ultrasound contrast-imaging system at different time points schematically described in FIG. 10. Visual imaging of contrast is obtained with intensity scales optimized for visual (only) overlay of contrast and 2D-B-mode images, but quantitation is performed on identical scales. The average contrast intensity signals (CIS) are obtained at different ROIs (n = 10) showing peak CIS among extratumoral feeder vessels at the base of tumors or in areas of intratumoral microvessels at each time point.

[0191] After molecular imaging, *in vivo* sonoporation was performed using a 1-MHz SoniGene sonoprotector (Visual Sonics-Fuji™, Canada): 1 MHz, 1.5 Watt/cm² x 60 seconds, 50% duty cycle. Two days after sonoporation, *in vivo* imaging of RFP-mediated fluorescence was performed using IVIS (Xenogen™, Inc) per manufacturer's specifications.

Analysis of jNP[miRNA]MB-heteroplex delivery of miRNA-126 to tumors *in vivo*.

[0192] Assembly of miRNA payload into heteroplexes and *in vivo* delivery is done identical to jNP[DNA]MB-heteroplexes. The inventors use 27-30µg miRNA/10⁸-MBs per rat (21-bp double-strand miRNA-126 mimic, Stem Loop ID: hsa-miRNA-126-5p; Ambion Life Technologies™). Two-

days after miRNA-126 delivery by sonoporation of DEspR-targeted jNP[miRNA-126]MB-heteroplexes to nude rat heterotopic xenograft tumors, detection of full length miRNA-126 is performed by miRNA-126-specific quantitative real-time RT-PCR analysis. KRAS protein levels were determined by Western blot analysis.

Analysis of circulating cytokine levels after jNP infusion *in vivo*.

[0193] Safety studies are done in Dahl salt-sensitive hypertensive rats using 10^{12} - and 10^{13} -jNPs/rat infused via tail vein without microbubbles compared to age-matched controls. Daily health monitoring is performed, and analysis of serum cytokine levels is done 2 weeks later by ELISA following manufacturer's specifications (Rat Cytokine/Chemokine Array, RayBiotech™, GA). ELISA is done in duplicates per cytokine per rat, with 2 rats/group for 3 study groups.

Statistical analysis.

[0194] All data are checked for normality, and the appropriate statistical test applied. One-way ANOVA with Tukey's test for multiple pairwise comparisons was used to assess differences of pertinent study groups e 3, with (n = 8-10 measures/study group) in different experimental systems; students t-test (normality passed) or non-parametric Wilcoxon rank sum test were used accordingly for differences in group means. Two-way ANOVA repeated measures was used for 3-group time course analysis of T2* relaxivity (n = 20 measures/timepoint, n = 20 timepoints). GraphPad PRISM™ (Release 5.0, GraphPad™, CA) software was used for statistical analyses.

REFERENCES

- 1 Nguyen, J. & Szoka, F. C. Nucleic acid delivery: the missing pieces of the puzzle? *Acc. Chem. Res.* **45**, 1153-1162 (2012).
- 2 Chen, Y., Gao, D.-Y. & Huang, L. In vivo delivery of miRNAs for cancer therapy: Challenges and strategies. *Adv. Drug Deliv. Rev.* (2014).
- 3 Lammers, T., Aime, S., Hennink, W. E., Storm, G. & Kiessling, F. Theranostic Nanomedicine. *Acc. Chem. Res.* **44**, 1029-1038 (2011).
- 4 Gilleron, J. *et al.* Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotech.* **31**, 638-646 (2013).
- 5 Sahay, G. *et al.* Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nat. Biotech.* **31**, 653-658 (2013).
- 6 Lentacker, I., De Cock, I., Deckers, R., De Smedt, S. C. & Moonen, C. T. Understanding ultrasound induced sonoporation: definitions and underlying mechanisms. *Adv. Drug Deliv. Rev.* **72**, 49-64 (2014).
- 7 Sirsi, S. R. *et al.* Polyplex-microbubble hybrids for ultrasound-guided plasmid DNA delivery to solid tumors. *J. Control. Release* **157**, 224-234 (2012).

- 8 Ballarin-Gonzalez, B. & Howard, K. A. Polycation-based nanoparticle delivery of RNAi therapeutics: adverse effects and solutions. *Adv Drug Deliv Rev* **64**, 1717-1729 (2012).
- 9 Beyerle, A. *et al.* Inflammatory responses to pulmonary application of PEI-based siRNA nanocarriers in mice. *Biomaterials* **32**, 8694-8701 (2011).
- 10 Tros de Ilarduya, C., Sun, Y. & Duzgunes, N. Gene delivery by lipoplexes and polyplexes. *Eur. J. Pharm. Sci.* **40**, 159-170 (2010).
- 11 Zhou, J. *et al.* Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery. *Nat. Mater.* **11**, 82-90 (2012).
- 12 Hu, S.-H. & Gao, X. Nanocomposites with Spatially Separated Functionalities for Combined Imaging and Magnetolytic Therapy. *JACS* **132**, 7234-7237 (2010).
- 13 Lattuada, M. & Hatton, T. A. Synthesis, properties and applications of Janus nanoparticles. *Nano Today* **6**, 286-308 (2011).
- 14 Schick, I. *et al.* Multifunctional Two-Photon Active Silica-Coated Au@MnO Janus Particles for Selective Dual Functionalization and Imaging. *JACS* **136**, 2473-2483 (2014).
- 15 Vasquez, E. S., Chu, I. W. & Walters, K. B. Janus Magnetic Nanoparticles with a Bicompartamental Polymer Brush Prepared Using Electrostatic Adsorption to Facilitate Toposelective Surface-Initiated ATRP. *Langmuir* **30** (2014).
- 16 Xie, J., Lee, S. & Chen, X. Nanoparticle-based theranostic agents. *Adv. Drug Deliv. Rev.* **62**, 1064-1079 (2010).
- 17 Yen, S. K., Padmanabhan, P. & Selvan, S. T. Multifunctional iron oxide nanoparticles for diagnostics, therapy and macromolecule delivery. *Theranostics* **3**, 986-1003 (2013).
- 18 Barrefelt, A. *et al.* Biodistribution, kinetics, and biological fate of SPION microbubbles in the rat. *Int. J. Nanomedicine* (2013).
- 19 Yang, F. *et al.* Superparamagnetic iron oxide nanoparticle-embedded encapsulated microbubbles as dual contrast agents of magnetic resonance and ultrasound imaging. *Biomaterials* **30**, 3882-3890 (2009).
- 20 Budijono, S. J., Russ, B., Saad, W., Adamson, D. H. & Prud'homme, R. K. Block copolymer surface coverage on nanoparticles. *Colloids Surf., A* **360**, 105-110 (2010).
- 21 Jokerst, J. V., Lobovkina, T., Zare, R. N. & Gambhir, S. S. Nanoparticle PEGylation for imaging and therapy. *Nanomedicine* **6**, 715-728 (2011).
- 22 Migneault, I., Dartiguenave, C., Bertrand, M. J. & Waldron, K. C. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques* **37**, 790-802 (2004).
- 23 Cruz, L. J., Tacke, P. J., Fokkink, R. & Figdor, C. G. The influence of PEG chain length and targeting moiety on antibody-mediated delivery of nanoparticle vaccines to human dendritic cells. *Biomaterials* **32**, 6791-6803 (2011).

- 24 Li, M. *et al.* Comparison of Two Ultrasmall Superparamagnetic Iron Oxides on Cytotoxicity and MR Imaging of Tumors. *Theranostics* **2**, 76-85 (2012).
- 25 Duan, H. *et al.* Reexamining the Effects of Particle Size and Surface Chemistry on the Magnetic Properties of Iron Oxide Nanocrystals: New Insights into Spin Disorder and Proton Relaxivity. *J. Phys. Chem. Lett.* **112**, 8127-8131 (2008).
- 26 Torchilin, V. P. Multifunctional nanocarriers. *Adv. Drug Deliv. Rev.* **64**, Supplement, 302-315 (2012).
27. Tan, Y. H. *et al.* A nanoengineering approach for investigation and regulation of protein immobilization. *ACS Nano* **2** (2008).
28. Liu, J. *et al.* Computational model for nanocarrier binding to endothelium validated using in vivo, in vitro, and atomic force microscopy experiments. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 16530-16535 (2010).
- 29 Park, Y. *et al.* Stability of Superparamagnetic Iron Oxide Nanoparticles at Different pH Values: Experimental and Theoretical Analysis. *Langmuir* **28** (15) 6246-6255 (2012).
- 30 Mukhopadhyay, A., Joshi, N., Chattopadhyay, K. & De, G. A Facile Synthesis of PEG-Coated Magnetite (Fe₃O₄) Nanoparticles and Their Prevention of the Reduction of Cytochrome C. *ACS Appl. Mater. Interfaces* **4**, 142-149 (2011).
- 31 Wang, L., Neoh, K. G., Kang, E. T., Shuter, B. & Wang, S.-C. Superparamagnetic Hyperbranched Polyglycerol-Grafted Fe₃O₄ Nanoparticles as a Novel Magnetic Resonance Imaging Contrast Agent: An In Vitro Assessment. *Adv. Funct. Mat.* **19**, 2615-2622 (2009).
- 32 Kleemann, E. *et al.* Nano-carriers for DNA delivery to the lung based upon a TAT-derived peptide covalently coupled to PEG-PEI. *J. Control. Release* **109**, 299-316 (2005).
- 33 Taratula, O. *et al.* Multifunctional nanomedicine platform for cancer specific delivery of siRNA by superparamagnetic iron oxide nanoparticles-dendrimer complexes. *Curr. Drug Deliv.* **8**, 59-69 (2011).
- 34 Herrera, V. L. *et al.* DEspR Roles in Tumor Vasculo-Angiogenesis, Invasiveness, CSC-Survival and Anoikis Resistance: A 'Common Receptor Coordinator' Paradigm. *PLOS ONE* **9** (1) e85821 (2014).
- 35 Taurin, S., Nehoff H Fau - Greish, K. & Greish, K. Anticancer nanomedicine and tumor vascular permeability; Where is the missing link? *J. Control. Release* **164**, 265-275 (2012).
- 36 Soo Choi, H. *et al.* Renal clearance of quantum dots. *Nat. Biotech.* **25**, 1165-1170 (2007).
- 37 Decano, J. L., Moran, A. M., Ruiz-Opazo, N. & Herrera, V. L. Molecular imaging of vasa vasorum neovascularization via DEspR-targeted contrast-enhanced ultrasound micro-imaging in transgenic atherosclerosis rat model. *Mol. Imaging Biol.* **13**, 1096-1106 (2011).
- 38 Frampton, A. E. *et al.* Loss of miR-126 is crucial to pancreatic cancer progression. *Expert Rev. Anticancer Ther.* **12**, 881-884 (2012).

- 39 Hamada, S. *et al.* MiR-126 acts as a tumor suppressor in pancreatic cancer cells via the regulation of ADAM9. *Mol. Cancer Res.* **10**, 3-10 (2012).
- 40 Tavazoie, S. F. *et al.* Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451**, 147-152 (2008).
- 41 Marshall, J. S. Mast-cell responses to pathogens. *Nat. Rev. Immunol.* **4**, 787-799 (2004).
- 42 Kawakami, S., Ito, Y., Charoensit, P., Yamashita, F. & Hashida, M. Evaluation of proinflammatory cytokine production induced by linear and branched polyethylenimine/plasmid DNA complexes in mice. *J Pharmacol. Exp. Ther.* **317**, 1382-1390 (2006).
- 43 Lattuada, M. & Hatton, T. A. Preparation and Controlled Self-Assembly of Janus Magnetic Nanoparticles. *JACS* **129**, 12878-12889 (2007).
- 44 Wang, D. S. *et al.* Cationic versus neutral microbubbles for ultrasound-mediated gene delivery in cancer. *Radiology* **264**, 721-732 (2012).
- 45 Delalande, A., Kotopoulis S., Postema, M., Midoux, P., Pichon, C. Sonoporation: mechanistic insights and ongoing challenges for gene transfer. *Gene* **525** (10) 191-199 (2013).
- 46 Gao, X. *et al.* The association of autophagy with polyethylenimine-induced cytotoxicity in nephritic and hepatic cell lines. *Biomaterials* **32**, 8613-8625 (2011).
- 47 Hunter, A. C. & Moghimi, S. M. Cationic carriers of genetic material and cell death: a mitochondrial tale. *Biochim. Biophys. Acta* **1797**, 1203-1209 (2010).
- 48 Tay, C. Y., Menon, N., Leong, D. T., & Tan, L. P. Molecular architecture governs cytotoxicity and gene transfection efficacy of polyethylenimine based nanoplexes in mammalian cell lines. *J Inorg Organomet Polym* **25**, 301-311 (2015).
- 49 Caldorera-Moore, M. E., Liechty, W. B. & Peppas, N. A. Responsive Theranostic Systems: Integration of Diagnostic Imaging Agents and Responsive Controlled Release Drug Delivery Carriers. *Acc. Chem. Res.* **44**, 1061-1070 (2011).
- 50 Guo, S. & Huang, L. Nanoparticles Escaping RES and Endosome: Challenges for siRNA Delivery for Cancer Therapy. *J. Nanomat.* **2011**, 1-12 (2011).

EXAMPLE 3: SUPPLEMENTARY METHODS

Synthesis and surface modification of jNPs

[0195] Janus functionalized nanoparticles are prepared using the following materials: Iron tri(acetylacetonate) (99.9%), citric acid (CA; 99.5+%), methanol (99.8%), acetone, benzyl ether (99%), N-hydroxysuccinimide ester (NHS ester; 98%), oleic acid (OA; 90%), oleyl amine (OAm; 70%) 1-ethyl-3-(3-dimethylamino- propyl)carbodiimide hydrochloride (EDC; 97%) (Sigma™, St. Louis, MO); 1,2- dichlorobenzene (DCB; 99%), N,N2-dimethylformamide (DMF; 99.8%), diethyl

ether (99.9%), and hexane (99.9%) (Acros™, Morris Plains, NJ). Ethanol (ACS grade) (Pharmco™, Lees Summit, MO); amine-terminated polyethylene glycol (mPEG-NH₂) with molecular weight 2000Da (Laysan Bio™, Inc, Arab, AL); NH₂-mPEG-NH₂ with molecular weight 3400Da (Creative PEGWorks™, Winston Salem, NC); RFP minigene (pTurbo FP635-N cat# FP722, Evrogen™, Moscow, Russia), Alexafluor 594 or 568 labeling kits (Invitrogen™, Carlsbad, CA, USA); anti-rat DEspR monoclonal antibodies (ProMab™, CA) and IgG2 isotype (Santa Cruz Biotechnology™, Sta. Cruz, CA); MicroMarker microbubbles (Visual Sonics-Fuji™, Inc., Canada). Iron oxide NPs are first synthesized according to the method described by Sun et al¹. Briefly, NPs are produced by an organic phase process reacting Fe(acac)₃ and a long-chain alcohol, and then modified to yield monodisperse citric acid-coated Fe₃O₄ NPs about 10 nm in diameter. The NPs are then functionalized to contain a mixture of different polyethylene glycol (PEG) chains on their surface and attached as previously been described.² The mixed PEG brush is comprised of: molecular weight 2000 Da (PEG2000) and 3400 Da (PEG3400) in a 4:1 ratio. PEG2000 molecules are amine-terminated at one end, while PEG3400 molecules are amine-terminated in both ends. PEG chains are attached to carboxyl groups of citric acid on the USPIO surface through N-hydroxysuccinimide (NHS) ester and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) coupling². This results in a mixed PEG brush coat around the USPIO³, referred to as the precursor-USPIO for production of Janus-functionalized nanoparticles (jNPs).

[0196] The asymmetric functionalization method is based on previously reported layer-by-layer methods^{4,5}. In order to prepare jNPs, a freshly 3x cleaved mica surface is placed in a 100 mm-diameter petri dish. The square mica sheet is adjusted in size to fit in the dish. Magnets are attached to the underside of the petri dish in order to keep the magnetic USPIOs in place during the functionalization procedure. Upon placement in the petri dish, the mica sheet is incubated with 0.1 % solution of linear polyethyleneimine of MW 25kDa (PEI), followed by incubation with 5% glutaraldehyde solution to react with secondary amines on PEI. After a wash step, Mixed-brush PEGylated USPIOs are then introduced to the mica sheet with just enough SPIONs to cover the mica plate with a thin layer of USPIOs and held down by the magnet. The glutaraldehyde is allowed to react with amine groups on PEG3400, creating a monolayer of USPIOs bound on one side to the mica surface. The unbound face of the USPIOs oriented away from the mica is then reacted with N-hydroxysuccinimide maleimide (NHS-MAL) at 100 µg/mL to convert free amines on the PEG3400 to a MAL. Targeting antibodies (ab), anti-DEspR, or control non-targeting non-specific IgG are presented to USPIOs for test jNPs and control jNPs respectively, and allowed to attach overnight with gentle agitation. Attachment of the ab-protein to the USPIO is achieved by coupling MAL on USPIOs to thiol groups on cysteine residues in the antibodies. Based on exposed USPIO surface volume and antibody 12-nm diameter, approximately 9-13 antibodies are attached to each USPIO. Upon attachment of targeting antibodies to the USPIOs, the mica sheet is washed and the now functionalized Janus nanoparticles, jNPs, released using 0.2 M NaCl, disrupting the electrostatic

interaction between the mica sheet and PEI. Upon release, the solution is diluted with dI H₂O to yield a final NaCl concentration of 0.15 M. The resulting particles encompass asymmetric functionalization with one face, the payload-face, comprised of polycationic PEI, and the 2nd face, the targeting-face, presenting antibodies.

Verification of layer-by-layer functionalized jNPs

[0197] In order to verify different steps of the functionalization, tests are conducted at different points of the procedure. The synthesis of PEGylated USPIOs had previously been verified in the inventors' laboratory through transmission electron microscopy, dynamic light scattering, zeta potential, elemental analysis and iron quantification.⁶ In order to verify that the USPIO surface is functionalized with a mixed layer of PEG2000 and amine-terminated PEG3400, analysis of increases in hydrodynamic radius from a 100% PEG 2000 layer to a 80%/20% PEG2000/PEG3400-NH₂ layer are measured on a Brookhaven 90Plus (Brookhaven Instrument™, Holtsville, NY) using dynamic light scattering (DLS). A positive shift in the zeta potential using the same instrument also provides confirmation. In order to verify the attachment of PEI, USPIOs are released before attachment of the targeting antibody, and a further positive shift of the zeta potential are measured. In order to confirm attachment of targeting antibodies, the antibodies are fluorescently labeled, and fluorescence is measured upon release from mica and subsequent wash of the targeted jNPs. Antibodies are fluorescently labeled using the Alexa Fluor® Protein Labeling Kit from Invitrogen™.

Quantitation of jNPs

[0198] The number of jNPs produced by the method are quantified using the following materials: Thioglycolic acid 98%, Hydroxylamine hydrochloride, 1,10-Phenanthroline, sodium citrate, Concentrated H₂SO₄, Ammonium iron(II) sulfate hexahydrate (Sigma Aldrich™, MO, USA). jNPs are quantified using an adapted method from Nitin et al⁷. The number of jNPs is determined by CCD camera (Gauting, Germany) using EMMENU4 Program. A low dose kit minimizes irradiation damage to the sample.

Atomic Force Microscopy (AFM) Images of jNPs

[0199] AFM imaging is performed using an Agilent 5500 AFM operated in intermittent contact mode (Acoustic AC (AAC) mode) using a Mac II cantilever (Agilent Technologies™) with a resonant frequency of approximately 75 kHz and a spring constant of 2.8 N/m. AFM images are recorded at a resolution of 512x512 pixels at a line rate of 1 line/sec. Scan sizes ranged from 300 nm x 300 nm up to 1.5 μm x 1.5 μm.

Ethidium Bromide Dye Exclusion and Displacement Assays

[0200] The binding of jNPs to DNA is tested by ethidium bromide (EtBr) dye exclusion assay (Kleenman et al 2005) based on the principle that EtBr fluoresces only when it intercalates into DNA base pairs. Fluorescence therefore indicates free DNA base pairs that are not bound to jNPs. To demonstrate specificity, increasing amounts of jNPs (0, 0.2, 0.6, 1.8×10^{10} jNPs) are mixed together with two different amounts of DNA (100 ng and 300 ng) and tested for EtBr fluorescence at two time points: 10 min and at 1hr. Fluorescence reads are done at 260 nm excitation/590 nm emission, and background autofluorescence of non-intercalated EtBr is subtracted. Afterwards, samples are then run in 0.8% agarose gels and fluorescence from EtBr intercalated into DNA is visualized with UV 260 nm excitation and photographed.

[0201] To further test affinity of jNP for DNA, we use an EtBr displacement assay (Taratula et al 2011), based on loss of fluorescence upon displacement of EtBr from DNA base pairs (bp) by jNPs binding to DNA with deduced equal or greater affinity. Aliquots of DNA plasmid in a 96-well plate (100 ng or 250 ng plasmid GFP-DNA per well) in physiological salt solution (150 mM NaCl, pH = 7) are incubated for 10 min with a molar ratio of 4:1 bp:EtBr. Increasing amounts of a solution containing 1.22×10^{13} jNPs/ml in 150 mM NaCl is added to aliquots of DNA plasmid and brought to a final working concentration of 200 l with additional physiological salt solution. Fluorescence of intercalated EtBr is measured at Ex./Em. of 480/590 nm using a Spectramax™ M5 plate reader (Molecular Devices™, Sunnyvale, CA), and relative fluorescence values are reported as the ratio of fluorescence intensity of the sample containing jNP to samples containing EtBr/DNA plasmid alone (n=6).

Analysis of jNP stability in serum

[0202] Stability against aggregation and loss of particle functionalization is demonstrated by observing the effective diameter of jNPs in 85% rat serum solution using both Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). Briefly, 250 μ l of jNP in 150 mM NaCl is mixed with pure rat serum for a final jNP concentration of 8.5×10^{11} particles/ml in 75% rat serum. Mean jNP diameters are determined via DLS using a Brookhaven 90Plus (Brookhaven Instrument™, Holtsville, NY) over several time points: 0, 1 hr, 3 hr, and 24 hr. Additionally, at each time point, 10 μ l is removed from the sample and deposited on a mica slide under a magnet. After allowing jNPs to deposit on the surface for one hour, slides are rinsed with DI water and dried for 48 hr before imaging using AFM. Due to the nonsymmetrical shape of the jNP, AFM effective diameter is reported as the longest diameter through the SPION core.

AFM imaging of modeled jNP interaction with lambda phage DNA

[0203] To further document interaction of jNPs with DNA, we use AFM to directly image jNP binding to deposited lambda phage DNA strands. Both 1 μ l of a 1×10^{13} solution of jNP and 25 μ l of 1

mg/ml » phage DNA are co-deposited on a mica slide under a magnet. After allowing 1-hour for jNPs and DNA to interact and deposit, the slide is rinsed with deionized water and air dried for 48-hours prior to AFM imaging. Images are taken as described above.

Stepwise jNP[DNA]MB-heteroplex assembly

[0204] The heteroplex is assembled stepwise in physiological phosphate buffered saline under constant slow rotation. The inventors used commercially available MicroMarker™ microbubbles (VisualSonics-Fuji™, Inc) which have been characterized by others (Escoffre JM et al 2011; Escorffree et al 2013; Olive KP et al 2009; Sun C et al 2014) as follows.

Size: 2-3 microns

Shell: phospholipids, PEG, fatty acids

Gas: N₂C₄F₁₀

Surface charge: negative

[0205] MicroMarker™ MBs with streptavidin (Target-Ready MicroMarker™ MBs, Visual Sonics Inc™), used for control DEspR-targeted non-jNP-MBs for *in vivo* molecular imaging and used for control DEspR-targeted [DNA]MBs in DNA delivery studies, were characterized and described by different research groups (Helfield et al 2013; Lee DJ et al 2008; Loveless ME et al, 2009; Lyshchik et al 2007; Rychak JJ et al 2007; Willmann JK et al 2008; Willmann JK et al 2010) and found to have the following characteristics:

MicroMarker MBs with streptavidin: 1-2 microns or 2-3 microns

7,600 streptavidin molecules/2 (Willmann et al, 2008); and 4 moles of biotin bind 1 mole streptavidin (ProZyme)

Streptavidin-positive MicroMarker MBs have been validated for molecular imaging by different groups using antibodies targeting VEGFR2, and integrin.

