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COMPOSITIONS AND ARTICLES COMPRISING (NANO) DIAMOND PARTICLES

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Abstract

Compositions and articles comprising diamond particles, such as nanodiamond-based pharmaceutical compositions, are generally provided. In some embodiments, the articles and methods comprising (nano)diamond particles may be useful for monitoring and/or treating a disease (e.g., in a subject).

– 1 –

COMPOSITIONS AND ARTICLES COMPRISING (NANO)DIAMOND PARTICLES

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/862,802, filed June 18, 2019, and entitled “COMPOSITIONS AND ARTICLES COMPRISING NANODIAMOND PARTICLES,” which is incorporated herein by reference in its entirety for all purposes.

TECHNICAL FIELD

Compositions and articles comprising diamond particles, such as nanodiamond based pharmaceutical compositions, are generally provided.

BACKGROUND

The use of nanomaterials for novel diagnostics and therapeutic purposes is a fast progressing scientific discipline that builds on the bioengineering of biological and pharmaceutical entities in combinations with physical materials. However, improved articles and methods are needed.

SUMMARY

Diamond particles and related devices and methods, such as nanodiamond particles (e.g., fluorescent nanodiamond particles) for administration of a therapeutic agent to a subject and/or monitoring the progression of a disease within a subject.

The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In one aspect, articles (e.g., configured for administration of a therapeutic agent, for use with a subject) are provided. In some embodiments, the article comprises a plurality of fluorescent diamond particles and a therapeutic agent bound to at least a portion of the fluorescent diamond particles, wherein the article is configured for prolonged residence internal to an organ of a subject.

In some embodiments, the article comprises an injection component configured to administer a composition to the subject and a reservoir associated with the injection

– 2 –

component containing the composition, the composition comprising a plurality of fluorescent diamond particles.

In another aspect, pharmaceutical compositions are provided. In some embodiments, the pharmaceutical composition comprises an intravenous carrier fluid and a plurality of fluorescent diamond particles suspended in the intravenous carrier fluid.

In yet another aspect, methods (e.g., of treating a disease, of monitoring disease progression in a subject suspected of having a disease) are provided. In some embodiments, the method comprises administering intravenously, to a subject, a plurality of diamond particles and a therapeutic agent bound to at least a portion of the diamond particles, wherein the plurality of diamond particles is configured for prolonged residence internal to an organ of a subject.

In some embodiments, the method comprises administering to the subject a plurality of diamond particles, after the step of administering, obtaining a first image of a location internal to the subject suspected of containing the plurality of diamond particles, obtaining, after a predetermined period of time, a second image of the location internal to the subject suspected of containing the plurality of diamond particles, and measuring a morphological change of the location internal to the subject, between the first image and the second image, relative to the plurality of diamond particles, wherein the morphological change is associated with progression of the disease.

In some embodiments, use of a plurality of fluorescent diamond particles in the manufacture of a medicament for the treatment of liver disease and/or liver cancer, are provided. In some embodiments, use of a plurality of fluorescent diamond particles in the manufacture of a medicament for monitoring of disease progression, are provided.

Other advantages and novel features of the present invention will become apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures. In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical

– 3 –

component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the figures:

FIG. 1A is a schematic illustration of a system including fluorescent nanodiamond particles, according to one set of embodiments;

FIG. 1B is a schematic presentation of method used for quantification of FNDP-(NV) uptake into cells, according to one set of embodiments;

FIGS. 2A-2B show fluorescence microscope images of paraffin sections (5 μ m) of liver obtained from rats treated or not with FNDP-(NV)~700/800 nm (FNDP-(NV)), according to one set of embodiments. In FIG. 2A, images of tissue sections analyzed with 10x objective with 1.6x extension are shown and in FIG. 2B images of tissue sections analyzed with oil 40x objective with the left images showing overlapped three colors red (FNDP-(NV)), blue (DAPI-nuclei), Green (phalloidin –cytoskeleton) shown with different shades of grey while images on the right show overlapped two colors red (FNDP-(NV)), blue (DAPI-nuclei) also shown with different shades of gray and the upper images in each panel represent FNDP-(NV)-treated rats, lower images in each panel control (PBS- treated rats) and areas occupied by the particles are indicated by white arrows, according to some embodiments;