[0206] MicroMarker microbubbles have also been characterized for delivery of DNA via sonoporation, as well as acoustical behavior, and transfection efficiency *in vitro*. (Piron et al 2010; Escoffre JM et al 2013a; Delalande A et al 2013; Escoffre JM, et al 2013b).

[0207] For jNP[DNA]MB-heteroplex assembly, MBs are resuspended in normal saline (150 mM NaCl) for 10 min following manufacturer's specifications. Then 30 g of RFP-plasmid DNA, or 27 g of miRNA-126, or 15 g of single strand oligoDNA^{AF468} is incubated with 1 x 10⁸ microbubbles for 15 min, followed by incubation with jNPs calculated to cover 0.05%, 1%, 10%, or 50% (1 x 10¹² jNPs for 10% coverage of 10⁸MBs/rat, or 10⁴/MB) of the microbubble surface for another 45 min before further use or analysis.

FACS analysis of jNP[DNA]MB-heteroplex stepwise assembly

[0208] To document assembly of the jNP[DNA]MB-heteroplex, the inventors followed the identical stepwise assembly described above, and used fluorescent jNPs prepared using fluorescently labeled

anti-DEspR monoclonal antibodies (Alexa Fluor AF594) as the targeting moiety in the layering preparation of jNPs. Assembly of fluorescent jNPs with [DNA]MBs would therefore result in fluorescent jNP[DNA]MB-heteroplexes as distinguished from non-fluorescent jNP-negative [DNA]MBs on FACS analysis (LSRII flow cytometer, Becton Dickinson™, Inc). Increased granularity is assessed by side scatter. Increasing coverage (1%, 10% and 50% coverage) of the 2µm microbubble by jNPs is tested by FACs analysis.

[0209] To directly demonstrate binding of nucleic acid to microbubbles followed by complexing of jNPs onto [DNA]MBs, the inventors used double-labeled FACS analysis using commercially available 50 nucleotide-long AF488-labeled oligoDNA (DNA^{AF488}) and fluorescent jNPs with non-targeted AF568-labeled IgG (goat anti-rabbit IgG, Pierce Thermo Scientific™, Rockford IL). After resuspension of commercially available, non-fluorescent microbubbles (MicroMarkers™, VisualSonics-Fuji™, Inc., Canada), an aliquot of 10⁸ MBs is mixed with 15 ug oligoDNA^{AF488} for 15 min with gentle mixing on a rotator, and an aliquot analyzed by FACS analysis to document fluorescence of MBs upon DNA. Then fluorescent [oligoDNA^{AF488}]MBs are mixed with fluorescent jNPs (jNP^{AF568}) for 45 min and then analyzed by FACS analysis (t-1 hr; t-24 hr) for double fluorescent fully assembled jNP[DNA]MB-heteroplexes to distinguish from single fluorescent oligo-DNA bound MBs and non-fluorescent free MBs. Although the measured zeta potential of MBs alone is ~ -20 mV, FACS analysis show efficient and stable (up to 24 hr) binding of DNA to MBs (50-90% of MBs bound), thus indicating that MBs have amphoteric surfaces.

FACS of jNP[DNA]MB-heteroplex binding to cells

[0210] Human pancreatic cancer (Panc1) tumor cells are cultured as specified (ATCC™) and maintained at 37C in a humidified 5% CO₂ incubator. Panc1 tumor cells are isolated, washed then incubated with fluorescent jNP^{AF594}[DNA]MB-heteroplexes at room temperature with gentle rotation at 1:1 and at 1:5 cell-to-jNP[DNA]MB-heteroplex ratio for 45-60 min. Targeting efficiency is analyzed comparing the % of Panc1 tumor cells that gain fluorescence between the two ratios by FACS analysis (LSR II, BD Sciences, USA). Detection of fluorescent cells would indicate successful targeting of cells by fluorescent jNP[DNA]MB-heteroplexes through anti-DEspR ab interactions with the cognate receptor (DEspR) on Panc1 tumor cells¹⁰. Quantitation of relative amounts of gain-of-fluorescence by Panc1 tumor cells bound to fluorescent jNP[DNA]MB-heteroplexes to non-fluorescent Panc1 tumor cells, and free jNP[DNA]MB-heteroplexes is achieved using FACS analysis and FloJo™ analysis software.

***In vitro* analysis of jNP[DNA]MB-heteroplex binding to Panc1 cell under flow**

[0211] Panc1 cells are grown as described above in IBIDI perfusion 6-lane micro-slide VI (ibidiGmbH, Germany), and the DEspR-targeted jNP[DNA]MB-heteroplex labeled with AlexaFluor 594 on the targeting antibody moiety is introduced into the chamber and incubated for 45 min with

vertical rotation. Afterwards, the media is flushed with 1x phosphate buffered saline to wash off free fluorescent jNP[DNA]MB-heteroplexes. Cell nuclei are counterstained with DAPI (blue fluorescence). Imaging is done using a Nikon epi-fluorescence microscope (Nikon, Japan) for red fluorescence (AlexaFluor-labeled DEspR-targeted jNP*[DNA]MB), blue fluorescence (DAPI nuclear stain), as well as phase-contrast imaging to delineate cell membranes. Non-specific binding of the heteroplex is analyzed using jNPs functionalized with AlexaFluor 594-labeled IgG2 isotype antibody.

Analysis of jNP[DNA]MB-heteroplex payload delivery

[0212] Panc1 tumor cells are grown in OptiCell closed cell-culture system (Nunc/Thermo Scientific™, USA) with 75-μm thick gas-permeable membrane walls. The inventors used a Vevo770 ultrasound imaging system with contrast-enhanced capabilities (Visual Sonics-Fuji™, Inc., Canada) to demonstrate echolucency of membrane walls for sonoporation and to be able to detect and sequence-destruct bound microbubbles (Micromarkers™, VisualSonics™, Inc). Following identical conditions, test and control jNP[DNA]MB-heteroplexes, prepared as described above, and non-jNP[DNA]MB controls are allowed to interact with cells for 45 min with constant vertical rotation of OptiCell™ systems to simulate shear. Excess unbound targeted and non-targeted jNP[DNA]MB-heteroplexes and control non-jNP targeted ‘standard’ [DNA]MBs, as well as any free jNPs, DNA and MBs are removed, followed by one wash with media after 45 min, and new media added. Sonoporation is performed using a 1 MHz SoniGene™ sonoporation (VisualSonics-Fuji, Inc™, Canada) at 1.5 watts/cm² for 60 sec, 50% duty cycle (n = 8 independent sonoporation-sites in 2 different OptiCell™ culture systems per group, with a designated non-sonoporated area as a control in each OptiCell™ culture system.)

[0213] After 48 hr, analyses of cell health and RFP-mediated fluorescence are done using a Nikon™ epi-fluorescent microscope as described above. Targeted jNP[DNA]MBs had anti-human DEspR monoclonal ab (mAb) (in-house development) as the targeting moiety for the jNP. DEspR is present on Panc1 tumor cells (Herrera et al., 2014). The inventors compared DEspR-targeted jNP[DNA]MB-heteroplexes to non-targeted [DNA]MBs, non-jNP/DEspR-targeted[DNA]MBs, and control non-specific ab-jNP[DNA]MB-heteroplexes to determine efficiency of targeted-delivery. The inventors also compared sites in the same OptiCell™ culture system with and without sonoporation. Quantitative analysis is performed by determining the %fluorescent RFP-positive cells over total cells in two high power fields (HPF) per 8 independent sonoporation sites and 4 independent non-sonoporation sites, (n = 8-16 HPF sites).

MR-imaging analysis of tumors and normal liver and kidney

[0214] MRI is performed in an 11.7 Tesla vertical-bore Bruker Avance spectrometer. The inventors use a clinical MRI sequence called susceptibility weighted imaging (SWI) to analyze tumor tissue samples. SWI is a unique pulse sequence which combines both the phase and magnitude information,

which has been proven to show sensitivity to iron particles – detected in venous blood, hemorrhage, iron storage or nanoparticles. For MRI of liver and kidney, the **Fast Low Angle SHot™ (FLASH)** clinical MRI sequence is used.

MR-imaging comparing jNPs and SPION-PEG-NH₂.

[0215] Three 5mm NMR tubes are filled with agar alone, or with agar containing jNPs or SPION-PEG-NH₂ at a concentration of 0.5×10^{12} jNPs/ml in 1% agar. These tubes are then embedded in 2% agar inside a 26mm diameter plastic tube, with their long axes parallel to the long axis of the outer tube. This assembly is placed at the isocenter of a 9.4T horizontal axis MRI scanner (Bruker Biospec™, Billerica, MA) with the tubes oriented parallel to the magnetic field. Axial and coronal T2-weighted rapid acquisition with relaxation enhancement (RARE) images are acquired with TR/TE=3300/22ms, echo train length = 4 ms.

[0216] T2* relaxation times in the three vials are assessed using a multi-gradient echo sequence with TR=300ms, 26 degree flip angle, and 20 echoes per image acquired at echo times ranging from 4.08 to 99.08ms in 5ms steps. To minimize effects of macroscopic magnetic field variations (which can shorten T2*), two steps are taken. First, prior to each measurement, the magnetic field is shimmed locally using a point-resolved spectroscopy (PRESS) sequence to minimize the proton linewidth in a 5-6 mm cube centered on the vial of interest. The line width (FWHM) ranged from 7-13Hz in the three vials prior to T2* measurement in each vial. Second, each multi-gradient echo image series is acquired with two voxel sizes. A first acquisition is performed with 1mm slice thickness and 0.312mm in-plane resolution, and a second is then acquired with 0.5mm slice and 0.156mm in-plane resolution. The second acquisition had half the voxel size of the first, and hence will have approximately half as much intravoxel dephasing due to macroscopic magnetic field inhomogeneities. Under these conditions, the T2* measurements are consistent (within error) between the two data sets. T2* in each voxel is determined by fitting the pixel intensities within a region of interest centered within the vial to a function of the form $Ae^{-TE/T2^*}+b$, where b is an overall offset representing the noise floor in these magnitude images.

Analysis of jNP[DNA]MB-heteroplex targeted molecular imaging

[0217] After obtaining baseline ultrasound 2-dimensional B-mode imaging and baseline IVIS documentation of zero-fluorescence of tumors, experiments are done using jNP[DNA]MB-heteroplexes with an estimated 10% jNP-coverage of microbubbles. Heteroplexes comprise: [30 ug DNA], [1×10^8 MBs], and [1×10^{12} jNPs]. Rats are infused with either test DEspR-targeted jNP[DNA]MB-heteroplex or control non-jNP DEspR-targeted DNA/MBs in which the same anti-DEspR antibody is linked to MBs via biotin-streptavidin coupling.¹¹ After allowing most microbubbles to clear the circulation (about ~5 min),¹¹ target-bound microbubbles are visualized by contrast-enhanced imaging of tumor feeder vessels at the base of the tumor and intratumoral

microvessels, using the Vevo770 contrast-enhanced imaging program and software analysis at 10- 20- , and 30-min post-infusion (t-20, t-30).

[0218] The inventors performed contrast-enhanced ultrasound imaging using with identical settings for all tumors following precautions to minimize movement artifacts: rats were anesthetized, scanhead was immobilized using the Vevo770™ scanhead-specific rail-system, and imaging was gated to respirations. The inventors used the Vevo770™ Contrast Mode parameters as per manufacturer's specifications similar to published description by Lee et al., 2008, and Willmann et al., 2008 and 2010, except that the inventors use a rat-appropriate scanhead – the RMV16, and modified the contrast-imaging protocol in terms of timing as described below.

[0219] More specifically, the inventors used a Vevo770™ (VisualSonics-Fuji Inc™) ultrasound imaging system and RMV-16 with broadband frequency up to 23.5 MHz, axial resolution of 85 microns and lateral resolution of 215 microns at focal length 17.5 mm, field of view up to 33 mm. B-mode imaging was optimized with acoustic focus entered at the level of the spontaneous mammary tumor. Rats were maintained on 1.2-1.5% isoflurane during scanning; and all imaging settings were kept constant for all imaging sessions. Tail vein infusions of test and control MBs (total 10^8 MBs/rat) were done respectively with documentation of increased contrast intensity signals (CISs) from the inflow of MBs. After 5 minutes, ascertainment of clearance of most, if not all, MBs from the circulation was checked. At t-10, the first molecular imaging tumor scan was done gated to respirations, and with the scanhead immobilized on the Vevo770™ rail system to eliminate movement artifacts manifested as speckle variance. 200 ultrasonographic frames were obtained at a temporal resolution of 10 seconds, followed by a destruction pulse [20 cycles, 10 MHz, mechanical index of 0.59] for 3 seconds followed by imaging for another 20 seconds (~ 400 frames) post-destruction.

[0220] Image processing and quantification are done using the Vevo770™ contrast Mode analysis offline for regions of interest (ROI) corresponding to feeder vessels and intratumoral vessels. As described Lyshchik et al., 2007, “image processing in the Vevo770™ system relies on 2 sets of images: a predestruction set and a postdestruction data set. ... Images from the predestruction set were paired to their partner images in the postdestruction set using an absolute sum-of-differences technique. ... Once the image pairs were calculated, the subtracted image was generated and displayed in shades of green on top of the B-mode image by a blending algorithm to provide a map of the spatial distribution of the ultrasound contrast agents retained by the tissues.” (Lyshchik et al., 2007) Offline analysis with the Vevo770™ Contrast Enhanced Imaging software of operator-selected ROIs then plots contrast intensity signals against time (time intensity plots) demarcating predestruction and postdestruction CISs.

This protocol is repeated in 2-3 other regions of the tumors and the sequence repeated detecting stable receptor-targeted adherent MBs. Comparative analysis was then done using 10 ROIs per study group comprised of: the later two time points (t20, t30), two vessel types (extratumoral feeder vessels and

intratumoral microvessels, and analysis of test (DEspR-targeted jNP[DNA]MBs), and control non-jNP DEspR-targeted MBs.

***In vivo* DNA/miRNA-payload delivery by sonoporation using jNP[DNA/miRNA-126]MB-heteroplexes**

[0221] After contrast-enhanced ultrasound molecular imaging, sonoporation is performed using the SoniGene sonoporation with a 1 cm-diameter 1 MHz probe with a half-value depth of 2.3 cm.

Sonoporation is performed with the following settings: 50% duty cycle, for 1.5 watts/cm², for 60 seconds, 153 kPa (Sonigene, Visual Sonics Inc, Canada).

[0222] Delivery and functional expression (transcription, translation) of the RFP-minigene is determined by imaging red fluorescence in tumors by IVIS (Xenogen™, Inc) two days after sonoporation. Mock-sonoporated rats (sonoporation, no microbubbles) serve as negative controls and are imaged side-by-side with the jNP[DNA]MB-heteroplex-sonoporated rat to ascertain identical conditions and set-up for valid comparative fluorescence imaging studies.

RNA Isolation and quantitative real-time RT-PCR analysis for miRNA-126 levels

[0223] RNA is isolated from 50 mg of pulverized frozen xenograft tumor tissue using the miRNeasy™ Mini Kit (Qiagen™, Maryland USA) following the manufacturer's recommended protocol. Reverse transcription (RT) is performed using the miScript™ II Reverse Transcription Kit (Qiagen™, Maryland USA) with the following conditions: Two micrograms of total RNA are reverse transcribed in HiSpec™ Buffer, which facilitates selective conversion of mature miRNAs into cDNA. Twenty microliter reactions are set up following manufacturer's specifications and incubated at 37°C for 1 hr, then 95°C for 5 min. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for detection of mature miRNA-126 is performed using the miScript™ SYBR Green PCR Kit (Qiagen™, Maryland USA) together with the miRNA-126 miScript™ Primer Assay (Qiagen™, Maryland USA). Cycling is done in a StepOnePlus™ machine (Applied Biosystems™, California USA) with the following thermal profile: 95°C for 15 min; 40 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec with optics on; followed by a melt curve analysis. miRNA-126 sequences for rat and human are identical.

Western Blot Analysis of KRAS protein levels as a downstream target of delivered miRNA-126.

[0224] Cytosolic proteins are isolated from an aliquot of frozen pulverized xenograft tumors used for miRNA-126 real-time qRT-PCR analysis. Western blot analysis is done as described¹⁰ using equal amounts of cytosolic protein extract (30 µg) isolated from MDA-MB-231 and Panc1 CSC-derived xenograft tumors from test and control tumors. Cytosolic extracts are prepared by tissue homogenization in buffer containing 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 250 mM Sucrose,

protease inhibitors, followed by centrifugation (11000g x 10 min) and final collection of supernatant (cytosolic fraction). Proteins are separated on a 15% SDS-PAGE and are transferred to PVDF membrane (Bio-Rad™). The Western blot is reacted sequentially with anti-KRAS (Abcam™ cat# ab55391 at 20 µg/ml) followed by anti-β-actin (Santa Cruz™ cat# sc-47778 at 1 µg/ml) antibodies. Immunoreactive proteins are detected by chemiluminescence using the ECL Western Detection kit (GE Healthcare™).

References for Supplementary Methods

1. Sun, S. Monodisperse MFe₂O₄ (M = Fe, Co, Mn) Nanoparticles. **126**, 273-279 (2004).
2. Lattuada, M. & Hatton, T.A. Functionalization of monodisperse magnetic nanoparticles. **23**, 2158-2168 (2007).
3. Budijono, S.J. . Block copolymer surface coverage on nanoparticles. **360**, 105-110 (2010).
4. Sardar, R., & Shumaker-Parry, J.S. Asymmetrically functionalized gold nanoparticles organized in one-dimensional chains. **8**, 731-736 (2008).
5. Wang, B., Li, B., Zhao, B., & Li, C.Y. Amphiphilic Janus gold nanoparticles via combining "solid-state grafting-to" and "grafting-from" methods. **130**, 11594-11595 (2008).
6. Park, Y.C. Effect of PEG molecular weight on stability, T2 contrast, cytotoxicity, and cellular uptake of superparamagnetic iron oxide nanoparticles (SPIONs). **11**, 106-14 (2014).
7. Nitin, N. Functionalization and peptide-based delivery of magnetic nanoparticles as an intracellular MRI contrast agent. **9**, 706-712 (2004).
8. Dubochet, J., Groom, M. & Mueller-Neuteboom, S. The mounting of macromolecules for electron microscopy with particular reference to surface phenomena and the treatment of support films by glow discharge. **8**, 107-135 (1982).
9. Dubochet, J. Cryo-electron microscopy of vitrified specimens. **21**, 129-228 (1988).
10. Herrera VL, Decano JL, Tan GA, Moran AM, Pasion KA, Matsubara Y, Ruiz-Opazo N. 2014. DEspR roles in tumor vasculo-angiogenesis, invasiveness, CSC-survival and anoikis resistance: a 'common receptor coordinator' paradigm. **9**, e85821.
11. Decano, J.L., Moran, A.M., Ruiz-Opazo, N., Herrera, V.L.M. Molecular Imaging of vasa vasorum neovascularization via DEspR-targeted contrast-enhanced ultrasound micro-imaging in transgenic atherosclerosis rat model. **13**, 1096-1106 (2011).
12. Escoffre JM1, Novell A, Piron J, Zeghimi A, Doinikov A, Bouakaz A. "Microbubble attenuation and destruction: are they involved in sonoporation efficiency?" (2013) *IEEE Trans Ultrason Ferroelectr Freq Control*. 2013 Jan;60(1):46-52.
13. Kleemann, E. Nano-carriers for DNA delivery to the lung based upon a TAT-derived peptide covalently coupled to PEG-PEI. *J Controlled Release* 109:299-316 (2005).

14. Taratula, O. Multifunctional nanomedicine platform for cancer specific delivery of siRNA by superparamagnetic iron oxide nanoparticles-dendrimer complexes. *Current Drug Delivery* 8:59- 69 (2011).
15. Delalande A, Kotopoulis S, Postema M, Midoux P, Pichon C. Sonoporation: mechanistic insights and ongoing challenges for gene transfer. *Gene* (2013) 525:191-199.
16. Escoffre JM, Zeghimi A, Novell A, Bouakaz A. (2013-b). *In vivo* gene delivery by sonoporation: recent progress and prospects. *Current Gene Therapy* 13:2-14 (2013).
17. Escoffre JM, Piron J, Novell A, Bouakaz A. Doxorubicin delivery into tumor cells with ultrasound and microbubbles. *ACS Molecular Pharmaceutics* 8:799-806, 2011.
18. Escoffre JM, Novell A, Serrier S, Lecomte T, Bouakaz A. Irinotecan delivery by microbubble- assisted ultrasound: *in vitro* validation and a pilot preclinical study. *Mol Pharmaceutics* 10:2667- 2675, 2013.
19. Lentacker I, DeCock I, Deckers R, DeSmedt SC, Moonen CTW. Understanding ultrasound induced sonoporation: definitions and underlying mechanisms. *Advanced Drug Delivery Reviews*, 2014.
20. Loveless ME, Whisenant JG, Wilson K, Lyshchik A, Sinha TK, Gore JC, et al. Coregistration of ultrasonography and magnetic resonance imaging with a preliminary investigation of the spatial colocalization of vascular endothelial growth factor receptor 2 expression and tumor perfusion in a murine model. *Mol Imaging* 2009 Aug;8(4):187-98.
21. Mohamedi G, Azmin M, Pastoriza-Santos I, Huang V, Perez-Juste J, Liz-Marzan LM, Edinsinghe M, Stride E. 2012. Effects of gold nanoparticles on the stability of microbubbles. *Langmuir* 28:13808-13815.
22. Mullin LB, Phillips LC, Dayton PA. Nanoparticle delivery enhancement with acoustically activated microbubbles. *IEEE Trans Ultrason Ferroelect Freq Control* 60(1), (2013).
23. Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009 Jun 12;324(5933):1457-61.
24. Sun, C. et al., *In vitro* acoustic characterization of three phospholipid ultrasound contrast agents from 2-43 MHz. *Ultrasound Med Biol*. Mar 2014; 40(3): 541–550.
25. Willmann JK, Kimura RH, Deshpande N, Lutz AM, Cochran JR, Gambhir SS. Targeted contrast-enhanced ultrasound imaging of tumor angiogenesis with contrast microbubbles conjugated to integrin-binding knottin peptides. *J Nucl Med* 2010. 51:433-440.

EXAMPLE 4: Use of jNP as negative contrast agent for MRI of tumor enhanced permeability and retention (EPR) and for target-specific molecular MR-imaging of tumors.

[0225] Specifically contemplated herein are methods and compositions comprising janus nanoparticles (jNPs) as described herein for use in MRI imaging, particularly tumor imaging.

[0226] Provided herein, in one aspect, are methods relating to the use of non-targeted jNPs as negative contrast agents for MR-imaging of EPR in any tumor tissue type (*i.e.*, not specific to a particular receptor or target). Such methods and compositions are essentially contemplated for use as a universal MRI contrast agent for monitoring EPR.

[0227] Clinically, EPR is the phenomenon by which drugs enter tumors and are ‘enriched’ in tumors in contrast to normal tissues. Detection of EPR and monitoring of EPR using jNPs will permit one of skill in the art to determine drug response and tumor localization. Such methods are relevant to personalized medicine care, as well as in the testing of novel biotherapeutics to determine whether EPR is a factor in the efficacy conditions of a new drug or candidate agent.

[0228] Another aspect provided herein relates to methods and compositions related to the use of targeted jNP for MR-imaging of target-specific tumor localization (*e.g.*, a molecular signature of a tumor).

[0229] Target-specific tumor localization of jNPs and hence target-specific MR-imaging of tumors could give insight into the molecular signature of tumors non-invasively. In addition, this technique can provide key information akin to “personalized medicine biopsy information” in determining the right kind of therapy especially for tumors that are not easily biopsied.

[0230] FIG. 5A shows that jNPs shorten T2* relaxivity, thus distinguishing jNP+ tumors from jNP(-) tumors.