FIGS. 3A-3H show “panoramic” images of hepatic lobes demonstrate intra-lobule heterogeneity of particles distribution, according to one set of embodiments, FIG. 3A and (FIG. 3B depict total panoramic view of a sagittal section from representative hepatic lobes from two animals with these figures constructed by ‘stitching’ 4x images using FSX100 microscope with the Phalloidin stained sections (5 μ m) imaged in the green channel (show in a shade of grey), and presence of FNDP-(NV) imaged in the red (shown in grey) channel; particles in the image have been magnified by thresholding and repeated dilations for visualization at very low resolution; hexagons are over-laid in the figure to indicate example hepatic lobules with areas indicated in gold are magnified in other panels and (FIG. 3C) presents four hepatic lobules demonstrating preferential particle distribution at the boundaries of the ‘hexagonal’ lobules format and (FIG. 3D) present 10x image of a single hepatic lobule showing preferential FNDP-(NV) deposition; large FNDP-(NV) aggregates are seen distributed non-uniformly with hepatic lobule indicated by dashed hexagon and (FIG. 3E), (FIG. 3F) present 10x image of a

2026202273 24 Mar 2026

– 4 –

single hepatic lobule after thresholding and dilating to improve visibility of very small aggregates, to demonstrate zonal deposition and (FIG. 3G), (FIG. 3H) providing magnified images of areas of vasculature from panel (FIG. 3A) indicated by gold dashed square and (FIG. 3I) as a schematic illustration of hepatic lobule that demarcates the various metabolic zones, according to some embodiments;

FIGS. 4A-4B show mathematical plots of size distribution of FNDP-(NV) aggregates in liver lobules; figures of one entirely liver lobule from two animals were stitched from 10x images on an FSX100 microscope; Maximum Entropy criteria was used to threshold stitched figures in ImageJ and the resulting detected FNDP-(NV) assemblies were sized and counted; (FIG. 4A) Distribution of FNDP-(NV) assembly sizes. (FIG. 4B) Distribution of total particle mass estimated by the area of each assembly, according to some embodiments;

FIGS. 5A-5D show laser scanning confocal microscope images of liver sections (50 μ m) obtained from rats treated with FNDP-(NV), according to one set of embodiments. (FIG. 5A) Parenchymal area of liver with indicated cells in yellow circles with up-taken particles. Inserts on the bottom and on the right of the photo represent vertical projection of images performed along the yellow lines. Yellow arrows indicate location of particles. (FIG. 5B) Parenchymal area of liver where yellow circles suggest aggregates of particles within liver sinusoids/venues. Inserts on the bottom and on the right represent vertical projection of images performed along the yellow lines. Yellow arrows indicate particles localized in sinusoids/venules. (FIG. 5C) Area of abundantly vascularized segment of the hepatic lobule where white circles particles suggest sub-endothelial and adventitial location of particles. Parenchymal cells with supposedly internalized particles are indicated in yellow circles. Inserts on the bottom and on the right represent vertical projection of images performed along the yellow lines. Yellow arrows indicate particles internalized in parenchymal cells. (FIG. 5D) Area of the liver hilum where white circles indicate particles associated with adventitial cellular elements. Inserts on the bottom and on the right represent vertical projection of images performed along the yellow lines. Yellow arrows indicate internalized particles into the vascular cells;

FIG. 6 shows confocal 3D reconstruction of hepatocytes with differing amount of incorporated FNDP-(NV), according to one set of embodiments. Confocal image stacks from 50 μ m sections stained with DAPI (blue) and phalloidin (green) with incorporated

– 5 –

nanodiamonds (red) shown in different shades of grey with image stacks were taken on a Fluoview F1000 confocal microscope and reconstructed using volume viewer in ImageJ. Particles inclusions within these cells (indicated by yellow arrow) include both sparse and dense FNDP-(NV) collections internalized in the cells. Left panel represents vehicle control. Middle panel represent low load particle and right panel represent high load particle in 2 separate cells.