[0231] FIG. 5B shows that non-targeted jNPs localize to tumors in a heterotopic subcutaneous xenograft tumor model.

[0232] FIG. 18 shows that non-targeted jNPs localize to multiple tumors in xenograft tumor model of pancreatic cancer peritoneal metastasis. This technique fulfills an unmet need in terms of early detection and treatment of such cancers.

EXAMPLE 5: *In Vitro* Cell Transfection using jNPs

[0233] Data provided herein show that janus nanoparticle coated microbubbles (jNP-MBs) deliver DNA *in vitro* (% cell transfection) at least 2-fold better than previously published reports of cationic microbubbles and with less cell loss. Thus, transfection with the jNP-MBs results in less cell loss and provides a wider safety margin in terms of % cell viability. See *e.g.*, FIGs. 17K, 17L, and 19.

[0234] In addition, the inventors have determined that DNA payloads on jNP-MBs are comparable to that reported for CMBs in the literature. Additionally, while CMB-DNA payloads are measured after 15 minutes, jNP-MB DNA payloads were measured at 1 hour and 6 hours showing stable DNA-payload binding. Although different targets and different cell lines were used in the comparator CMB studies, there is no evidence that CMBs are better than jNP-MBs, in fact data show that jNP-MBs can bind DNA equivalently and deliver DNA more efficiently and safely.

[0235] The efficacy and safety of the jNP-MBs were compared to the CMBs in the following references:

- [0236] **CMB-1** [VCAM-targeted; in vitro only]: Phillips LC, Klibanov AL, Wamhoff BR, Hossack JA. 2012. Intravascular ultrasound detection and delivery of molecularly targeted microbubbles for gene delivery. *IEEE Transaction on Ultrasonics, Ferroelectrics, and Frequency Control* 59:1596-1601.
- [0237] **CMB-2** [P-selectin-targeted] Jin Q, Wang Z, Yan F, Deng Z, Ni F, Wu J, Shandas R, Liu X, Zheng H. 2013. A novel cationic microbubble coated with stearic acid-modified polyethylenimine to enhance DNA loading and gene delivery by ultrasound. *PLOS One* 8:e76544.
- [0238] **CMB-3** [CD105-targeted]: Zhou Y, Gu H, Xu Y, Li F, Kuang S, Wang Z, Zhou X, Ma H, Li P, Zheng Y, Ran H, Jian J, Zhao Y, Song W, Wang Q, Wang D. 2015. Targeted antiangiogenesis gene therapy using targeted cationic microbubbles conjugated with CD105 antibody compared with untargeted cationic and neutral microbubbles. *Theranostics* 4(4): 399-417.
- [0239] **CMB-4** [P-selectin-targeted]: Xie A, Belcik T, Qi Y, Morgan TK, Champaneri SA, Taylor S, Davidson BP, Zhao Y, Klibanov AL, Kuliszewski MA, Leong-Poi H, Ammi A, Lindner JR. 2012. Ultrasound-mediated vascular gene transfection by cavitation of endothelial-targeted cationic microbubbles. *JACC Cardiovasc Imaging* 5:1253-1262.
- [0240] **CMB-5** [non-targeted]: Panje CM, Wang DS, Pysz MA, Paulmurugan R, Ren Y, Tranquart F, Tian L, Willmann JK. 2012. Ultrasound-mediated gene delivery with cationic versus neutral microbubbles: effect of DNA and microbubble dose on in vivo transfection efficiency. *Theranostics* 2012; 2:1078-1091.
- [0241] **CMB-6** [non-targeted]: Sun RR, Noble ML, Sun SS, Song S, Miao CH. 2014. Development of therapeutic microbubbles for enhancing ultrasound-mediated gene delivery. *J Control Release* 182:111-120.
- [0242] **CMB-7** [non-targeted]: Wang DS, Panje C, Pysz MA, Paulmurugan R, Rosenberg J, Bambhirk SS, Schneider M, Willmann JK. 2012. Cationic versus neutral microbubbles for ultrasound-mediated gene delivery in cancer. *Radiology* 264:721-732.

EXAMPLE 6: *In vivo* ultrasound molecular imaging

[0243] Data provided herein show that jNP-MBs exhibit greater molecular imaging efficacy, for example, by reaching higher peak contrast intensity signals (CIS) *in vivo* compared to CMB-3 and CMB-4 despite using much lower doses of MBs/gram body weight.

[0244] The dose of MBs used per gram body weight for jNP-MB experiments done in rats is 50-fold less than CMB-3 and 20-fold less than CMB-4 done in mouse models, thus indicating greater relative functional imaging efficiency (See FIG. 20; dotted → ~ f_{rxn}).

[0245] Although different *in vivo* models and targets are used in comparator CMB studies, there is no evidence that CMBs are better than jNP-MBs in molecular imaging. In fact, data show that jNP-MBs

exhibit better ultrasound contrast-enhanced imaging as measured by CIS and relative to dose of MBs used per gram body weight.

[0246] Importantly, given that reflectivity is to the 4th power of MB diameter, greater peak CIS cannot be attributed to size of MBs. Neither can it be attributed to absolute number of MBs used, or dose per gram body weight.

[0247] The time of sonoporation for jNP-MBs is also greater than in CMB studies. Given that MBs only last in the circulation < 5-7 minutes, the ability to form molecular imaging from 10-20 minutes when using jNP-MBs indicates that jNPs impart stability in targeting and maintenance of acoustic properties of MicroMarker MBs.

EXAMPLE 7: *In vivo* DNA delivery

[0248] Data provided herein (see *e.g.*, FIGs. 21A-21C) show that jNP-MBs exhibit greater efficacy in delivery of DNA *in vivo*, for example, by reaching higher peak fluorescence or luminescence measured as photons/sec/cm²/sr by IVIS, compared to targeted CMB-4, and non-targeted CMB-5, CMB-6, and CMB-7, despite using > 10-fold less DNA/g body weight, and lower doses of MBs/gram body weight.

[0249] The dose of MBs used per gram body weight for jNP-MB experiments performed in rats is 50-fold less than CMB-3 and 20-fold less than CMB-4 performed in mouse models, thus indicating greater relative functional imaging efficiency (dotted ➔ ~ f_{fxn}).

[0250] Although different *in vivo* models and targets are used in comparator CMB studies, there is no evidence that CMBs are better than jNP-MBs in *in vivo* delivery of DNA. In fact, data show that jNP-MBs exhibit better delivery of DNA *in vivo*.

[0251] Importantly, greater DNA delivery cannot be attributed to the use of more DNA or MBs per g/BW.

[0252] The time of sonoporation for jNP-MBs is also greater than in CMB studies. Given that MBs only last in the circulation < 5-7 minutes, the ability to delivery DNA at 25 minutes indicates that jNPs impart stability in targeting, DNA payload, and maintenance of acoustic properties of MicroMarker MBs for ultrasound-mediated delivery (sonoporation).

All references cited herein are hereby incorporated by reference in their entirety.

CLAIMS

1. A composition comprising: a microbubble with a Janus nanoparticle associated with the surface, wherein the Janus nanoparticle comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.
2. The composition of claim 1, wherein the Janus nanoparticle is less than 75 nm in size.
3. The composition of claim 1 or 2, wherein the superparamagnetic iron oxide nanoparticle is less than 20 nm in size.
4. The composition of claim 1, 2, or 3, wherein the Janus nanoparticle is asymmetrically shaped.
5. The composition of claim 4, wherein the Janus nanoparticle is avocado shaped.
6. The composition of claim 5, wherein the targeting face is situated at the upper part of the avocado shaped nanoparticle, and the carrier face is situated at the round, bottom part of the avocado shaped nanoparticle.
7. The composition of any one of claims 1-5, wherein the targeting face comprises a targeting moiety selected from the group consisting of: a targeting peptide, a polyclonal antibody, a monoclonal antibody, an antibody binding fragment, a composite antibody, a recombinant antibody, a cell penetrating peptide and a matrix interacting peptide.
8. The composition of claim 7, wherein the targeting moiety binds to a cell surface or cell matrix protein.
9. The composition of any one of claims 1-8, wherein the carrier face comprises a polymer.
10. The composition of claim 9, wherein the polymer comprises a polyelectrolyte.
11. The composition of claim 10, wherein the polyelectrolyte is a cationic polyelectrolyte or an anionic polyelectrolyte.
12. The composition of any one of claims 1-11, wherein the Janus nanoparticle comprises an iron oxide core with a PEG brush.

13. The composition of any one of claims 1-12, wherein the PEG brush is a mixed 3:1 short-long PEG brush.
14. The composition of any one of claims 1-13, wherein the moieties in the targeting and carrier faces are covalently coupled or tethered to the long PEG in the mixed PEG-brush.
15. The composition of any one of claim 1-14, wherein the iron oxide core is encapsulated within a polymer.
16. The composition of claim 15, wherein the polymer-encapsulated iron oxide core further comprises a bioactive agent.
17. The composition of any one of claims 1-16, wherein the composition further comprises a bioactive agent.
18. The composition of any one of claims 1-17, wherein the bioactive agent comprises a nucleic acid molecule.
19. The composition of claim 18, wherein the nucleic acid molecule is sandwiched between the microbubble and the carrier face of the Janus nanoparticle.
20. The composition of any one of claims 1-18, wherein the composition is formulated for use as a magnetic resonance imaging (MRI) contrast agent.
21. The composition of any one of claims 1-20, wherein the MRI contrast agent comprises a shorter T2*-relaxivity.
22. The composition of claim 20, wherein the MRI contrast agent is a negative contrast agent.
23. A composition for use as an MRI contrast agent comprising:
a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another.
24. The composition of claim 23, wherein the MRI contrast agent is a negative contrast agent.

25. The composition of claim 23 or 24 for use in detection and/or monitoring of electron paramagnetic resonance (EPR) imaging.
26. A composition for use as an MRI contrast agent for target-specific imaging, the composition comprising:
a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another,
and wherein the targeting face comprises an antibody directed to a target to be imaged.
27. The composition of claim 26, wherein the target to be imaged is a tumor.
28. A method for imaging, the method comprising:
(a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, and wherein the nucleic acid delivery face and the targeting face are distinct from one another, and
(b) performing an MRI on the subject.
29. The method of claim 28, wherein the MRI comprises electron paramagnetic resonance (EPR) imaging.
30. A method for target-specific imaging, the method comprising:
(a) administering to a subject an MRI contrast agent comprising: a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged, and
(b) performing an MRI on the subject.
31. Use of a composition of any one of claims 1-27 as an MRI contrast agent.
32. Use of a composition of any one of claims 1-27 as a drug delivery device.
33. Use of a composition of any one of claims 1-27 as a nucleic acid delivery device.

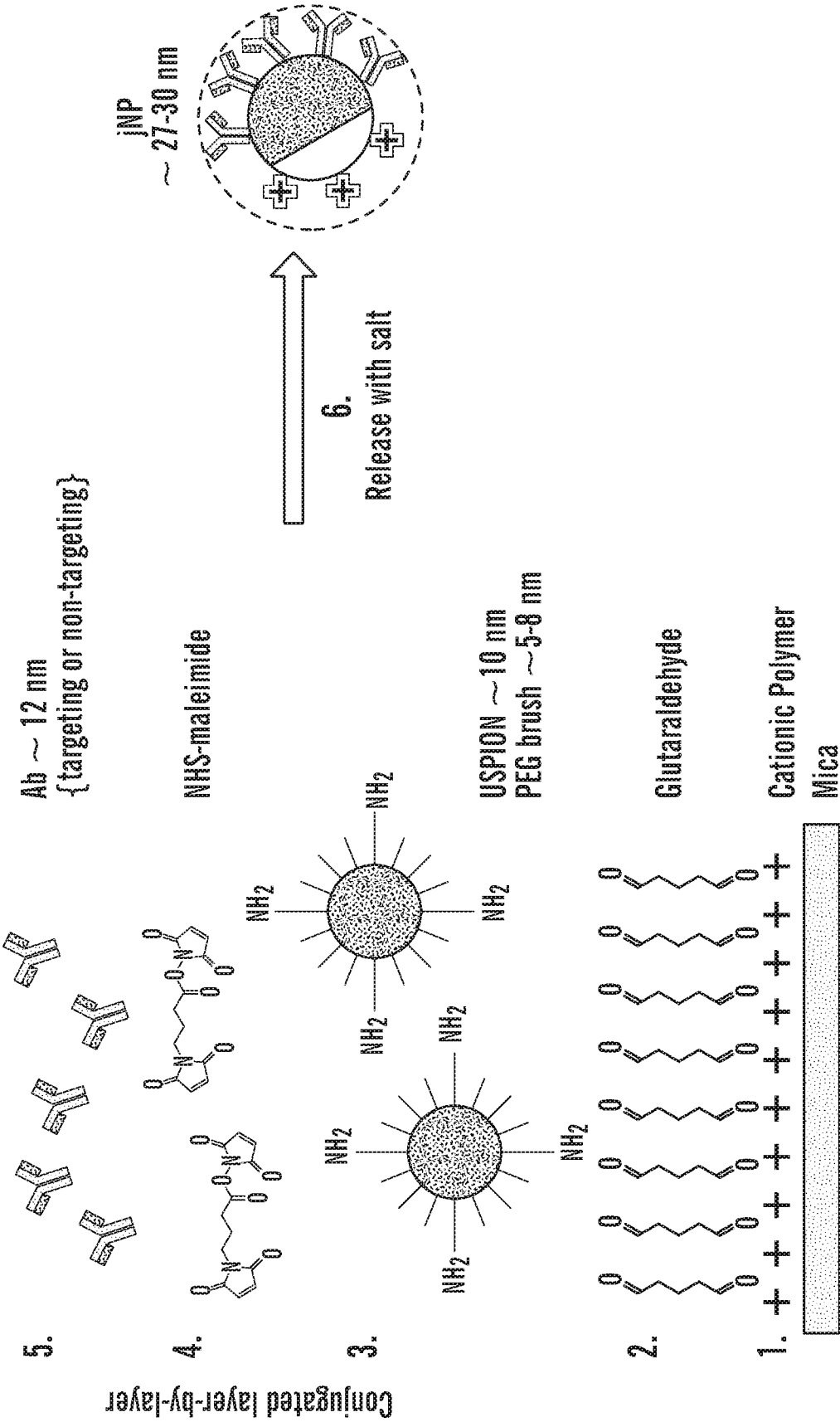
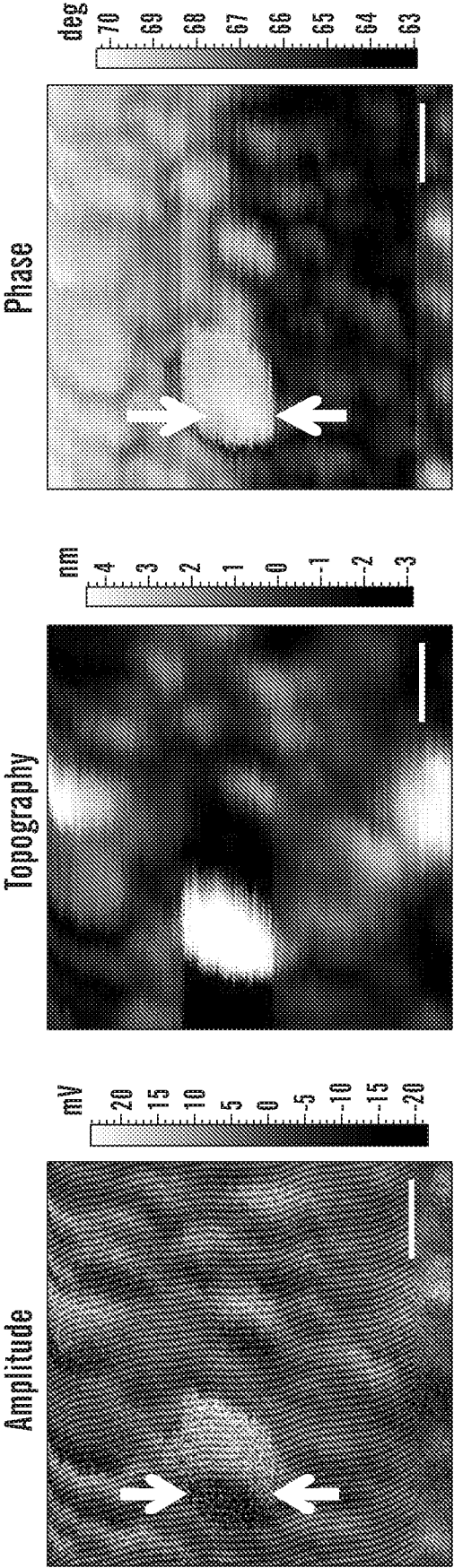
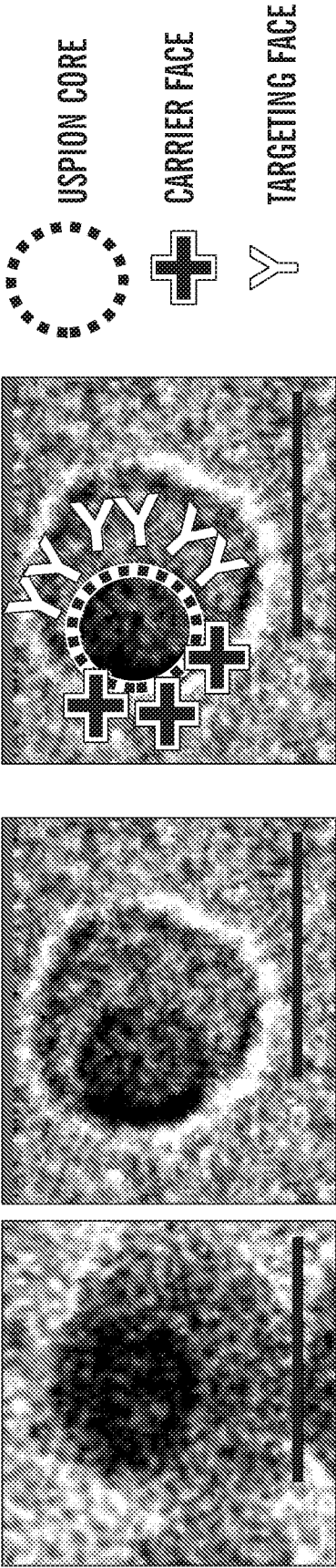