FIGS. 7A-7D show plots related to internalization of different concentrations of FNDP-(NV) into HepG-2 and HUVEC cells over time, according to one set of embodiments. (FIG. 7A), (FIG. 7B), (FIG. 7C) depict dose and time dependent uptake of FNDP by HepG-2 cells and HUVEC exposed to various concentrations of FNDP-(NV). Exponential curves were fitted for all three doses (high-dose 0.1 mg/ml; medium- dose 0.05 mg/ml, low-dose 0.025 mg/ml) of particles. (FIG. 7D) Total uptake of FNDP after 20 hours by HepG-2 cells and HUVEC exposed to various concentrations of FNDP. Error bars for all panels represent SD from quadruplicated samples. (*) $P < 0.001$ compared to 0.025 mg/ml by two-tailed Student's test; (†) $P < 0.001$ compared to 0.05 mg/ml by two-tailed Student's test;

FIGS. 8A-8B show fluorescence microscope of images of HepG-2 cell and HUVEC obtained after 2 and 20 hours incubation with FNDP-(NV), according to one set of embodiments. Images of HepG-2 cells (FIG. 8A) and HUVEC (FIG. 8B) obtained from fluorescence microscope analysis using 160x and 400x magnification after 2 or 20 hours of exposure to FNDP-(NV). Images of 160x magnification are presented in overlapped three colors fluorescence (green – FITC-phalloidin, red – FNDP-(NV), blue – DAPI) shown in different shades of grey with images of 400x magnification are presented in overlapped three colors fluorescence (green, red, blue) (left panels), and two colors fluorescence (red and blue) (right panels). White arrows denote example of the cytoplasmic phase of particles transition; Grey arrows indicated peri-nuclear assembly of large number of particle;

FIG. 9 shows representative images demonstrating various stages of HUVEC division in the presence of FNDP-(NV), according to one set of embodiments. HUVEC were treated with 0.05 mg/ml of FNDP-(NV) for 20 hours. Images of 400x or 640x magnifications are presented in overlapped three colors fluorescence (green – FITC-phalloidin, red – FNDP-(NV), blue – DAPI). Titles of the various phases noted are visual images of predicted cell replication mechanism;

2026202273 24 Mar 2026

– 6 –

FIGs. 10A-10B show the effect of passive adsorption of BSA on aggregation and surface potential of FDP-NV functionalized with carboxyl groups and suspended in water, culture medium and biological buffers where the particles were suspended in the various dispersants, applied into capillary cuvettes, and positioned into a Zetasizer instrument (Malvern Inc.) for measurement Z-average, diameter size (FIG. 10A) and ζ -potential (FIG. 10B) and where error bars represent SD from three measurements of independent samples. (*) $P < 0.01$ and (**) $P < 0.001$ for difference between FDP-NV-BSA and native FDP-NV, in particular dispersant, calculated using One Way ANOVA., according to one set of embodiments;

FIGs. 11A-11D show effect of FDP-NV on cell proliferation determined by evaluation of direct cell number, where the graphic presentation of numbers of HepG-2 cells (FIG. 11A) and HUVEC (FIG. 11B) obtained after incubation or not with FDP-NV-BSA, or vincristine and where error bars represent SD from 5 independent wells, and application for 7 observation fields for each well. (*) $P < 0.001$ between control and treated group calculated using One Way ANOVA, and where representative images of observation fields of HepG-2 cells (FIG. 11C) or HUVEC (FIG. 11D) applied for determination of cell numbers using ImageJ software with images that were obtained using fluorescence microscope (Olympus IX81) with application 10x objective and DAPI (blue) and TRITC (red) filters (shown in different shades of grey) with white arrows indicating internalized particles into flanking cells of HepG-2 colonies, according to some embodiments;

FIGs. 12A-12B show the effect of FNDP-(NV) on HepG-2 (FIG. 12A) and HUVEC (FIG. 12B) Redox state tested in MTT assay where error bars represent one SD from three independent experiments with One-way ANOVA calculated between control and compound treated group, (*) $P < 0.01$ and (**) $P < 0.001$, according to some embodiments;

FIGs. 13A-13B shows the effect of FNDP-(NV) on HepG-2 (FIG. 13A) and HUVEC (FIG. 13B) esterase activity monitored calcein AM assay where the graphic presentation of conversion of calcein AM to green-fluoresce calcein by esterases present in live HepG-2 cells (FIG. 13A) and HUVEC (FIG. 13B) is shown with error bars representing SD from three independent experiments and where One-way ANOVA was calculated between control and compound treated group, (*) $P < 0.01$ and (**) $P < 0.001$, according to some embodiments;

– 7 –

FIG. 14A shows the effect of FNBP-(NV) on migration of HUVEC stimulated by 2% FBS in scratch assay showing scratch closure” stimulated by 2% FBS in the presence or absence of FNBP-NV-BSA with non-stimulated cells (negative control) treated with a medium containing 0.1% FBS and error bars representing SD from three independent experiments, (*) $P < 0.001$ for comparison with control (2% FBS treated) in One-way ANOVA, according to one set of embodiments;

FIG. 14B shows the effect of FNBP-(NV) on migration of HUVEC stimulated by 2% FBS in scratch assay with images of scratches obtained using fluorescence microscope (Olympus IX81) with application 20x magnification and DAPI (blue) and TRITC (red) filters shown in different shades of grey, according to one set of embodiments;

FIGS. 15A-15B show the effect of FDP-NV on phosphorylation of MAPK Erk1/2 induced by FBS with 24 hour serum-starved HepG-2 cells (FIG. 15A) or HUVEC (FIG. 15B) stimulated with 2% FBS by 10 and 20 minutes and total MAPK Erk1/2 re-probed in PVDF membrane after stripping anti-phospho antibody with right plot bars presenting a ratio of intensity of total protein bands to phosphorylated protein bands and green bars presenting ratios for control (non-treated cells), whereas red bars for FDP-NV treated cells and left panes showing representative blot images for each cell type with error bars representing SD for three independent experiments, (*) $P < 0.01$ for comparison between treated or non-treated cells with FDP-NV-BSA 0.1mg/ml by ‘One-way ANOVA’, according to one set of embodiments;

FIGS. 16A-16B shows the identification of phospho- and total-MAPK Erk1/2 in cytoplasm and nuclei of HepG-2 cells and HUVEC in the presence and absence of FDP-NV and TPA, HepG-2 cells (FIG. 16A) or HUVEC (FIG. 16B) were treated or not with FDP-NV-BSA (0.1 mg/ml), and after 24 hour serum-starvation, stimulated or not with TPA with cells lysed and fractionated into cytoplasmic and nuclear fractions and fractions that are subjected to WB using indicated antibodies; Mek-1 was used as marker for cytoplasm fraction, whereas HDAC1 as nucleus fraction;

FIGS. 16C-16D shows the identification of phospho- and total-MAPK Erk1/2 in cytoplasm and nuclei of HepG-2 cells and HUVEC in the presence and absence of FDP-NV and TPA with HepG-2 cells (C) or HUVEC (D) grown on chamber slides and serum-starved for 24 hours, following exposed to FDP-NV-BSA. After treatment or not with TPA, cells were immune-stained with anti-phospho-MAPK Erk 1/2, following with

2026202273 24 Mar 2026

– 8 –

goat anti-rabbit tagged with FITC. slides analyzed under fluorescence microscope (Olympus IX81) using 400x magnification with oil objective and FITC (green) and TRITC (red) filters with overlapped areas of green and red in yellow shown with various shades of grey and with white arrows indicating high accumulation of particles in TPA-treated cells if compare with non-treated cells and blue arrows indicate nuclei of cell non-treated with TPA with red arrows indicating nuclei of cell treated with TPA, according to one set of embodiments;

FIG. 17A shows the effect of FDP-NV on induction of apoptosis and ER stress in HepG-2 cells and HUVEC with a Western blot analysis of cleavage of caspase 3 in the presence or absence of FDP-NV (0.1 mg/ml) in HepG-2 and HUVEC. Vincristine was used as positive control for apoptosis. Localization of molecular weight markers is indicated by arrows on the left side of images, according to some embodiments; and

FIG. 17B shows the effect of FDP-NV on induction of apoptosis and ER stress in HepG-2 cells and HUVEC with a Western blot analysis of expression of chaperons in ER in the presence or absence of FDP-NV (0.1 mg/ml) in HepG-2 cells and HUVEC. Tunicamycin was used as positive control for ER-stress, according to some embodiments.