FIG. 1A



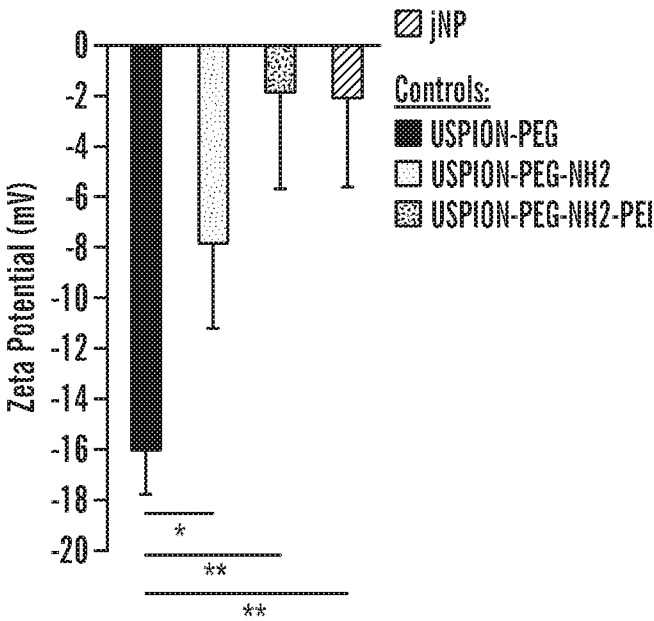


FIG. 2A

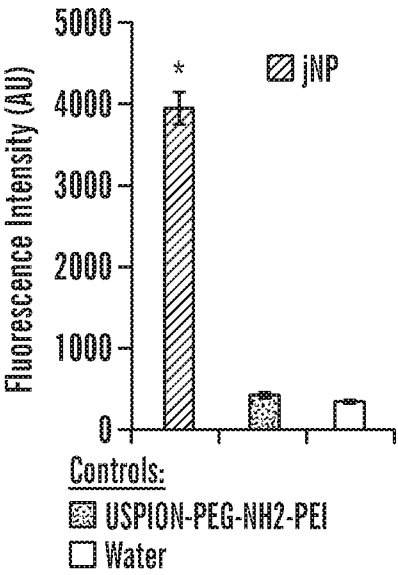


FIG. 2B

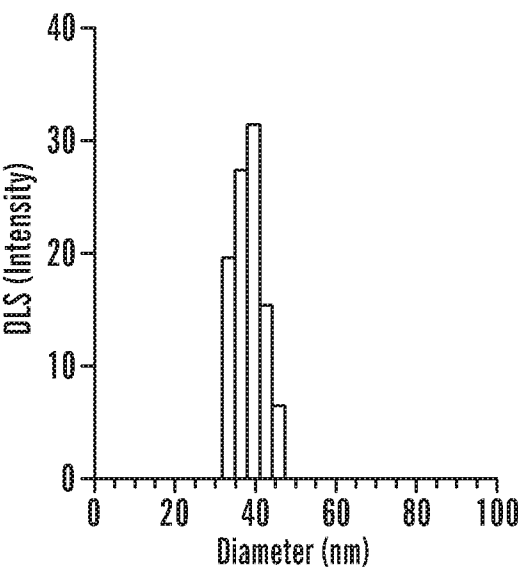


FIG. 2C

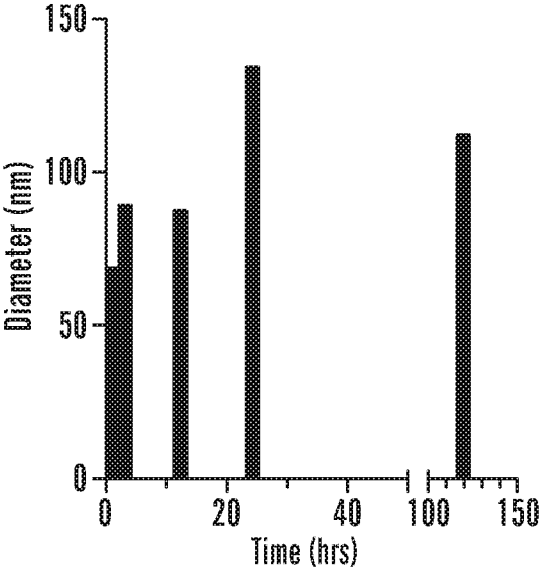
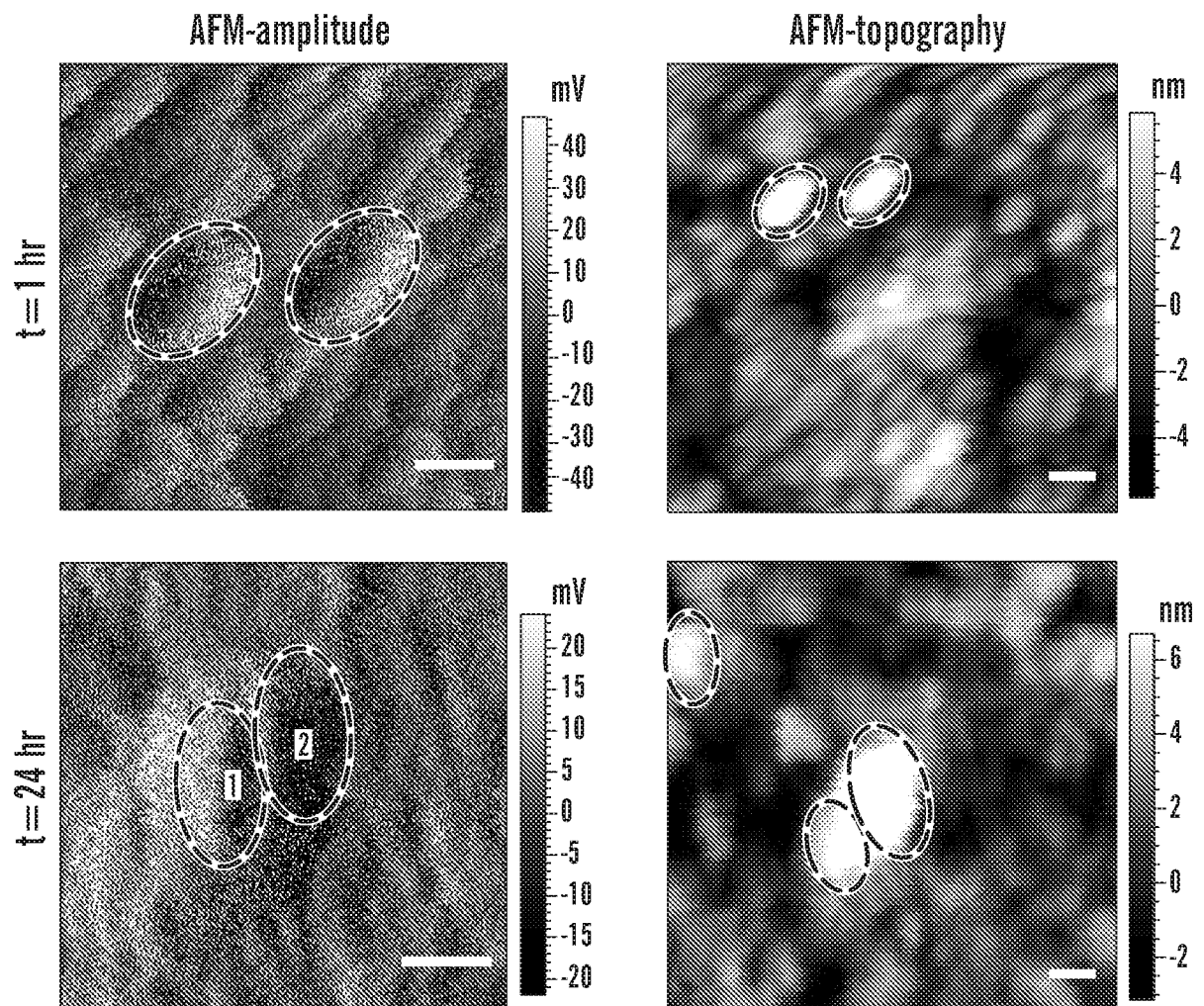
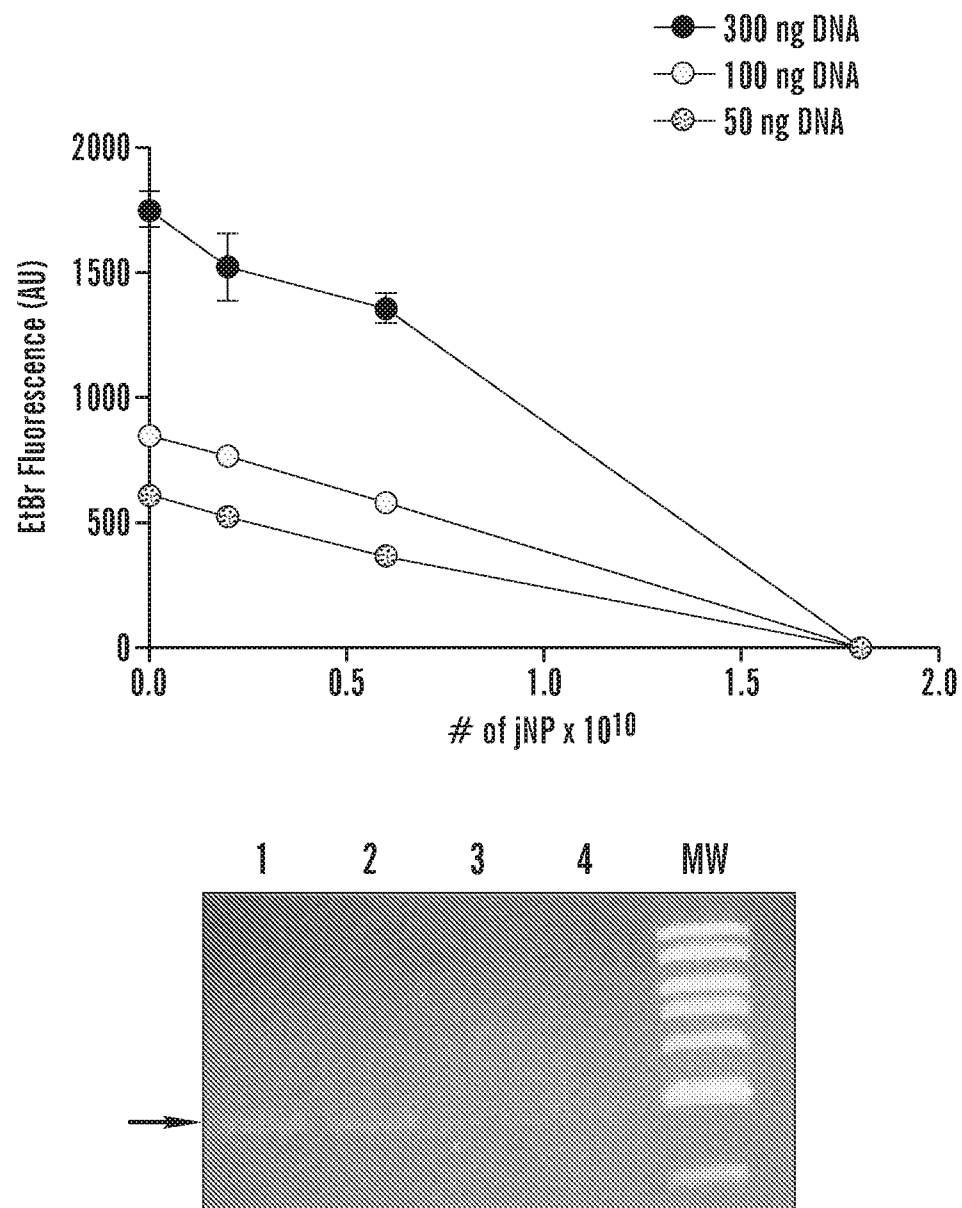


FIG. 2D

4/39



5/39

**FIG. 2F**

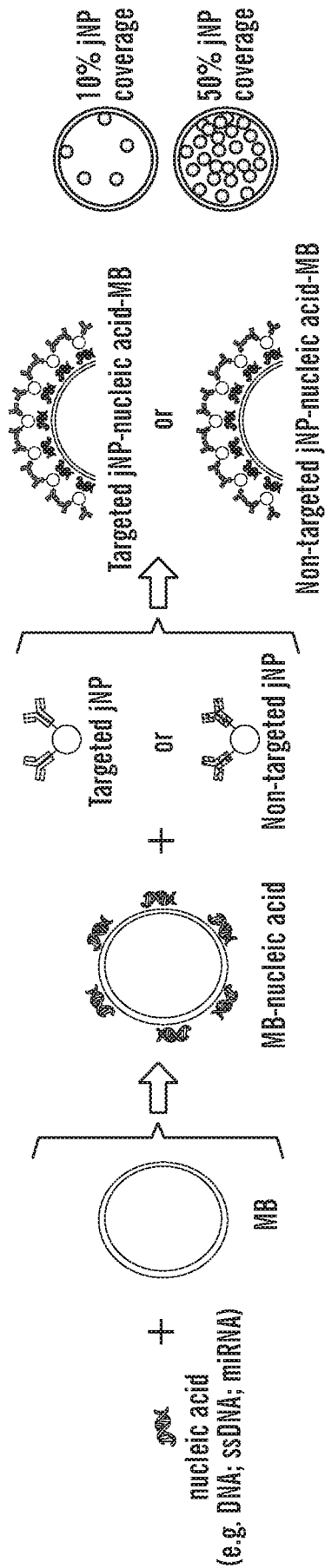


FIG. 3A

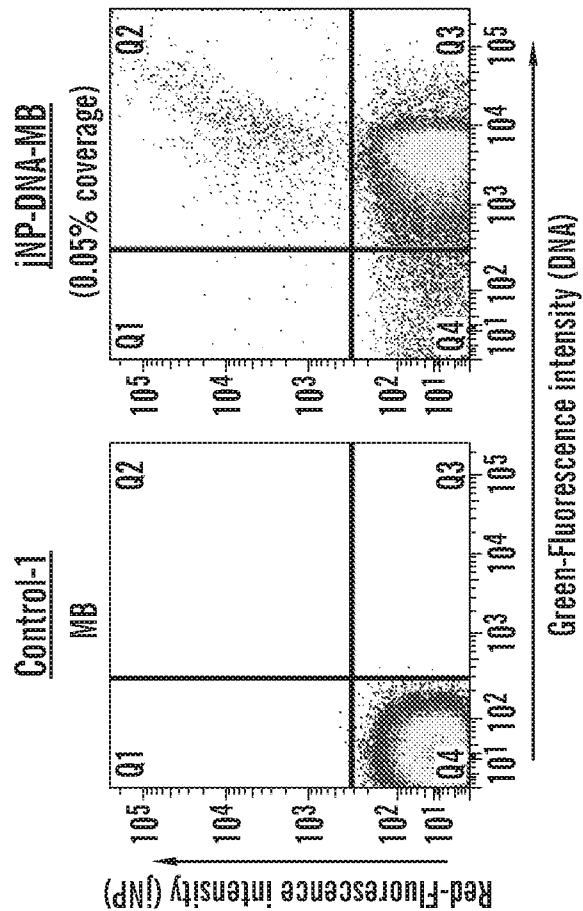


FIG. 3B

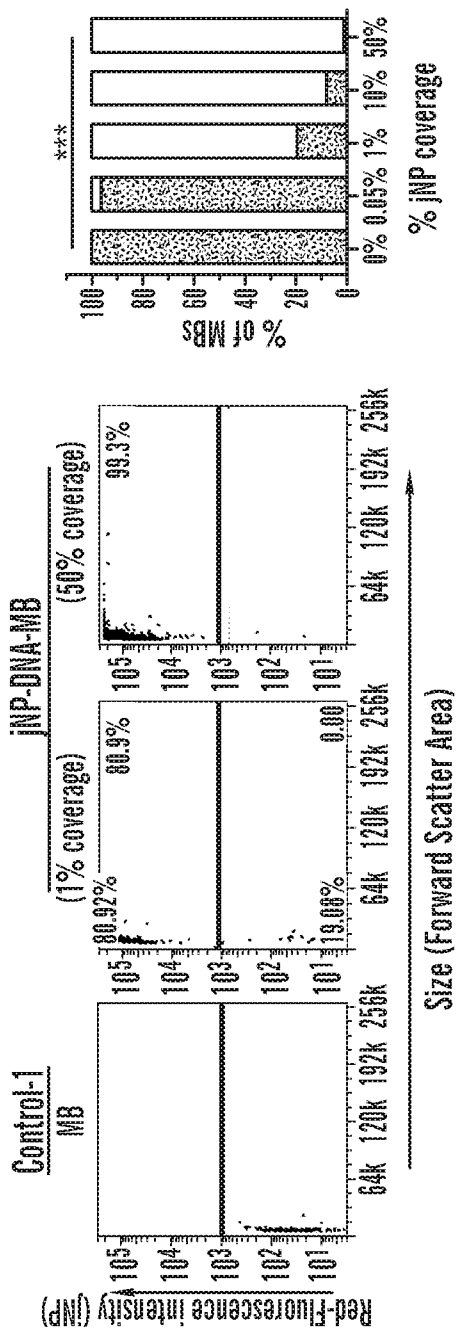


FIG. 3C

8/39

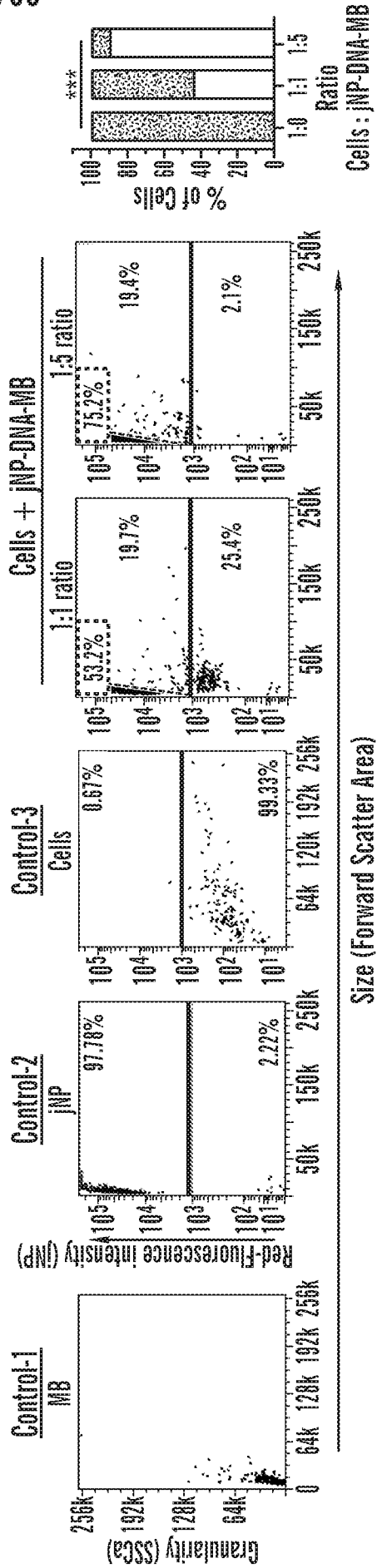


FIG. 3D

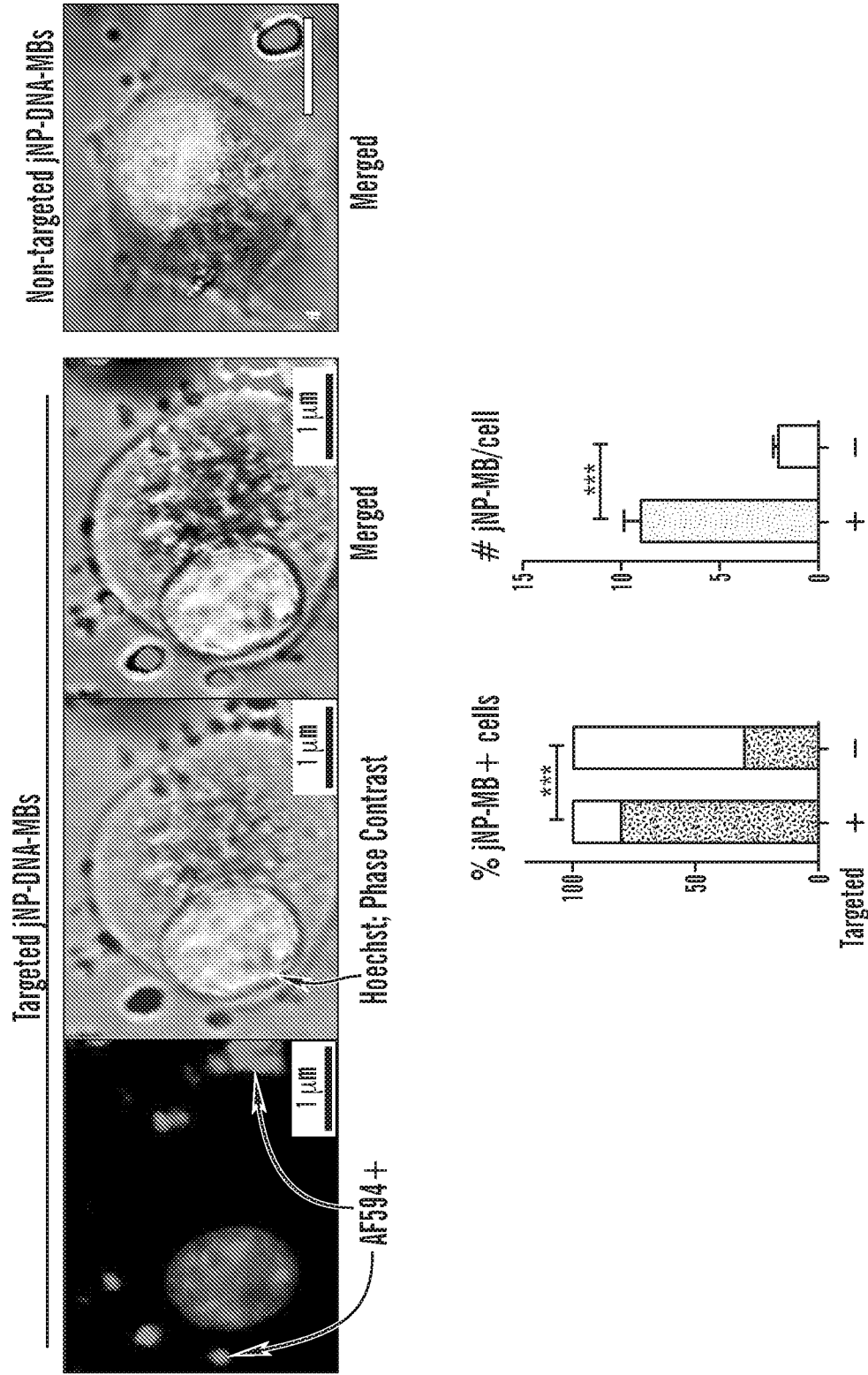
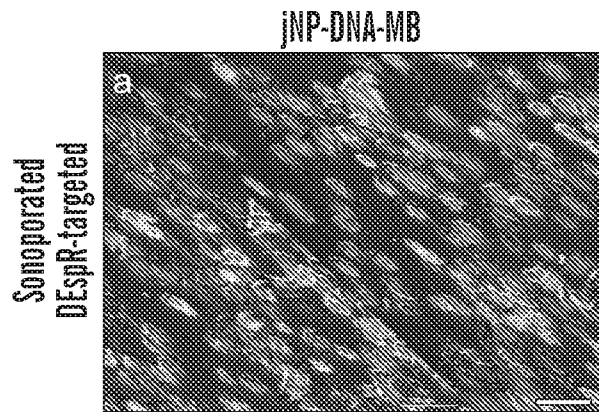
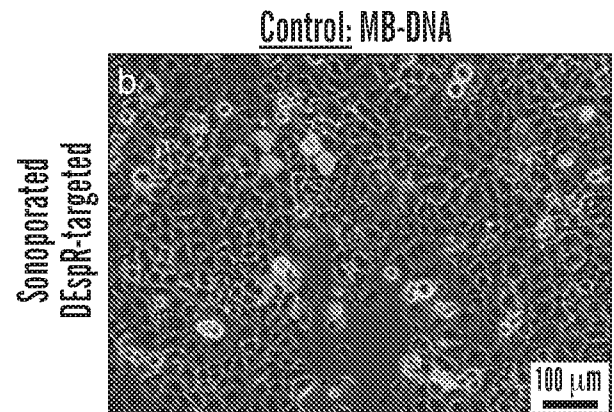
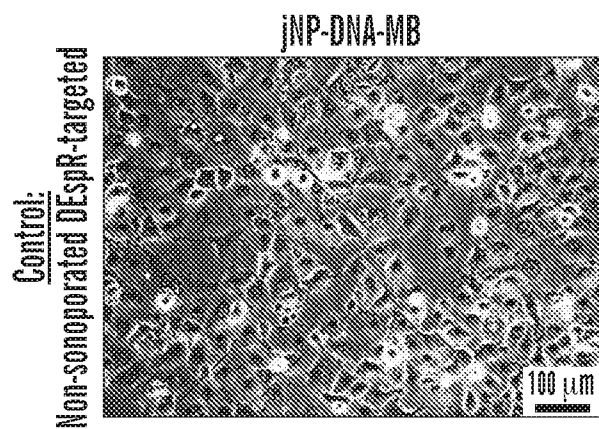
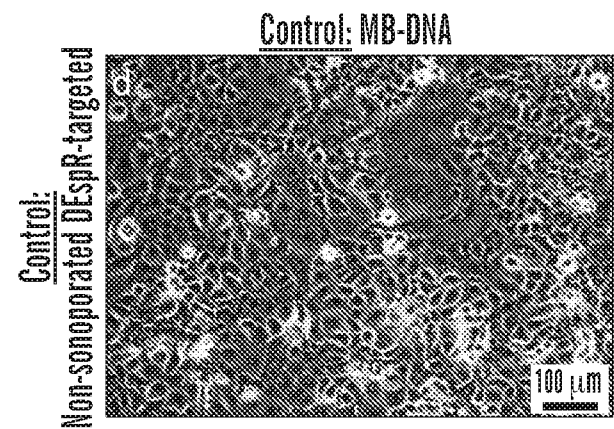


FIG. 3E

10/39

**FIG. 4A****FIG. 4B****FIG. 4C****FIG. 4D**

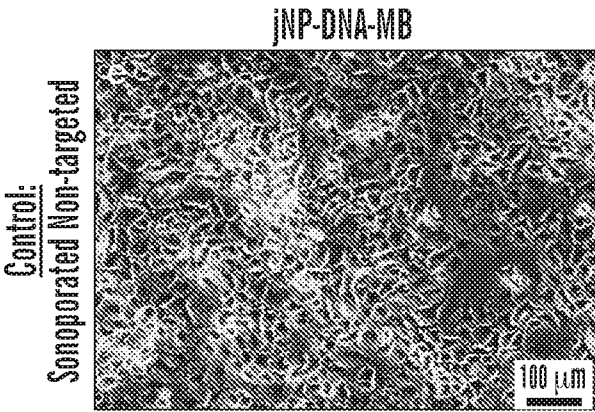


FIG. 4E

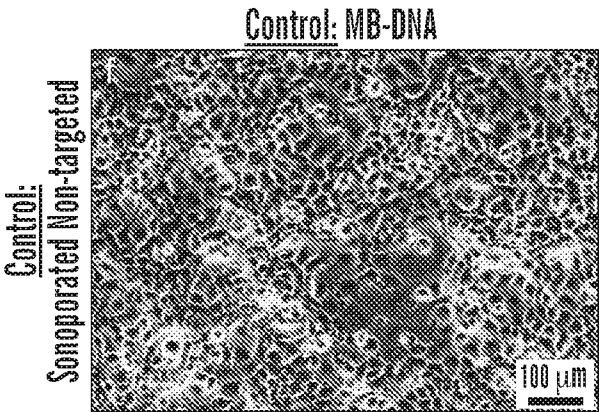


FIG. 4F

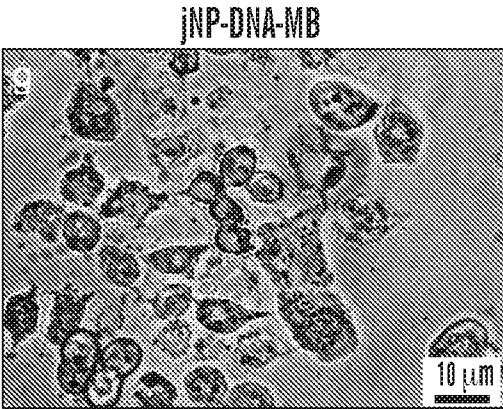


FIG. 4G

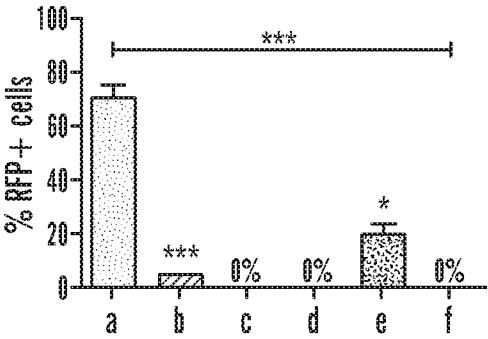
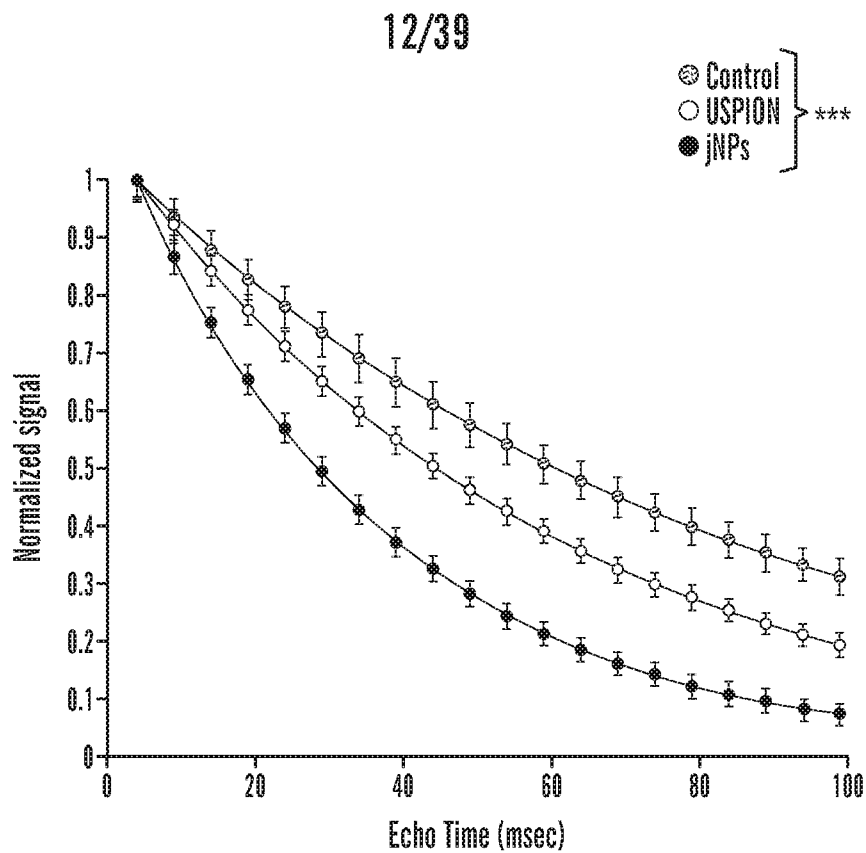
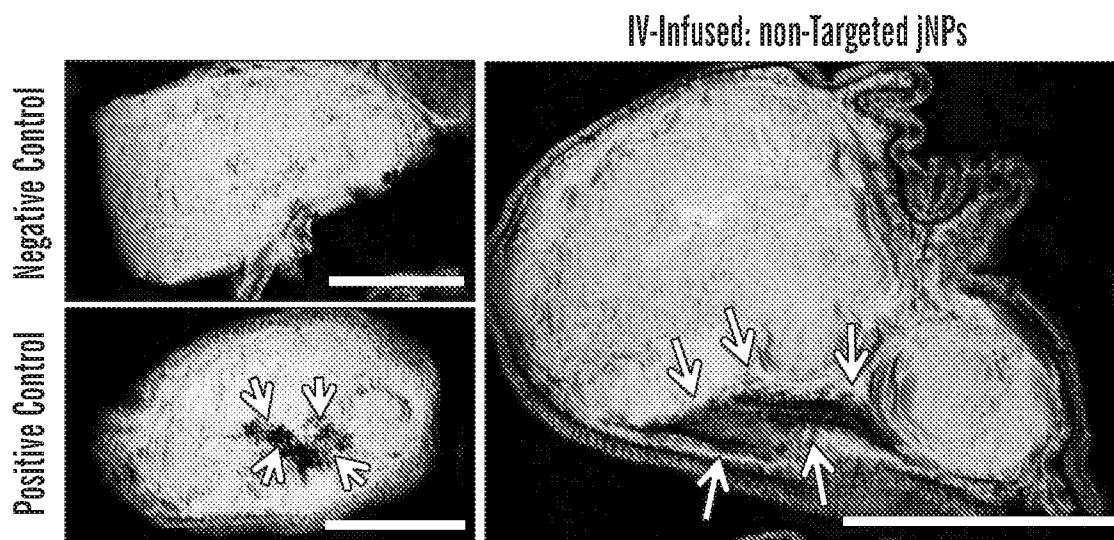