DETAILED DESCRIPTION

Compositions and articles comprising diamond particles, such as diamond based pharmaceutical compositions, are generally provided. In some embodiments, the articles and methods comprising diamond particles may be useful for monitoring and/or treating a disease (e.g., in a subject). In some embodiments, an article may be configured to administer a plurality of diamond particles (e.g., fluorescent (nano)diamond particles) that can be used to deliver a therapeutic agent bound to the (nano)diamond particles. For example, the plurality of (nano)diamond particles may be administered to a subject such that at least a portion of the plurality of (nano)diamond particles reside at a location internal to the subject (e.g., within an organ such as the liver). In some embodiments, the (nano)diamond particles may be used as a diagnostic tool. For example, in some embodiments, a plurality of (nano)diamond particles may be administered (e.g., via intravenous injection) to a subject. In some such embodiments, an image of the location suspected of containing the plurality of (nano)diamond particles may be obtained, and, after a diagnostically relevant period of time, a second image of the same location

internal to the subject suspected of containing the plurality of (nano)diamond particles may be obtained. In some embodiments, the first image and/or the second image is based on near infrared and/or fluorescent emissions (e.g., by the (nano)diamond particles). In some embodiments, a comparison of the first image and the second image may provide diagnostic information including, for example, progression of a disease state (e.g., cancer). For example, areas in the second image which comprise new tissue without the plurality of (nano)diamond particles may, in some cases, indicate malignant growth. As such, (nano)diamond particles, in some embodiments, may be useful for monitoring the progression of a disease. In some embodiments, the first image and the second image are obtained under similar (e.g., identical) conditions (e.g., same wavelength of excitation and/or emission).

A “subject”, as used herein, refers to any animal such as a mammal (e.g., a human). Non-limiting examples of subjects include a human, a non-human primate, a cow, a horse, a pig, a sheep, a goat, a dog, a cat or a rodent such as a mouse, a rat, a hamster, a bird, a fish, or a guinea pig. The embodiments described herein may be, in some cases, directed toward use with humans. The embodiments described herein may be, in some cases, directed toward veterinary use. In some embodiments, a subject may demonstrate health benefits, e.g., upon administration of the (nano)diamond particles.

In some embodiments, (nano)diamond particles described herein may be configured for prolonged residence time within one or more organs (e.g., the liver) of a subject. For example, the progression of tumor growth may be monitored by administering a plurality of (nano)diamond particles to a subject and imaging organs suspected of tumor growth as described above.

In some embodiments, (nano)diamond particles described herein may be configured to deliver a therapeutic agent (e.g., to an organ internal to a subject). In some embodiments, a therapeutic agent may be bound, at least partially, to a plurality of (nano)diamond particles. In some cases, the (nano)diamond particle bound to the therapeutic agent may be administered to a subject (e.g., to provide a therapeutic effect).

Because (nano)diamond particles may be configured to have a relatively prolonged residence internal to a location internal to the subject (e.g., an organ), therapeutic agents delivered using (nano)diamond particles may advantageously deliver a therapeutic agent over a prolonged period of time. In some embodiments, (nano)diamond particles are configured for prolonged residence in a subject or internal to

an organ of a subject. In some embodiments, the (nano)diamond particles are configured for residence (e.g., have a size and/or shape that facilitates residence). In some embodiments, the (nano)diamond particles are configured for residence in an organ for greater than or equal to 1 day, greater than or equal to 3 days, greater than or equal to 5 days, greater than or equal to 7 days, greater than or equal to 10 days, greater than or equal to 2 weeks, greater than or equal to 4 weeks, greater than or equal to 6 weeks, greater than or equal to 12 weeks, greater than or equal to 26 weeks, or greater than or equal to 52 weeks. In some embodiments, the (nano)diamond particles are configured for residence in an organ of a subject for less than or equal to 100 weeks, less than or equal to 52 weeks, less than or equal to 26 weeks, less than or equal to 12 weeks, less than or equal to 6 weeks, less than or equal to 4 weeks, less than or equal to 2 weeks, less than or equal to 10 days, less than or equal to 7 days, less than or equal to 5 days, or less than or equal to 3 days. Combinations of the above-referenced ranges are also possible (e.g., greater than 1 day and less than 100 weeks, greater than 5 days and less than 26 weeks, greater than 6 weeks and less than 52 weeks). Other ranges are also possible. In some embodiments, the (nano)diamond particles may be configured to reside in the organ of the subject for the lifespan of the subject. Advantageously, the (nano)diamond particles described herein may reside in an organ of a subject without toxic or detrimental physiological effects.