FIG. 4H

**FIG. 5A****FIG. 5B**

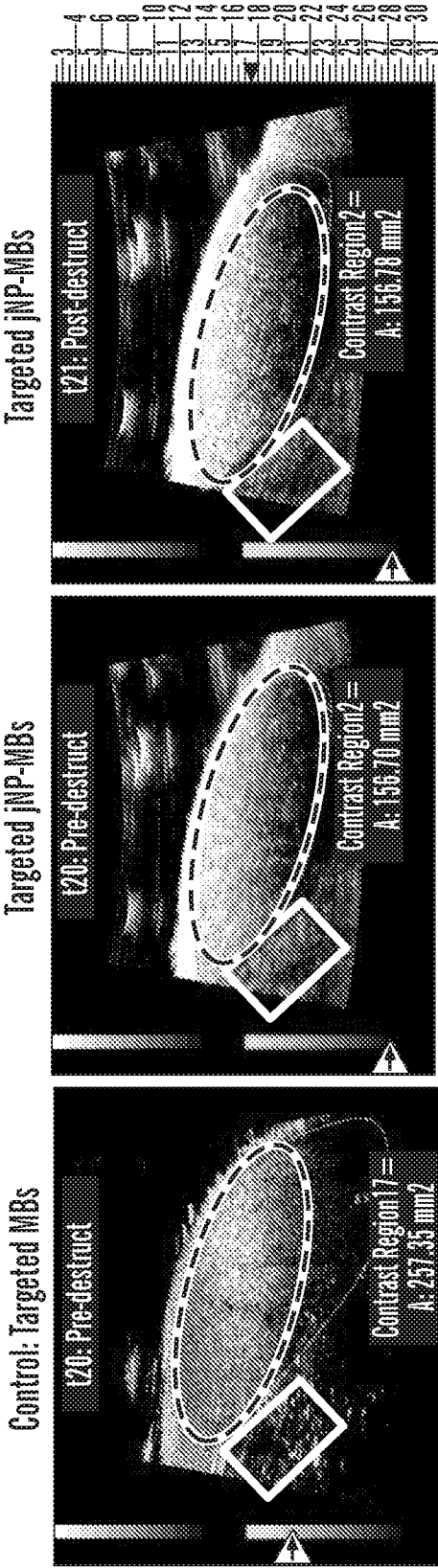


FIG. 5C

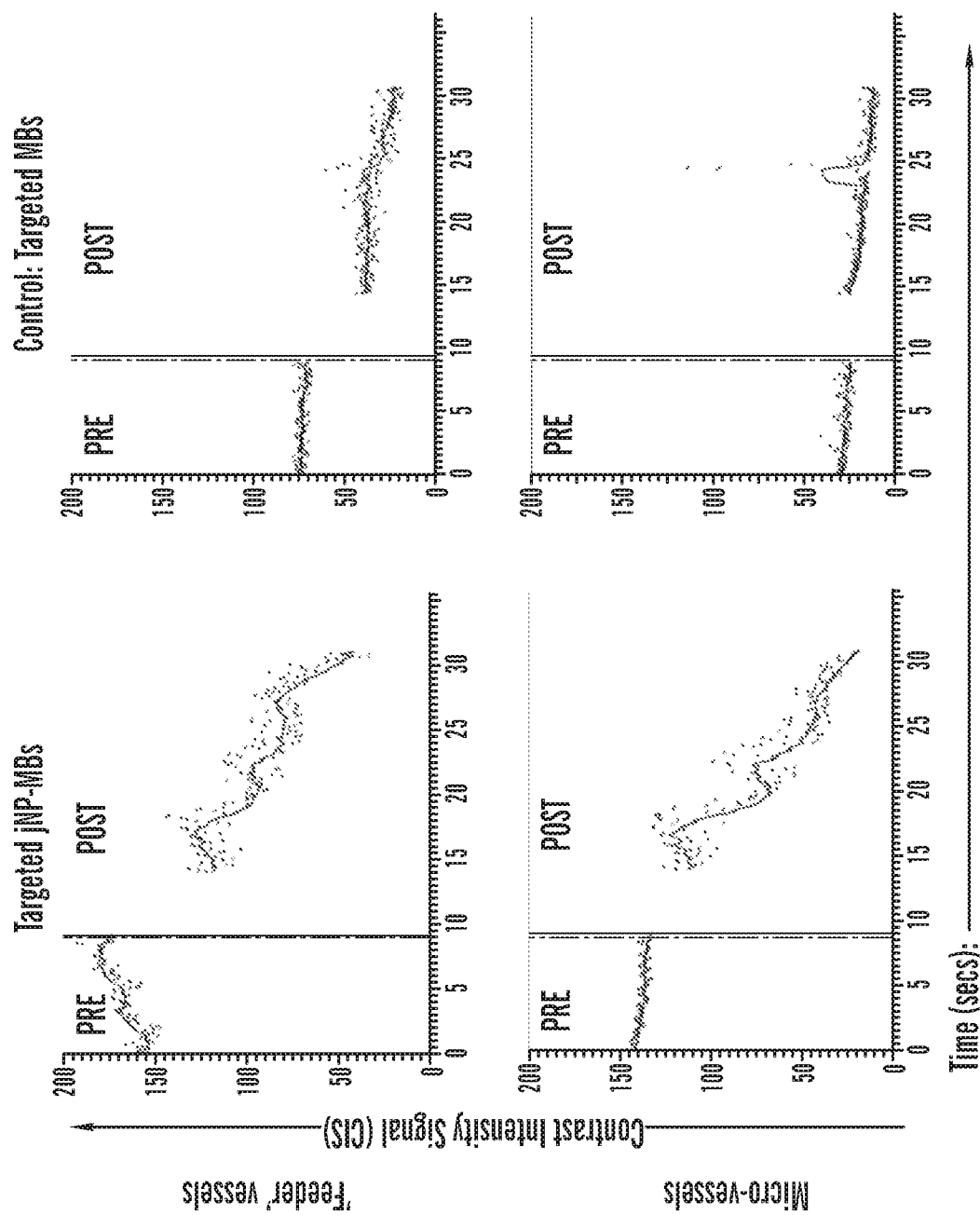
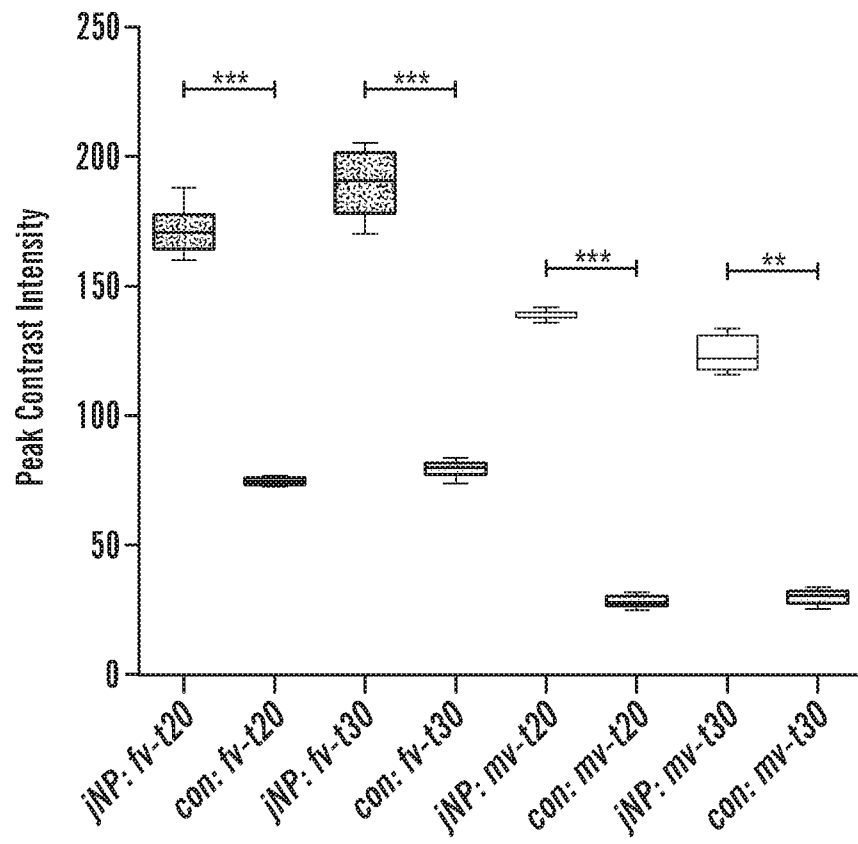


FIG. 5D

15/39

**FIG. 5E**

16/39

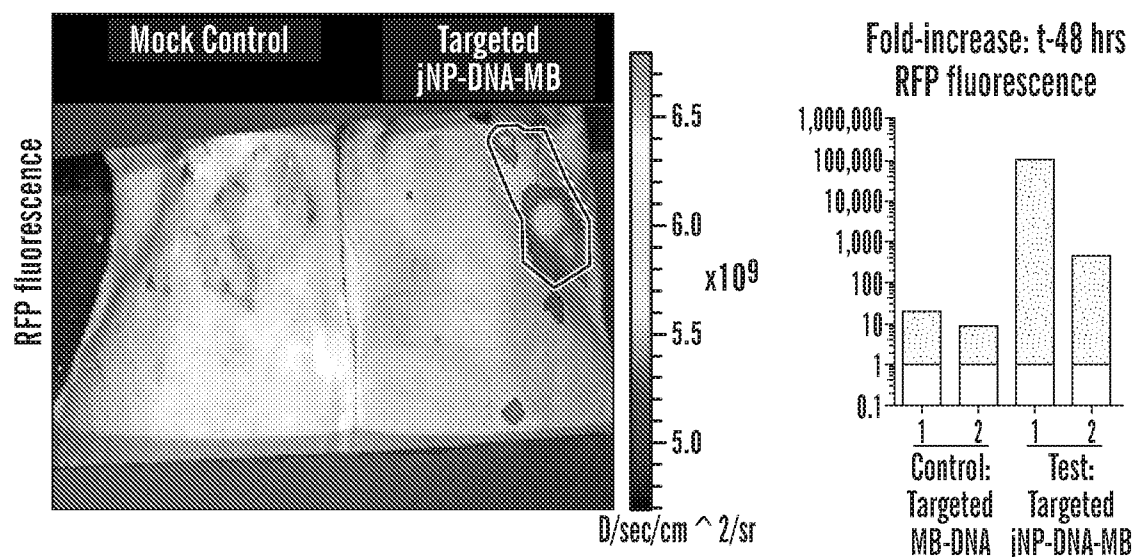


FIG. 6A

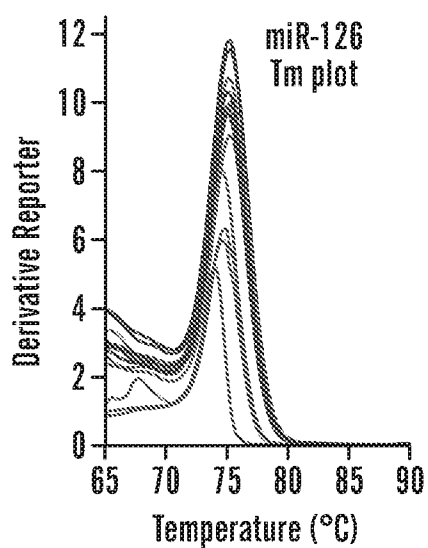


FIG. 6B

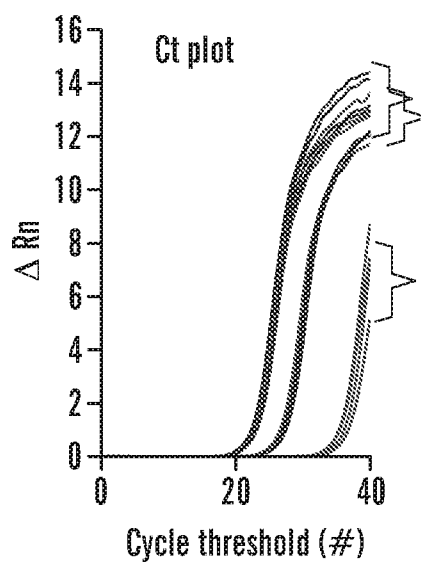


FIG. 6C

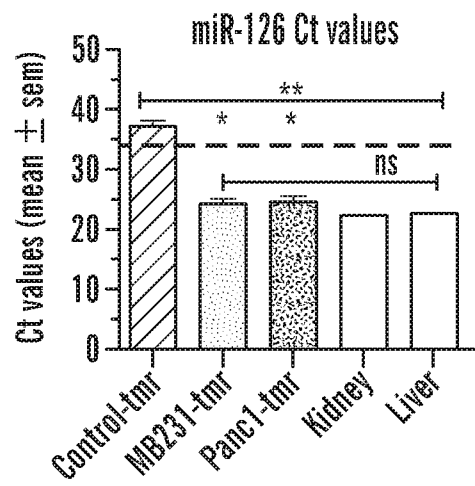


FIG. 6D

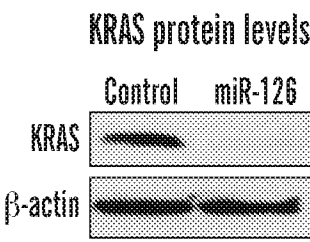


FIG. 6E

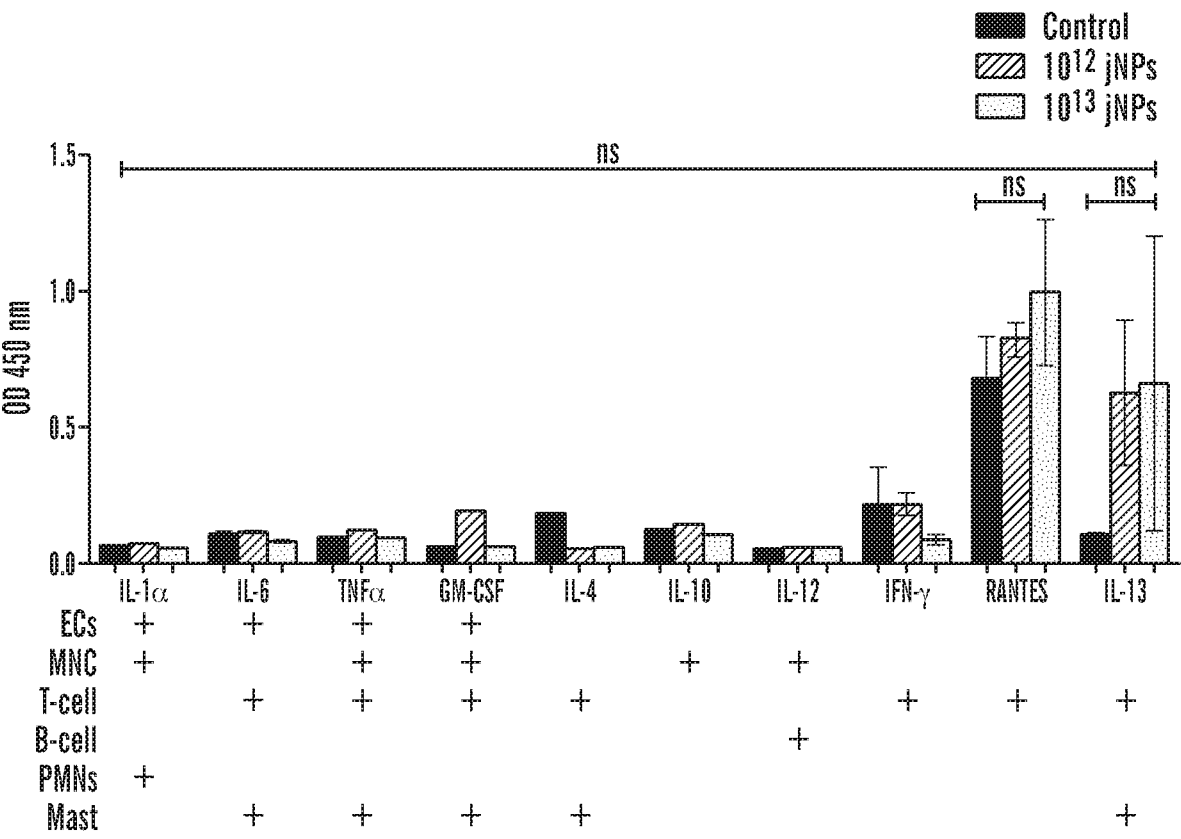


FIG. 6F

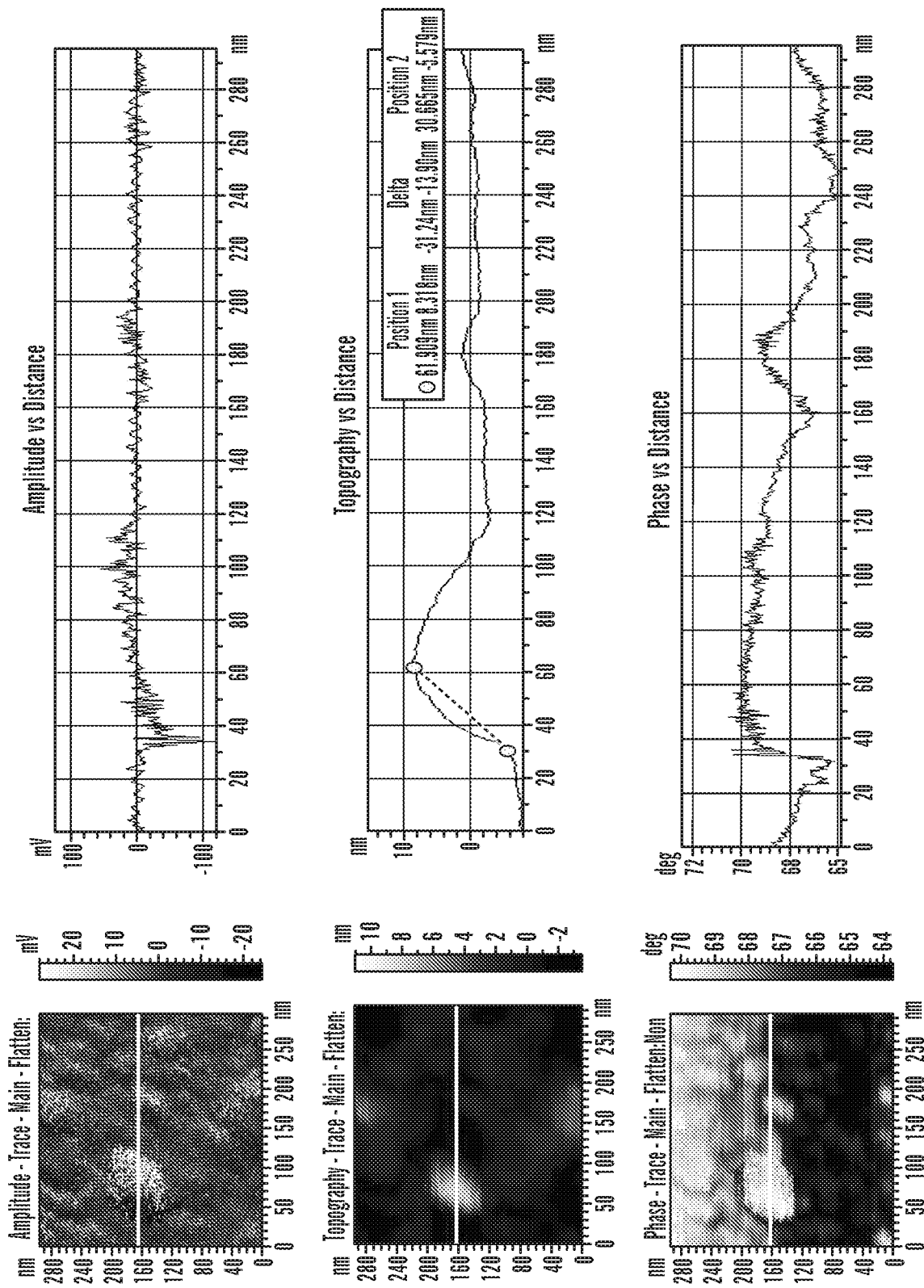


FIG. 7A

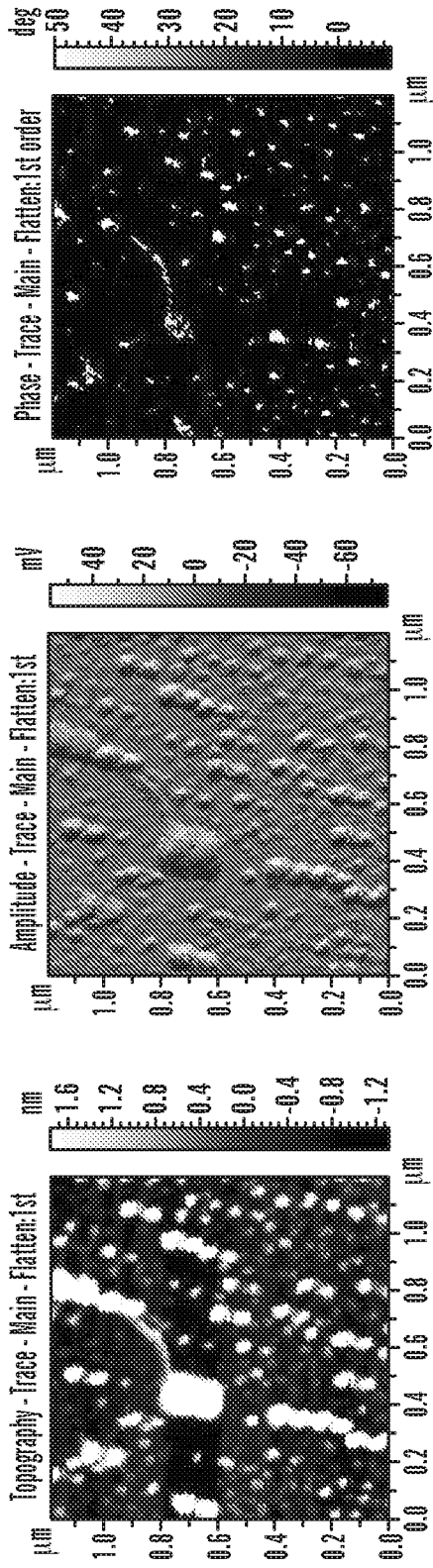


FIG. 7B

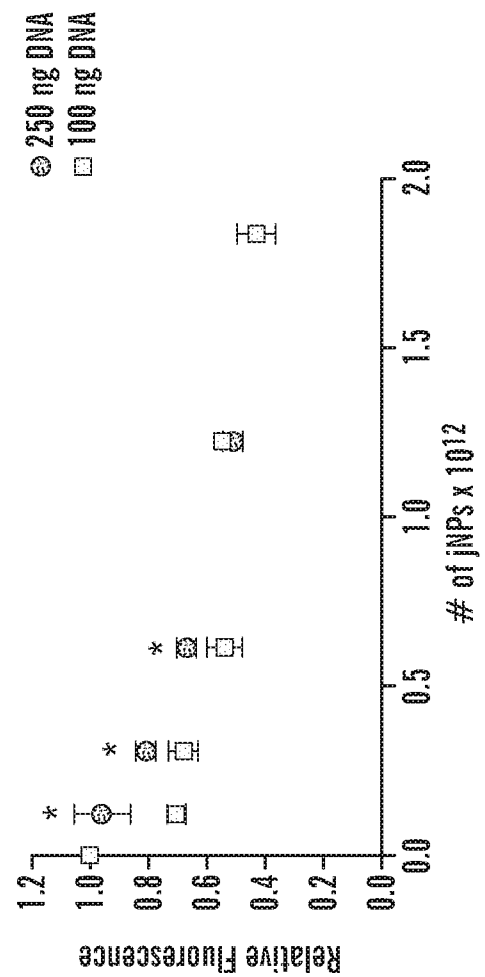


FIG. 8A

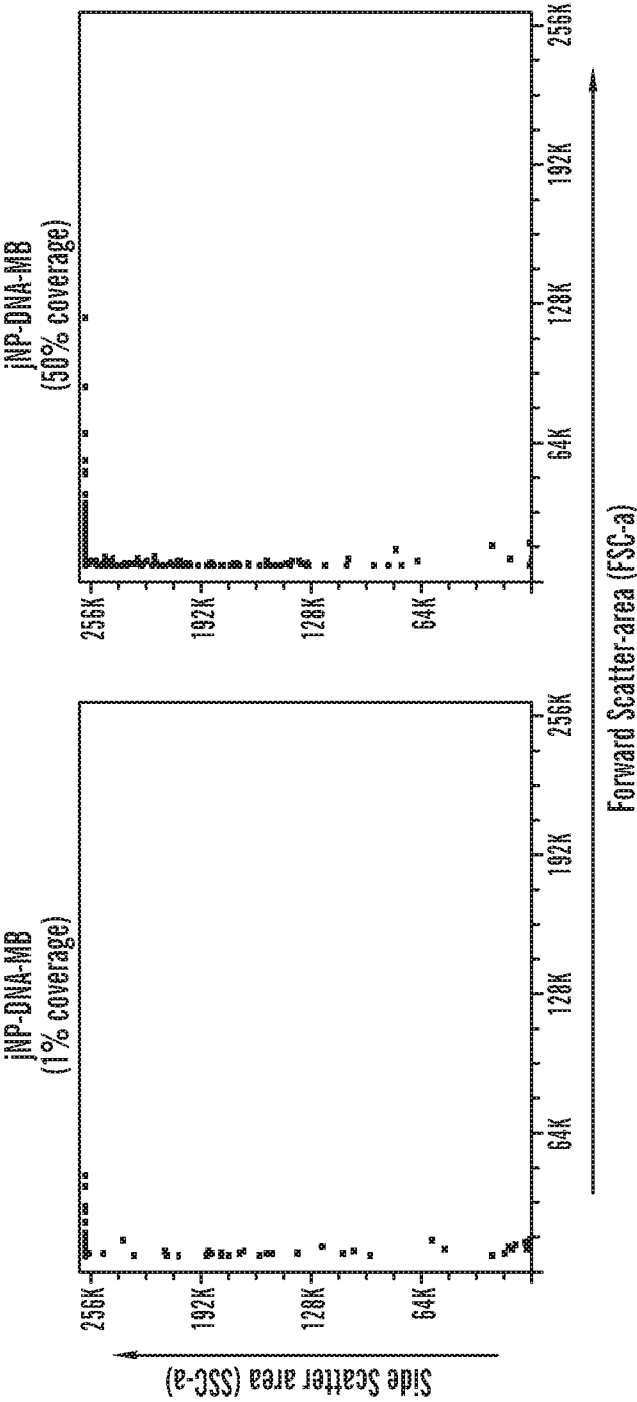


FIG. 8B

21/39

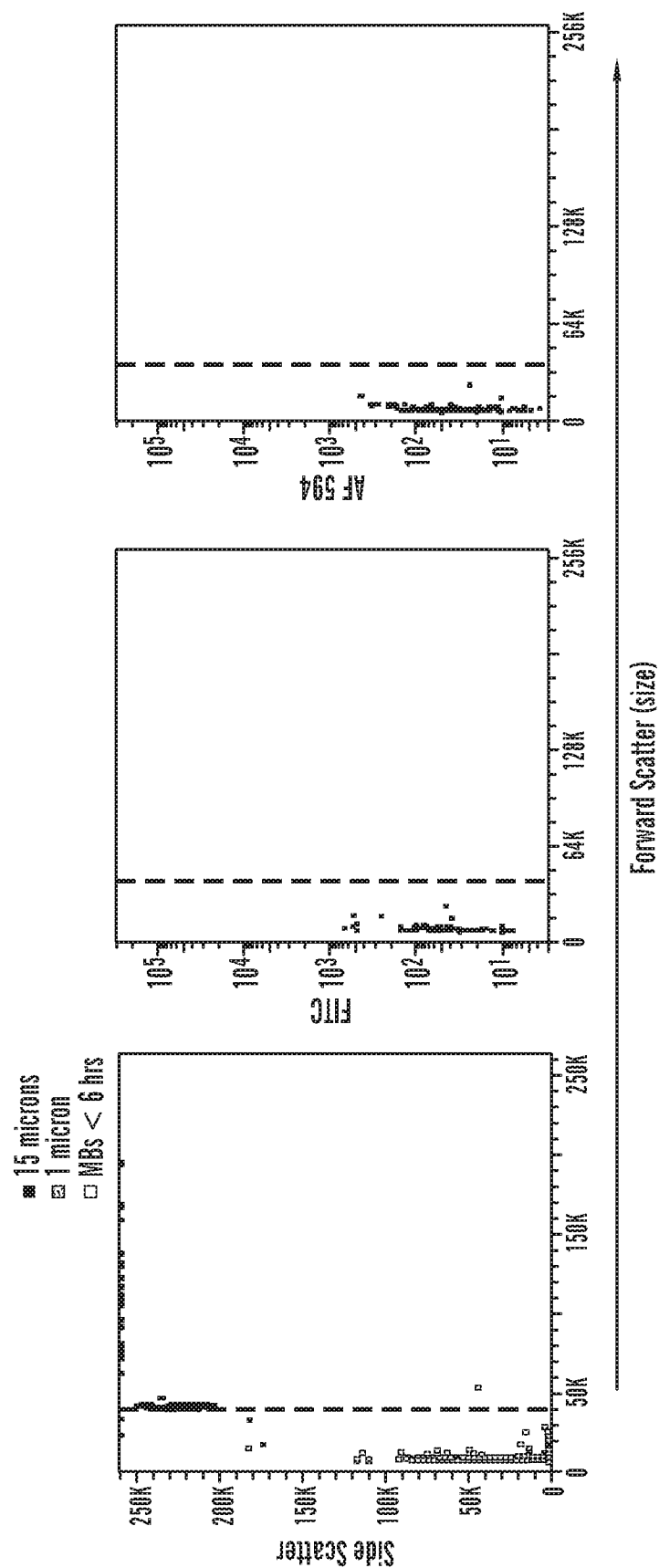
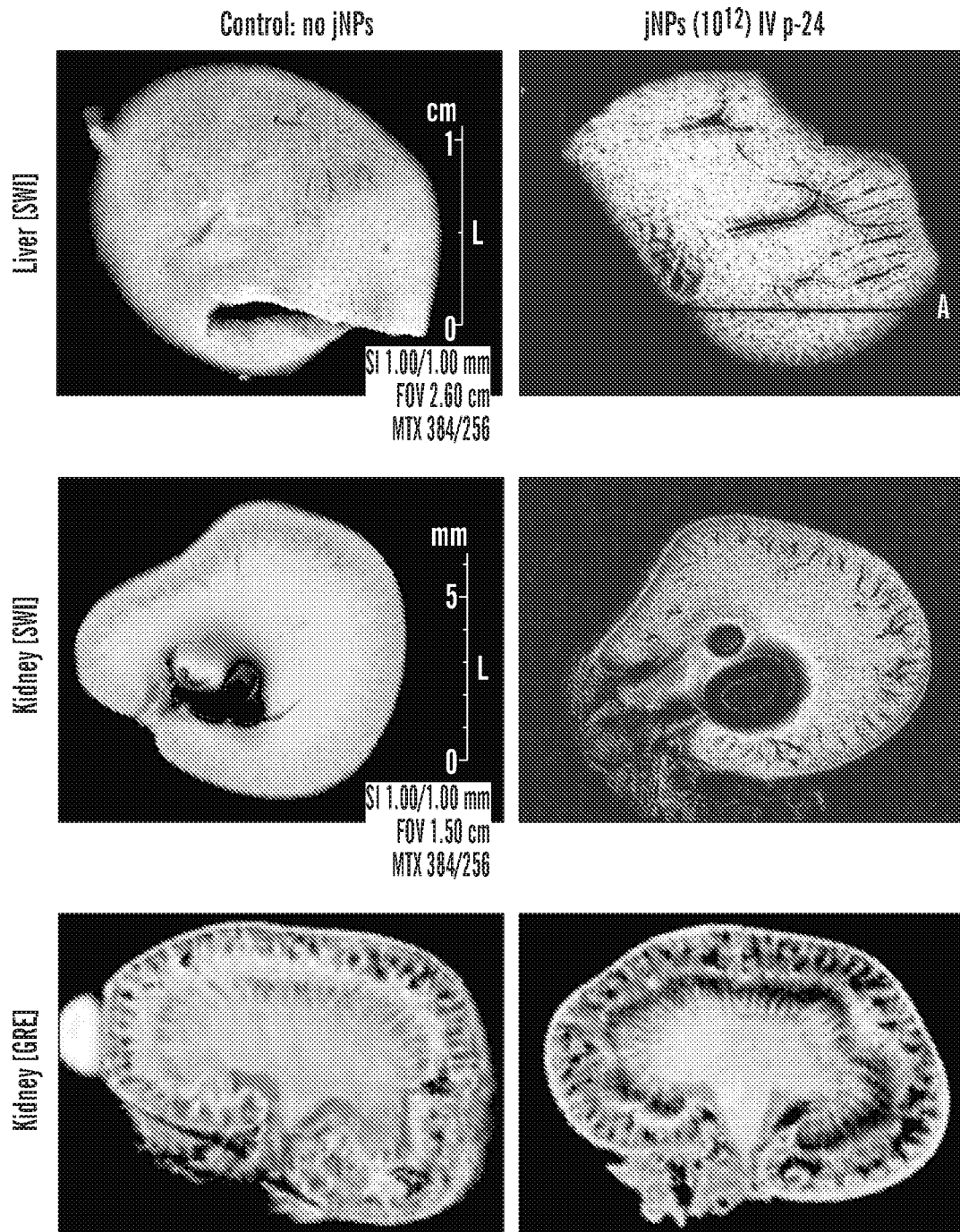