In certain embodiments, (nano)diamond particles may be captured by an organ internal to a subject. In some embodiments, the (nano)diamond particles may further organize or aggregate within a subject or within an organ internal to a subject. In some embodiments, (nano)diamond particles may form aggregates e.g., within an organ such as the liver. In some embodiments, diamond nanoparticles (e.g., (nano)diamond particles) may form aggregates within, e.g., the pancreas and/or pancreatic cells. In some cases, these aggregates advantageously may help to monitor the progression of a condition or disease within a subject and/or provide long term delivery of a therapeutic agent.

As described herein, (nano)diamond particles may be administered to a subject.

In some cases, the plurality of (nano)diamond particles are administered surgically (e.g., implanted) and/or injected (e.g., into the systemic circulation, intraocular, into the spinal system cord or fluids, e.g., via syringe). In certain embodiments, the plurality of

– 11 –

(nano)diamond particles may be administered orally, rectally, vaginally, nasally, or ureteral to the subject (e.g., within a capsule).

In some embodiments, administration of the (nano)diamond particles is via injection such as intravenous injection. For example, an injection component associated with a reservoir comprising the (nano)diamond particles may be used. In some embodiments, the injection component is a needle and the associated reservoir is a syringe. The needle may be of any size or gauge appropriate for administering a composition to a subject. The syringe may be of any size or volume appropriate for containing a particular amount of composition to be administered to a subject. In some embodiments, the injection component is a pipette. Those skilled in the art will be aware of other injection components suitable for administering a composition as described herein to a subject, as the disclosure is not so limited.

In some embodiments, the reservoir comprises an intravenous carrier fluid and a plurality of (nano)diamond particles suspended within the intravenous carrier fluid.

Non-limiting examples of suitable intravenous carrier fluids include saline (e.g., 9% normal saline, 45% normal saline), lactated Ringers, and aqueous dextrose (e.g., 5% dextrose in water).

In some embodiments, (nano)diamond particles (e.g., the plurality of (nano)diamond particles comprising a therapeutic agent bound to the (nano)diamond particles) may be administered to a subject (e.g., for the detection of an analyte (e.g., a biological element of physiological or pathological identity) suspected of being present in the subject). For example, in some cases, the plurality of (nano)diamond particles comprising the therapeutic agent may be administered to the subject and, upon detection of an emission (e.g., fluorescent emission, near infrared emission, etc.) of the (nano)diamond particles, confirm the presence of the therapeutic agent in the subject.

In some embodiments, a species (e.g. a therapeutic agent) is bound to a (nano)diamond particles or a plurality of (nano)diamond particles. In some embodiments, (nano)diamond particles are associated with (e.g., bound to) the species via functionalization of the (nano)diamond particle. For example, in some embodiments, a (nano)diamond particle is associated with a species via formation of a bond, such as an ionic bond, a covalent bond, a hydrogen bond, Van der Waals interactions, and the like. The covalent bond may be, for example, a carbon-carbon, carbon-oxygen, oxygen-silicon, sulfur-sulfur, phosphorus-nitrogen, carbon-nitrogen, metal-oxygen, or other

2026202273 24 Mar 2026

covalent bond. The hydrogen bond may be, for example, between hydroxyl, amine, carboxyl, thiol, and/or similar functional groups. For example, the species may further include a functional group, such as a thiol, aldehyde, ester, carboxylic acid, hydroxyl, and the like, wherein the functional group forms a bond with the (nano)diamond particle.

5 In some embodiments, a function group is bound to the (nano)diamond particles (e.g., capable of binding to the therapeutic agent). In some cases, the species may be an electron-rich or electron-poor moiety wherein interaction between the (nano)diamond particle and the species comprises an electrostatic interaction.

In some embodiments, a species (e.g. a therapeutic agent) is associated with a functionalized (nano)diamond particle comprising a -COOH, -OH, -NH₂, -SH, or -C=O functional group by reacting the functionalized (nano)diamond particle and the species in the presence of a cross-linking agent. Non-limiting examples of suitable cross-linking agents include carbodiimides such as 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC); amine-reactive compounds such as N-Hydroxysuccinimide ester, imidoester, and hydromethylphosphine; sulfhydryl-reactive compounds such as maleimide, pyridyl disulfides, and iodoacetyl; aldehyde-reactive compounds such as hydrazide and alkoxyamine; and photoreactive cross-linking agents such as aryl azides and diazirine. Other cross-linking agents are also possible. Those of ordinary skill in the art would be capable of selecting suitable cross-linking agents based upon the type of species selected and the teachings of this specification.