FIG. 8C

22/39

**FIG. 9**

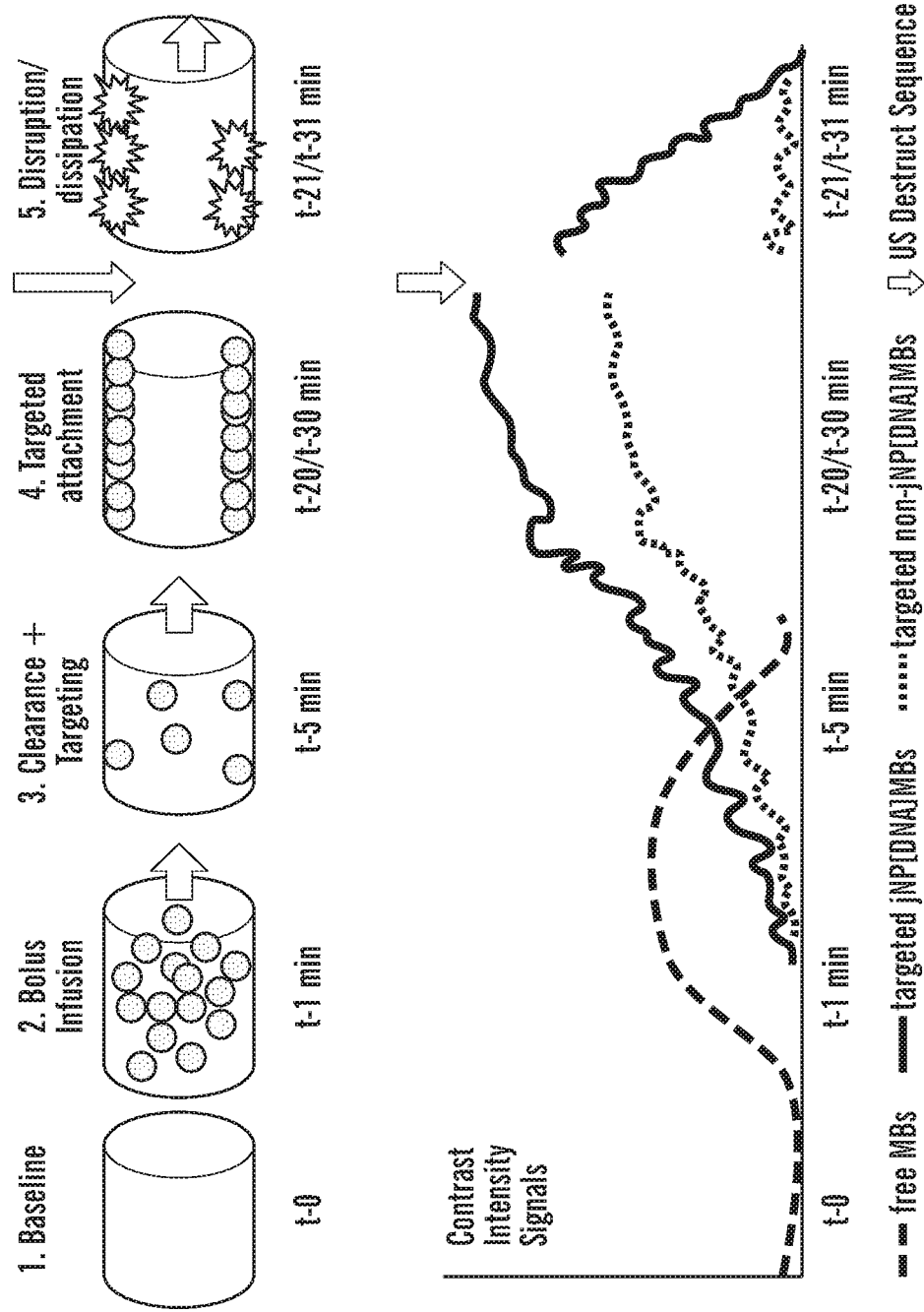


FIG. 10

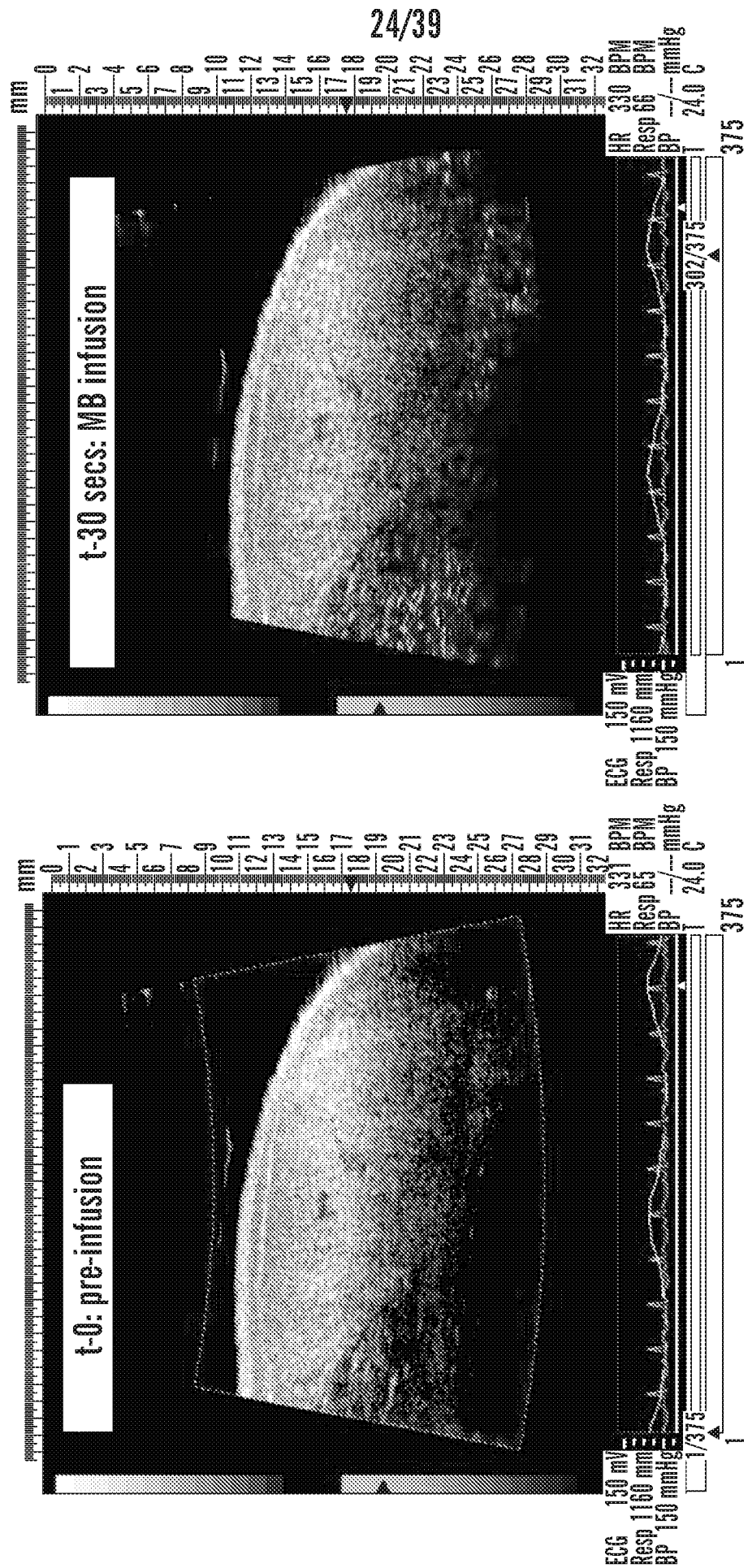


FIG. 11A

FIG. 11B

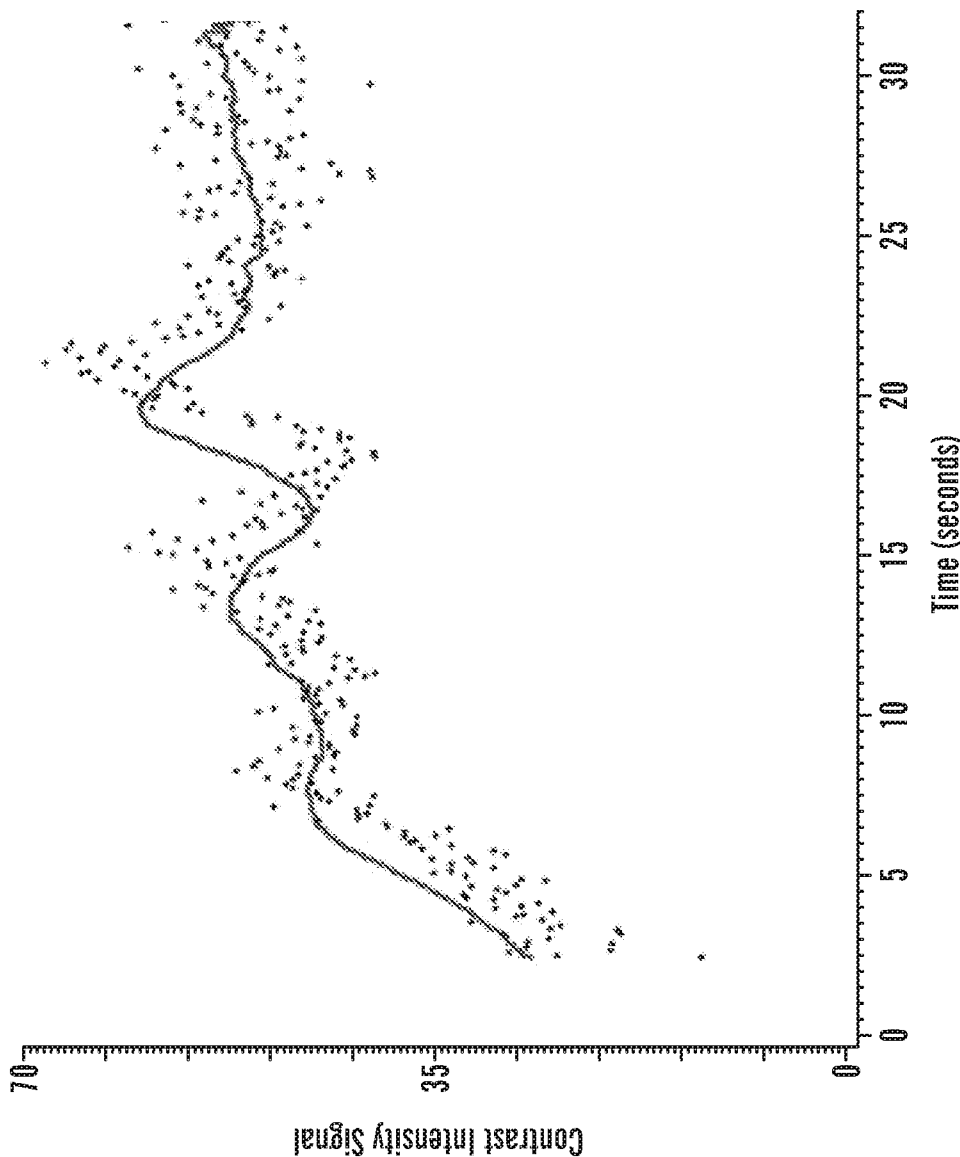


FIG. 11C

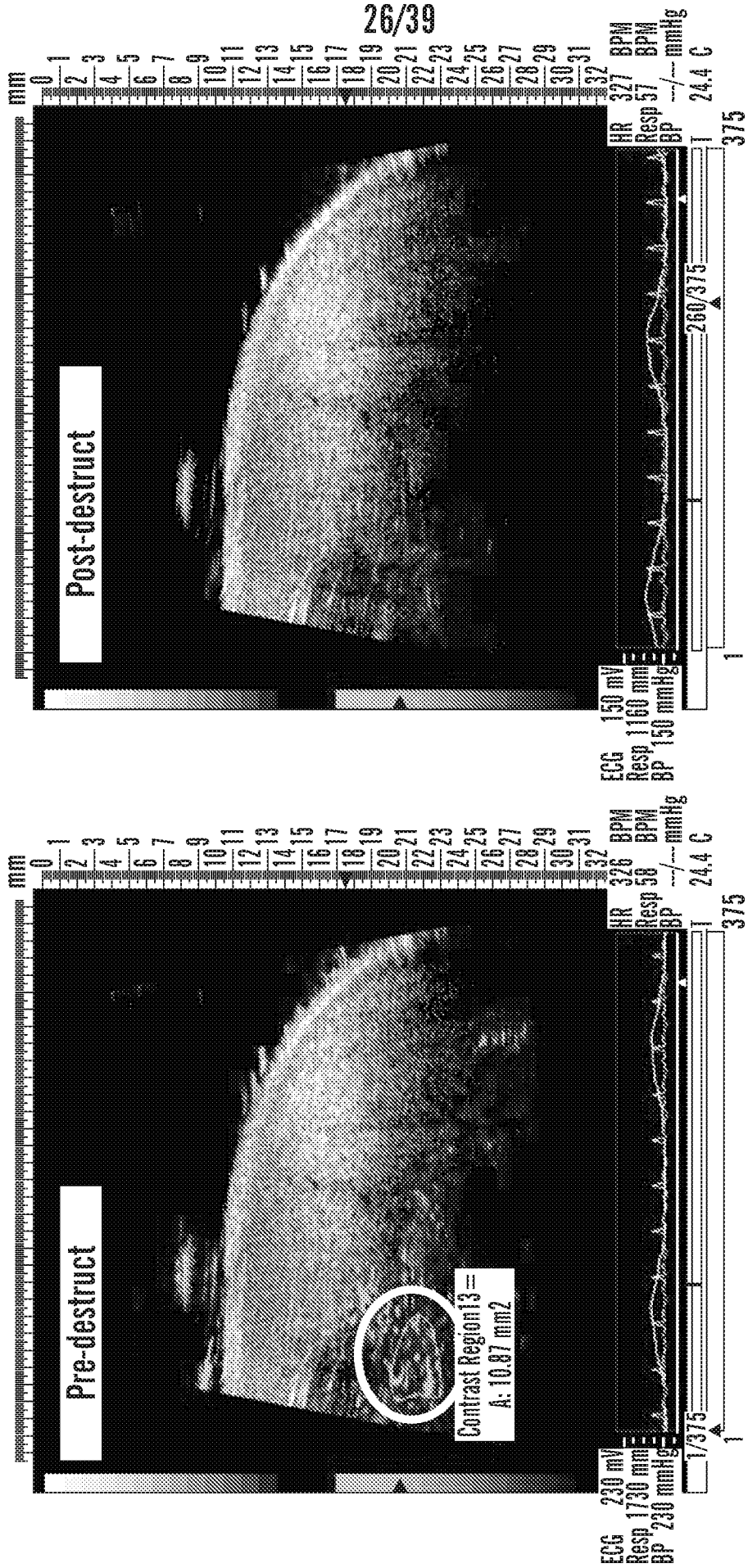


FIG. 11E

FIG. 11D

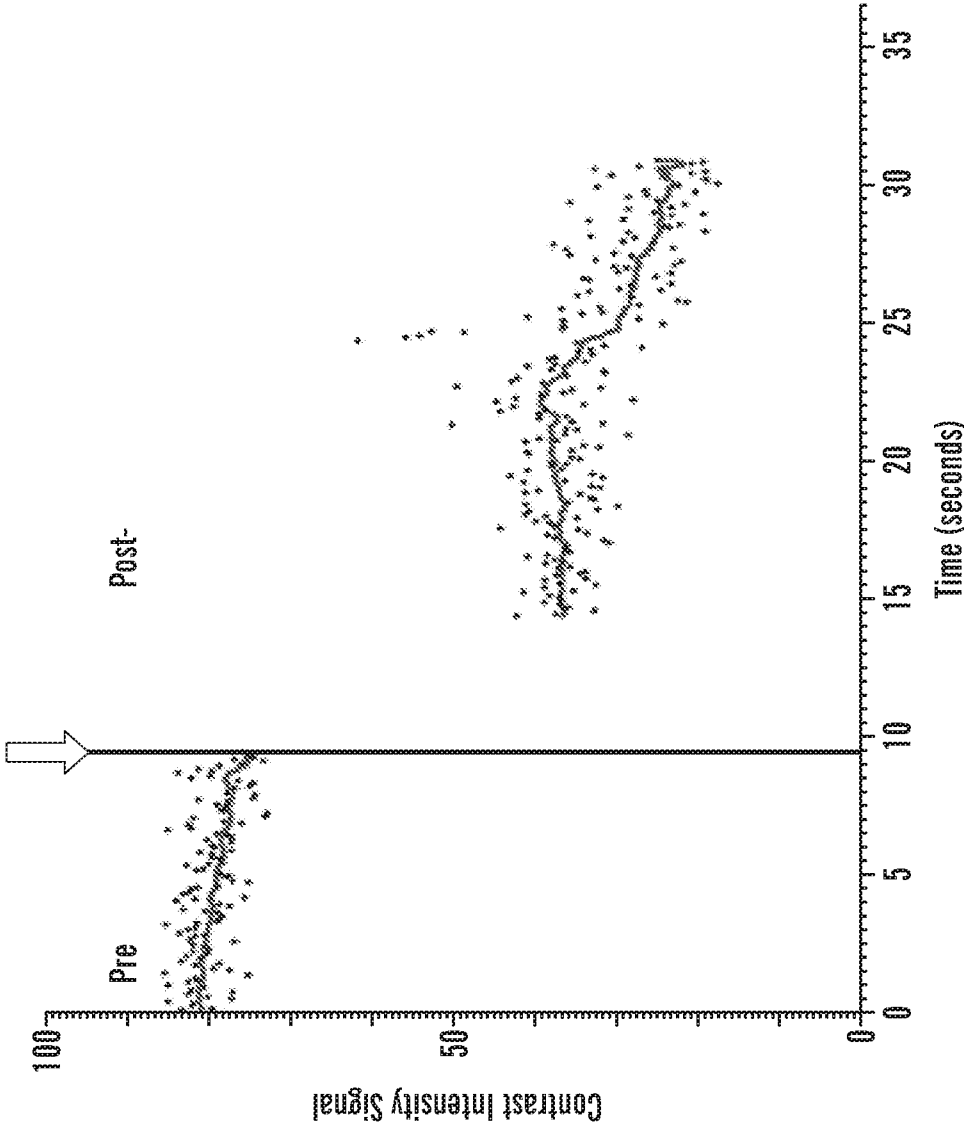
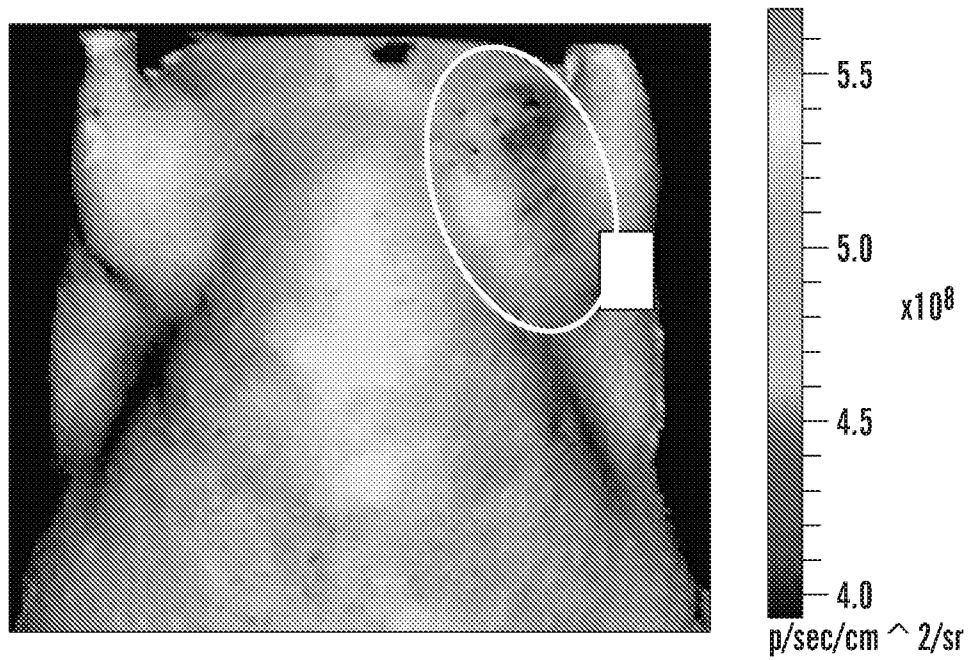
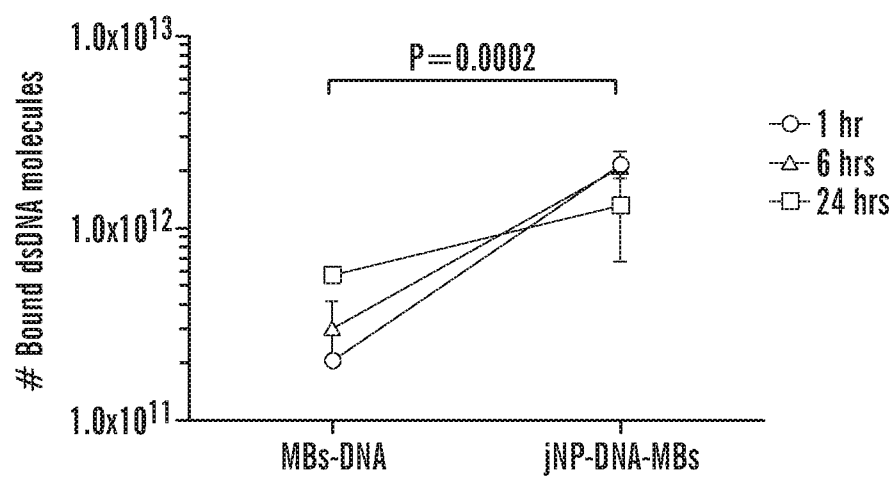


FIG. 11F

28/39

**FIG. 12****FIG. 13**

29/39

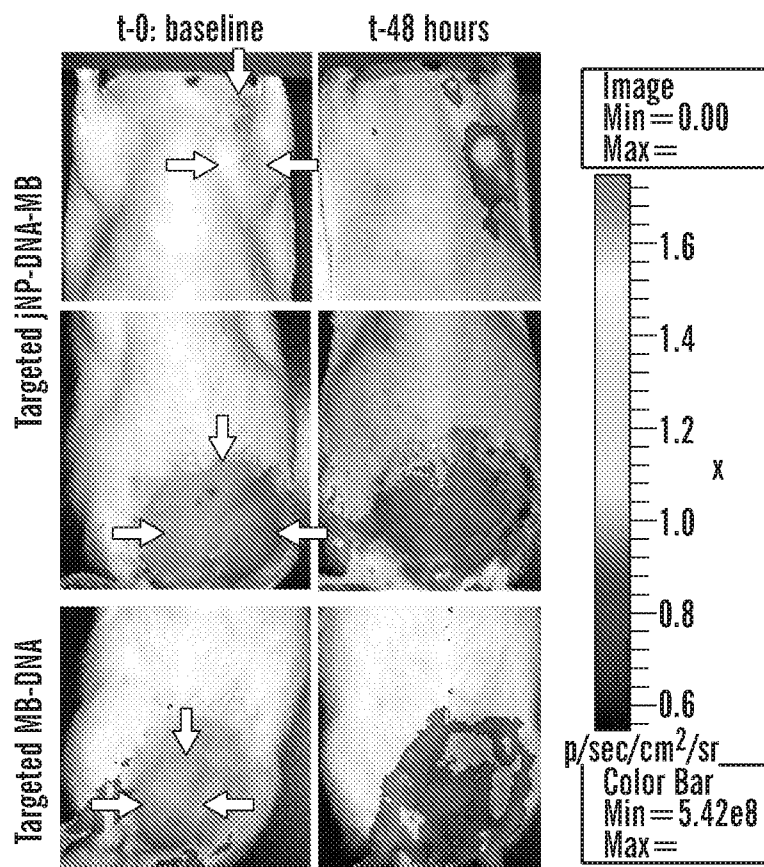


FIG. 14A

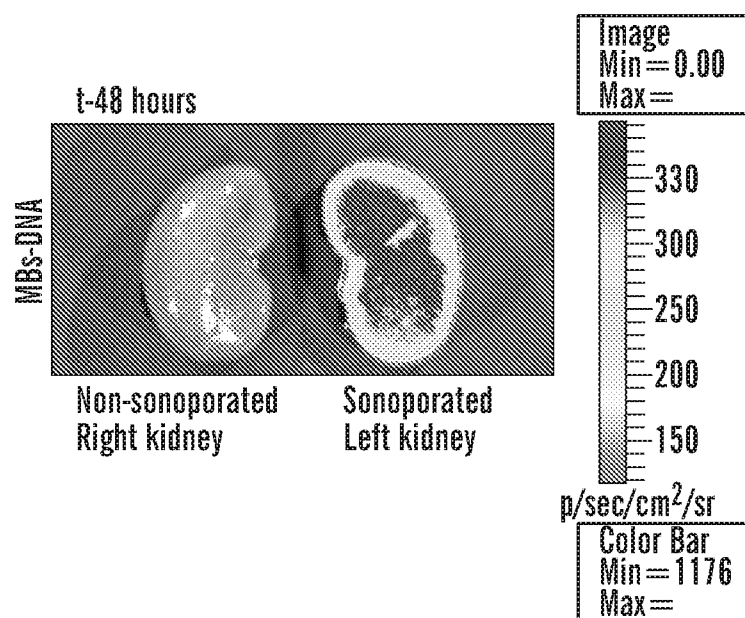
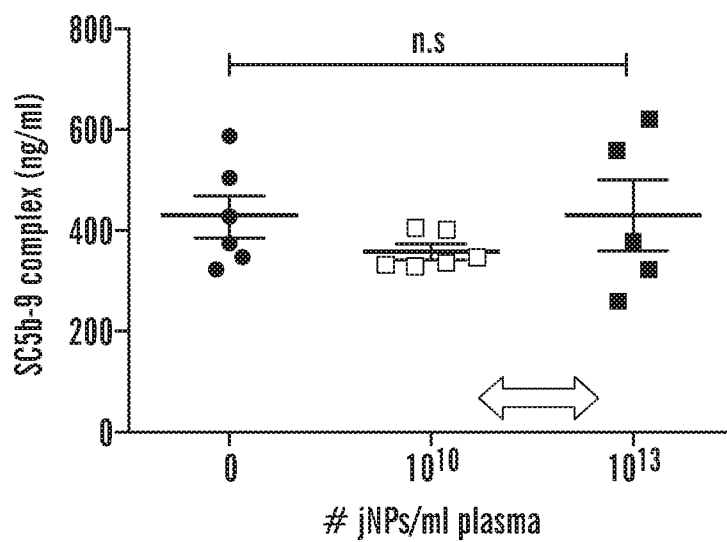
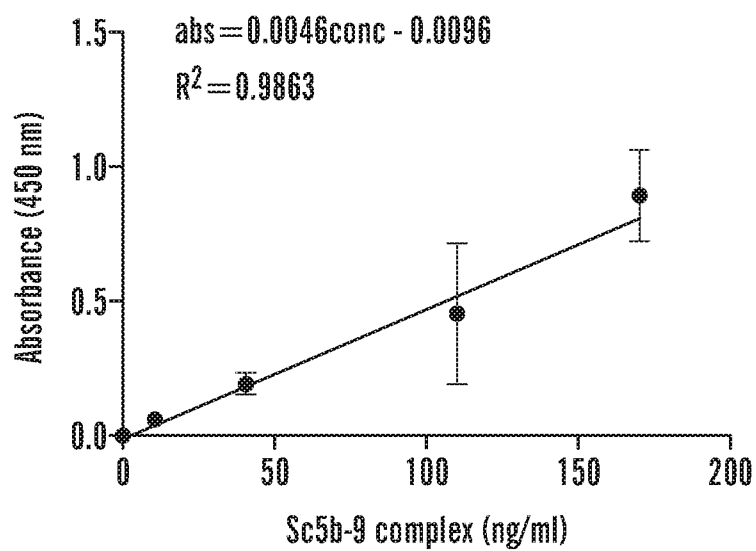


FIG. 14B

30/39

**FIG. 15A****FIG. 15B**

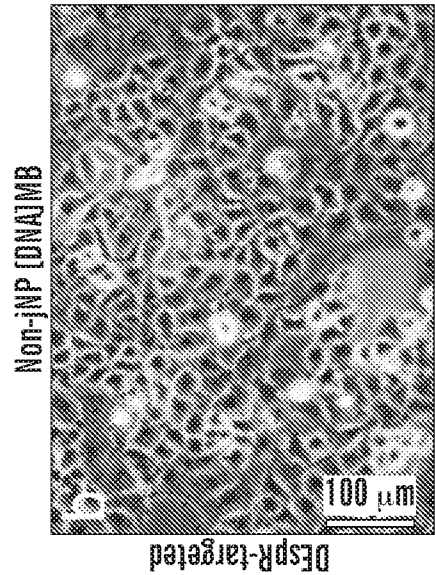


FIG. 16B

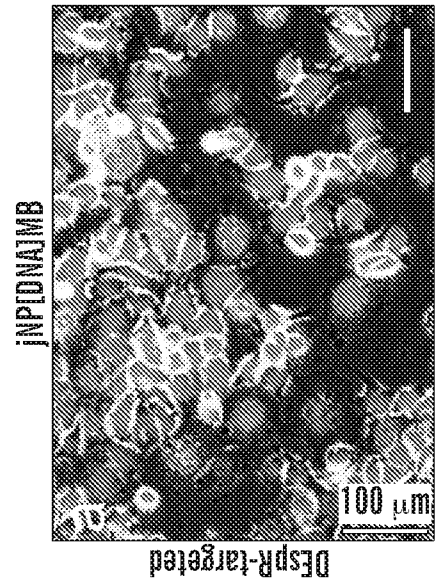


FIG. 16A

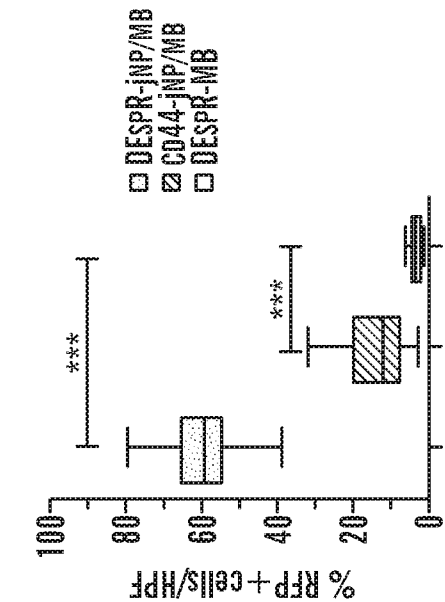


FIG. 16D

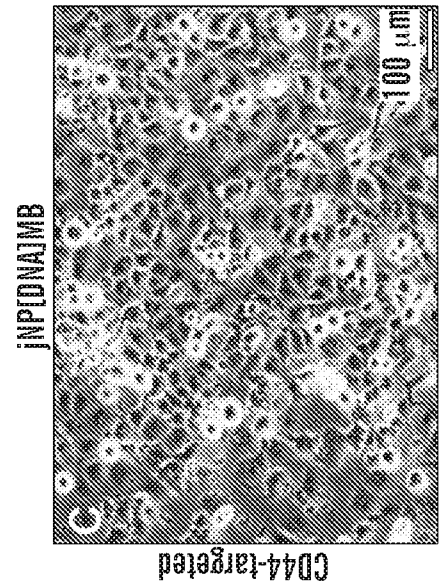


FIG. 16C

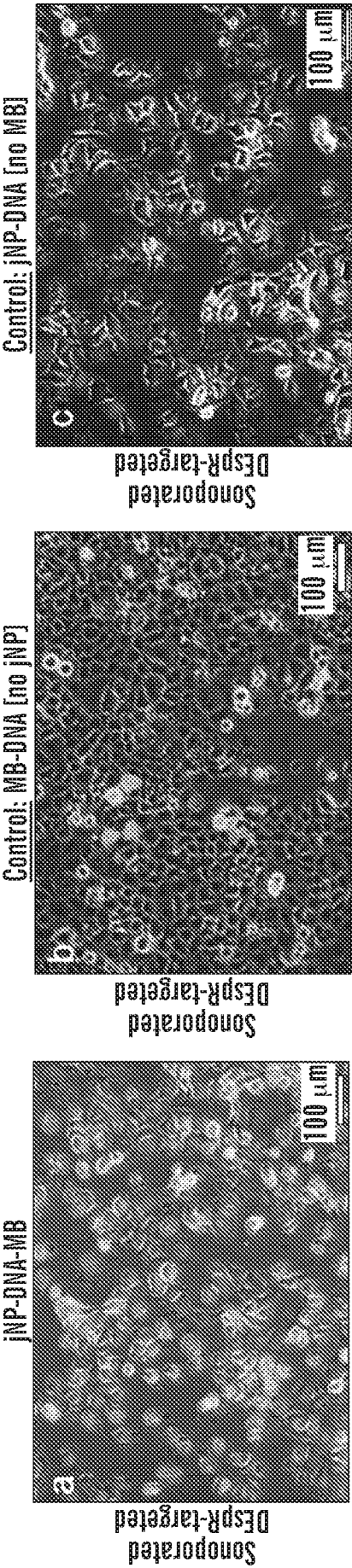


FIG. 17B

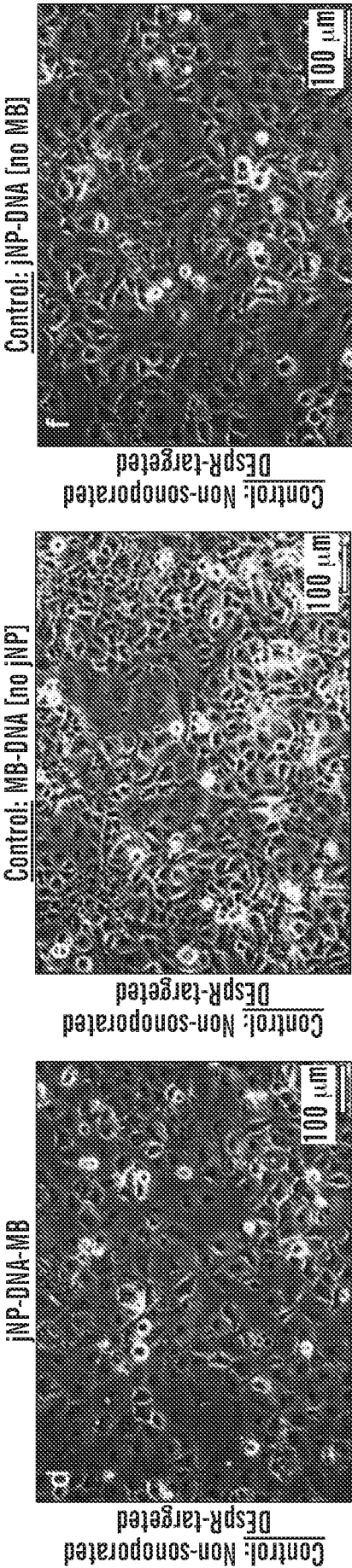
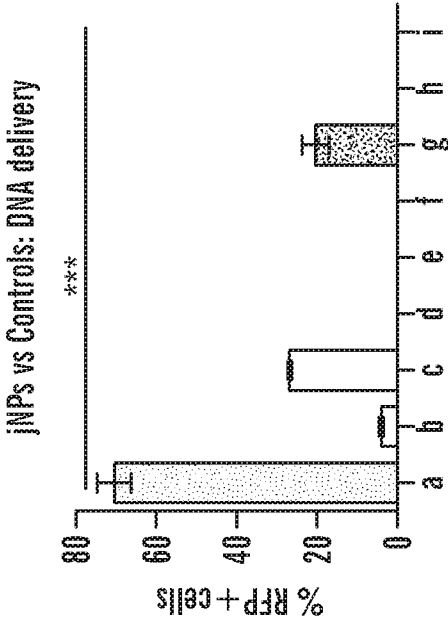
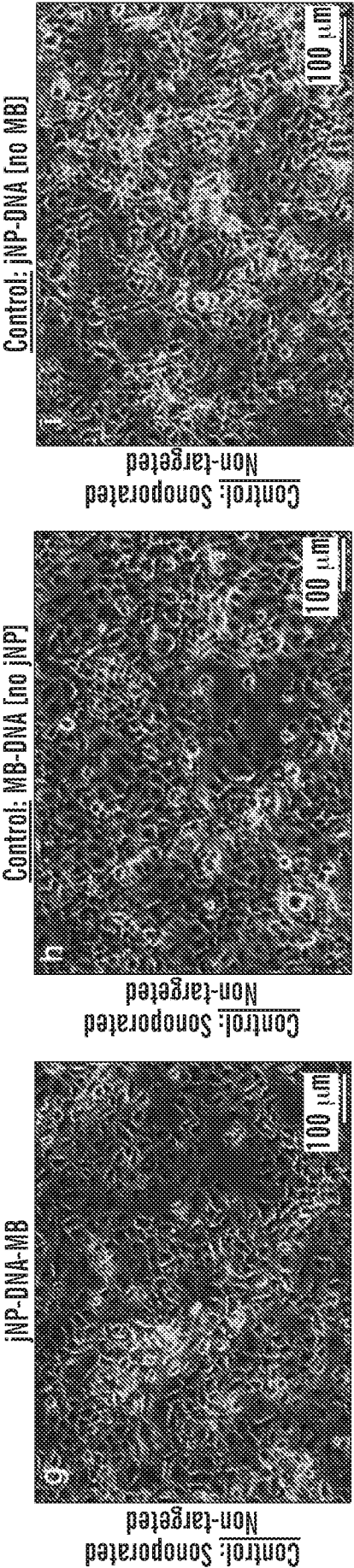


FIG. 17E

FIG. 17F



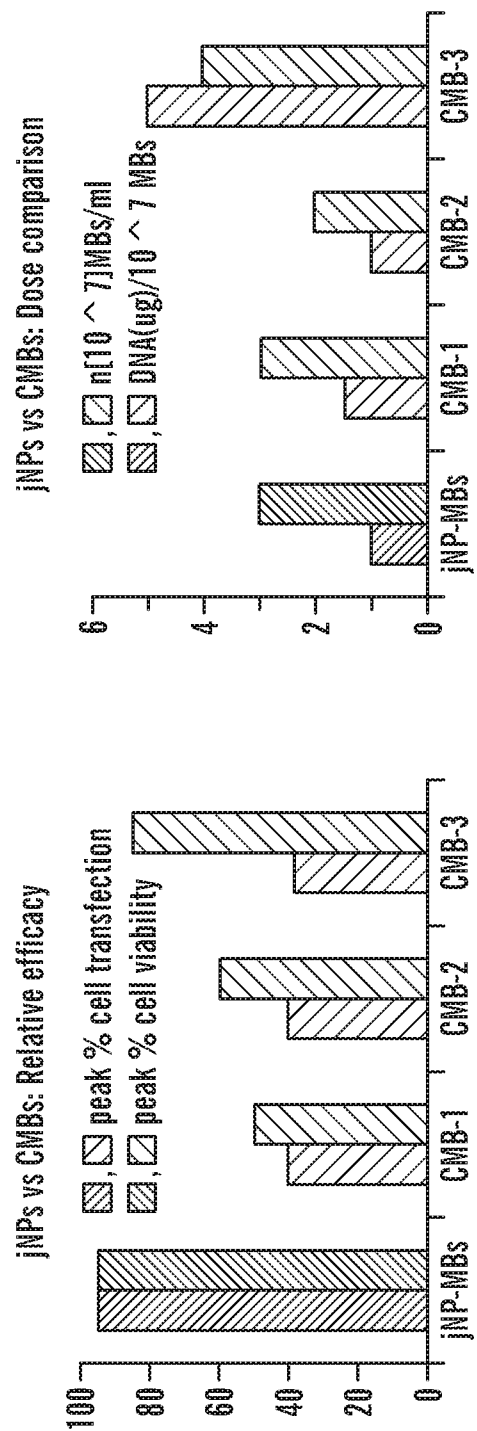


FIG. 17L

FIG. 17K

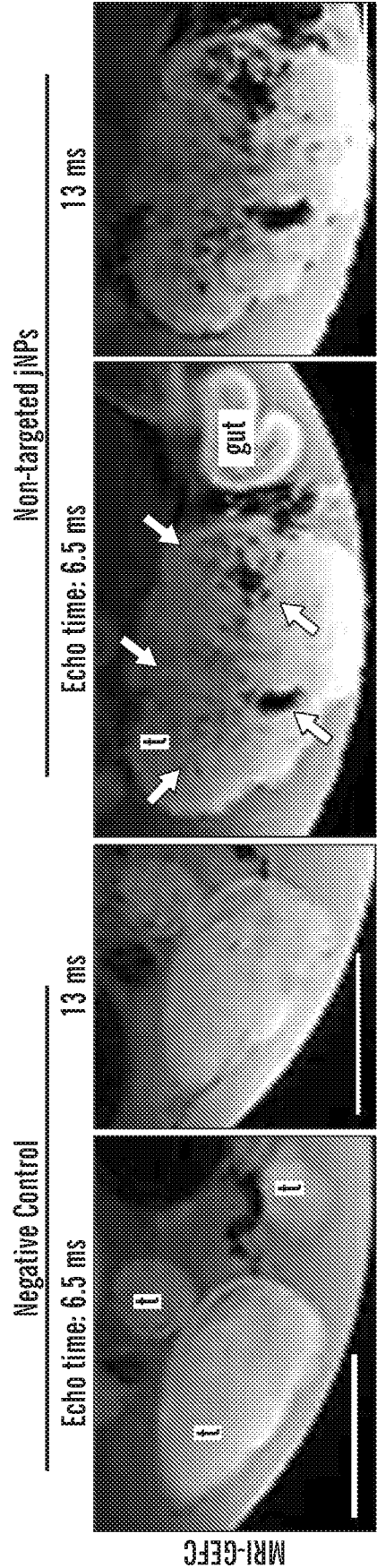
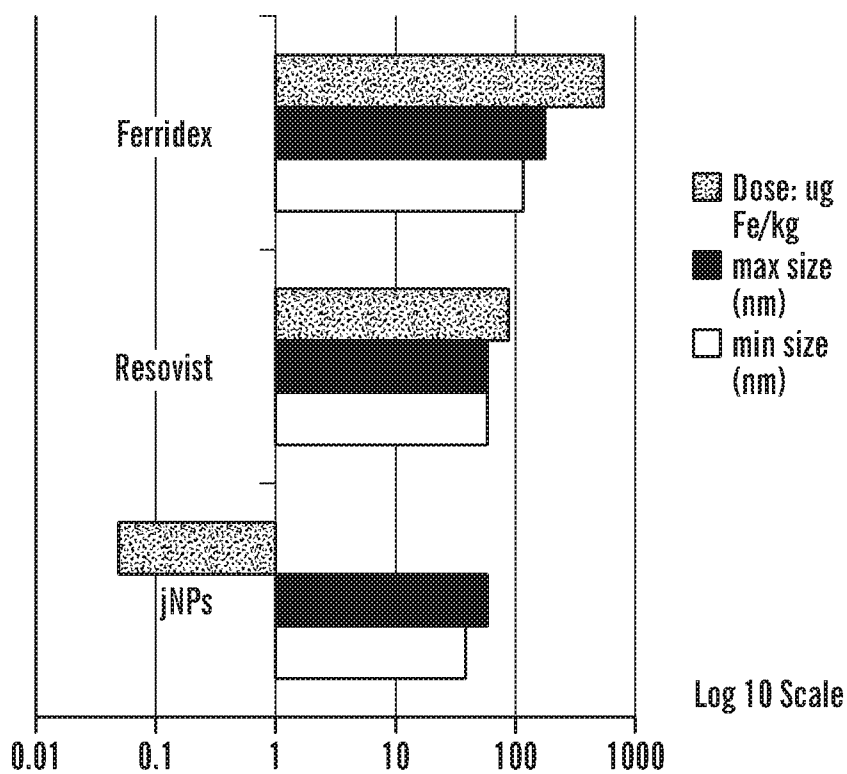
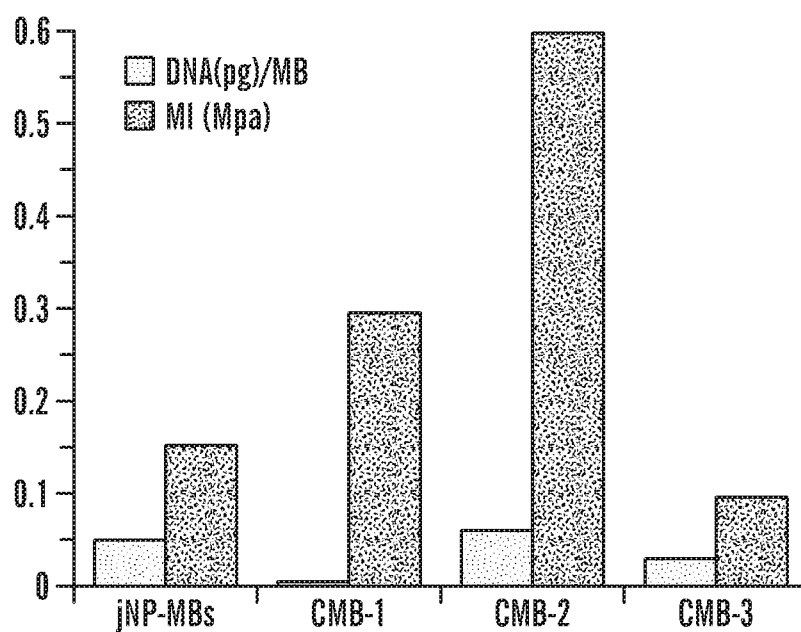


FIG. 18

36/39

**FIG. 19****FIG. 20**

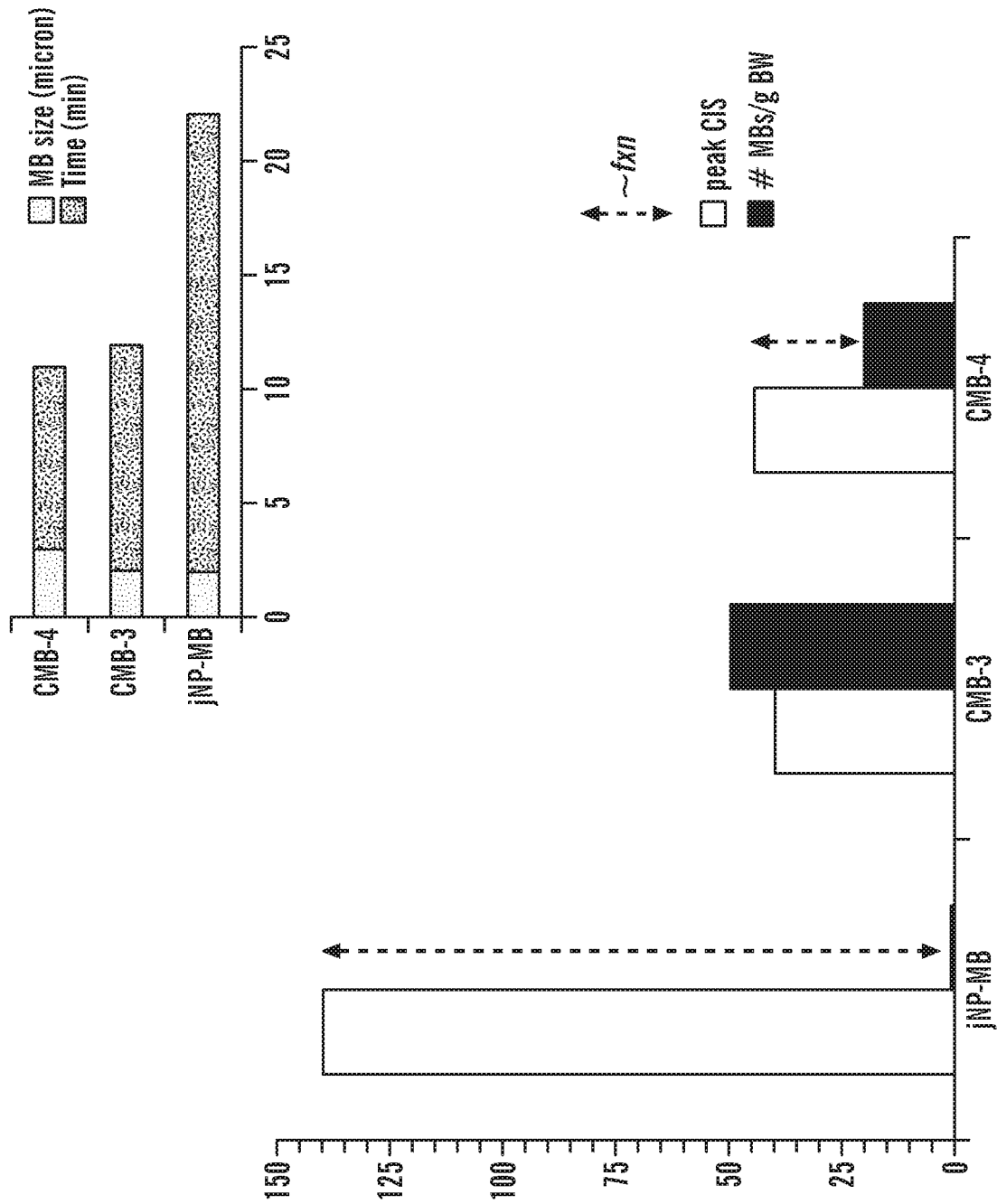
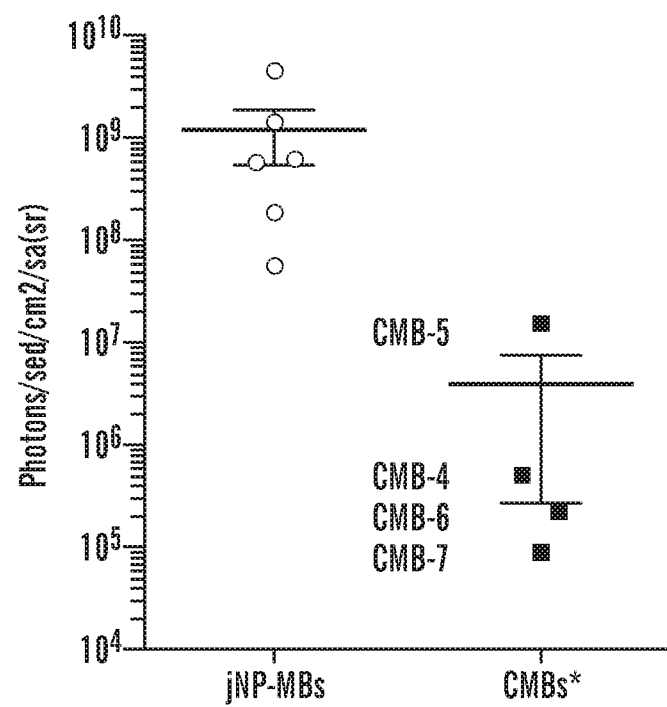
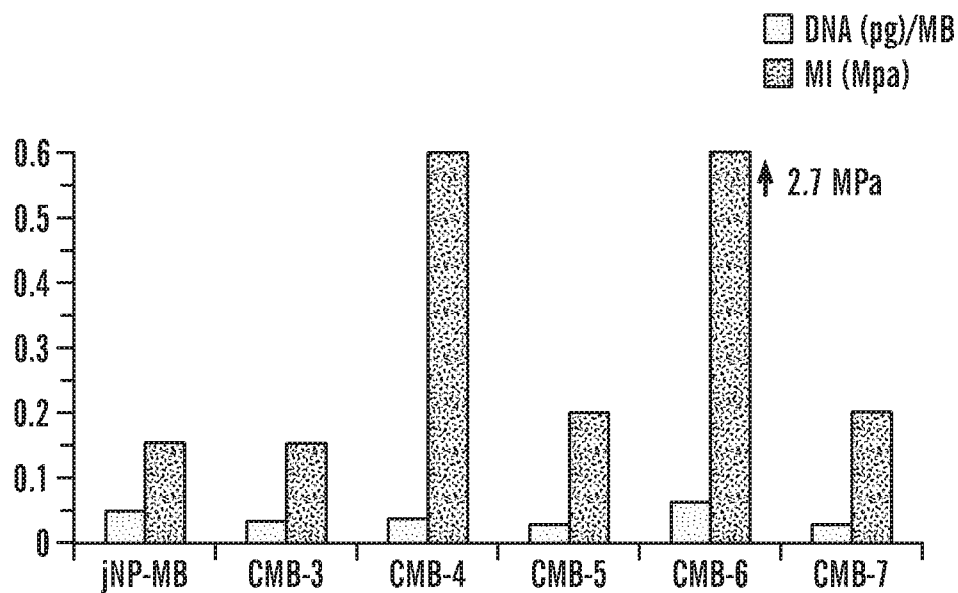
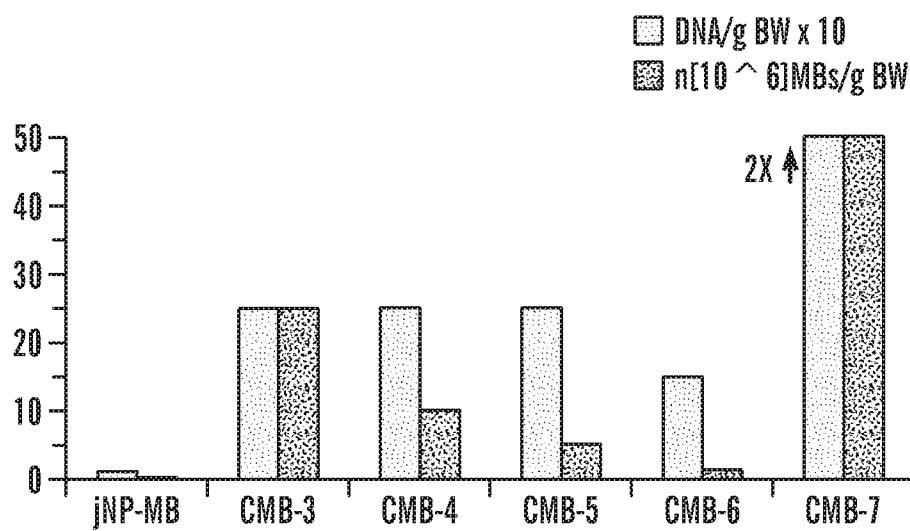


FIG. 21

38/39

**FIG. 22A**

39/39

**FIG. 22B****FIG. 22C**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/18417

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61B 5/05 (2016.01)

CPC - G01R 33/5601

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61B 5/05 (2016.01)

CPC - G01R 33/5601

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 600/420, 424/450, 422/119, 422/187

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

pubWEST; PatBase; Google Scholar

search terms - Nanoparticle, magnetic resonance, MRI, electron paramagnetic, epr, negative, contrast, janus, tumor, cancer, targeting, nucle*, DNA, delivery, microbubble

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEE et al. (2009) "All-in-one target-cell specific magnetic nanoparticles for simultaneous molecular imaging and siRNA delivery." Angew. Chem. Int. Ed. Engl. 48, 4174-4179. entire document, especially p. 4238 and 4239.	1-3, 23-27
Y	US 2013/0171208 A1 (SMITH et al.) 04 July 2013 (04.07.2013) abstract; para [0037]; [0098].	1-3, 23-27
Y	SEO et al. "Microfluidic assembly of monodisperse, nanoparticle-incorporated perfluorocarbon microbubbles for medical imaging and therapy." Langmuir 26.17 (2010): 13855-13860. entire document, especially abstract.	1-3
Y	US 2011/0054236 A1 (YANG et al.) 03 March 2011 (03.03.2011) para [0191]-[0199]; [0267].	24, 25

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 June 2016

Date of mailing of the international search report

14 JUL 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/18417

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-22, 31-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-3 and 23-27, directed to a composition comprising a Janus nanoparticle and a composition for use thereof.
Group II, claims 28-30, directed to a method for (target-specific) imaging.

The inventions listed as Groups I and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

--continued on first extra sheet attached hereto--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3, 23-27

Remark on Protest

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

--continued from box No III--

Special technical features

Group I has the special technical feature of a microbubble with a Janus nanoparticle associated with the surface, that is not required by Group II.

Group II has the special technical feature of a method comprising administering to a subject an MRI contrast agent and performing an MRI on the subject, that is not required by Group II.

Common technical features:

Groups I-II share the common technical feature of a composition comprising a Janus nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid delivery face and a targeting face, wherein the nucleic acid delivery face and the targeting face are distinct from one another, and wherein the targeting face comprises an antibody directed to a target to be imaged, and the composition for use as an MRI contrast agent for target-specific imaging. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is made obvious by the article by Lee et al., entitled 'All-in-one target-cell specific magnetic nanoparticles for simultaneous molecular imaging and siRNA delivery' (Angew. Chem. Int. Ed. Engl. 2009. 48, 4174-4179), (hereinafter Lee), in view of US 2013/0171208 A1 to Smith et al., (hereinafter Smith).

Lee teaches a composition comprising a nanoparticle, which comprises a superparamagnetic iron oxide nanoparticle having a nucleic acid component and a targeting component, and wherein the targeting component comprises an antibody directed to a target to be imaged (p. 4238, col 2, para 2-3 'The magnetic nanoparticle probes were conjugated with siRNAs, cancer-cell-specific targeting moieties, and fluorescent dyes...For the fabrication of multifunctional magnetic nanoparticles, manganese-doped magnetism-engineered iron oxide (MnMEIO) nanoparticles...their higher magnetic moment'; p. 4238, col 2, para 1 'Cell-specific targeting strategies for high-performance magnetic nanoparticles with dual (diagnostic and therapeutic) functions also include the immobilization of various target-specific moieties, such as antibodies, small peptides that can be recognized by cells, and aptamers').

Lee further teaches the composition for use as an MRI contrast agent for target-specific imaging (p. 4238, para 1 'use of magnetic nanoparticles for magnetic resonance imaging (MRI)'; p. 4238, col 2, para 2-3 'The magnetic nanoparticle probes were conjugated with siRNAs, cancer-cell-specific targeting moieties, and fluorescent dyes...For the fabrication of multifunctional magnetic nanoparticles, manganese-doped magnetism-engineered iron oxide (MnMEIO) nanoparticles...their higher magnetic moment'; p. 4238, col 2, para 1 'Cell-specific targeting strategies for high-performance magnetic nanoparticles with dual (diagnostic and therapeutic) functions also include the immobilization of various target-specific moieties, such as antibodies, small peptides that can be recognized by cells, and aptamers'). Lee does not specifically teach the nanoparticle is a Janus nanoparticle.

Smith teaches a composition comprising a Janus nanoparticle (abstract) 'that includes at least two physically or chemically differing components, where at least two of the components are exposed at the surface of the particle' (para [0098]). In view of Smith teaching use of the nanoparticles comprising an imaging agent and a second polymer (para [0037]), it would have been obvious to one of ordinary skill in the art to have combined the teachings of Lee and Smith to prepare Janus nanoparticles wherein the two physically or chemically differing components comprise imaging moieties and targeting moieties in order to generate diagnostically relevant images based on the target.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I-II lack unity of invention under PCT Rule 13.

NOTE, Claims 4-22 and 31-33 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).