Examples of suitable (nano)diamond particles are described in more detail in co-owned International Patent Application No. PCT/US2017/050257, filed September 6, 2017, entitled “(NANO)DIAMOND PARTICLES AND RELATED DEVICES AND METHODS” which is incorporated herein by reference in its entirety for all purposes.

25 As described above and herein, in some embodiments, (nano)diamond particles may be used for imaging. For example, in some embodiments, the (nano)diamond particles may emit (i.e. fluorescence) a characteristic emission which may be detected by a detector. In some embodiments, a detector may be positioned proximate a region of a subject suspected of containing the (nano)diamond particles. For example, the plurality of (fluorescent) (nano)diamond particles functionalized with a species may be administered to a subject, and the detector may be positioned proximate the subject such that any (nano)diamond particles may be detected (e.g., via an emission of the (nano)diamond particles).

– 13 –

Any suitable detector may be used with the devices and methods described herein. For example, in some embodiments, the detector may be an optical detector (e.g., fluorescence detectors, visible light and/or UV detectors, near infrared detectors, microscopes, MRI, CT scanners, x-ray detectors).

As described herein, a (nano)diamond particle is an aggregate of carbon atoms where at the core lies a diamond cage composed mainly of carbon atoms. Although (nano)diamond particles comprise diamond, other phases or allotropes of carbon may be present, such as graphite, graphene, fullerene, etc. A single (nano)diamond particle may comprise a single form of carbon in some embodiments. In other embodiments, more than one form of carbon may comprise a (nano)diamond particle.

In some embodiments, a plurality of diamond particles may have an average largest cross-sectional dimension (e.g. a diameter) of 2 μm or less. While much of the description is generally related to nanodiamond particles (i.e. diamond particles having a largest cross-sectional dimension of less than 1000 nm), those of ordinary skill in the art would understand, based upon the teachings of this specification, that diamond particles having larger cross-sectional dimensions (e.g., greater than or equal to 1000 nm) are also possible. For example, in some embodiments, the plurality of diamond particles may have an average largest cross-sectional dimension of less than 2 μm (e.g., less than or equal to 1800 nm, less than or equal to 1600 nm, less than or equal to 1400 nm, less than or equal to 1200 nm, less than or equal to 1000 nm, less than or equal to 900 nm less than or equal to 800 nm, less than or equal to 700 nm, less than or equal to 600 nm, less than or equal to 400 nm, less than or equal to 200 nm, less than or equal to 180 nm, less than or equal to 160 nm, less than or equal to 140 nm, less than or equal to 120 nm, less than or equal to 100 nm, less than or equal to 80 nm, less than or equal to 60 nm, less than or equal to 40 nm, or less than or equal to 20 nm). In some cases, the plurality of diamond particle may have an average largest cross-sectional dimension of greater than or equal to 10 nm, greater than or equal to 20 nm, greater than or equal to 40 nm, greater than or equal to 60 nm, greater than or equal to 80 nm, greater than or equal to 100 nm, greater than or equal to 120 nm, greater than or equal to 140 nm, greater than or equal to 160 nm, greater than or equal to 180 nm, greater than or equal to 200 nm, greater than or equal to 400 nm, greater than or equal to 600 nm, greater than or equal to 700 nm, greater than or equal to 800 nm, greater than or equal to 900 nm, greater than or equal to 1000 nm, greater than or equal to 1200 nm, greater than or equal to 1400 nm, greater

– 14 –

than or equal to 1600 nm, or greater than or equal to 1800 nm. Combinations of the above-referenced ranges are also possible (e.g., less than 2 μm and greater than or equal to 10 nm, less than or equal to 1400 nm and greater than or equal to 1000 nm). Other ranges are also possible. Those of ordinary skill in the art are capable of selecting suitable methods for determining the average cross-sectional dimension of a plurality of diamond based upon the teachings of this specification. In an exemplary set of embodiments, the plurality of diamond particles have an average largest cross-sectional dimension of less than or equal to 900 nm and greater than or equal to 700 nm. In some embodiments, diamond particles may form aggregate structures with other diamond particles (e.g., at a location internal to the subject). An aggregate of diamond particles, in some embodiments, may have a largest cross-sectional dimension greater than or equal to 1 μm (e.g. greater than or equal to 1 μm , greater than or equal to 5 μm , greater than or equal to 10 μm , greater than or equal to 20 μm , greater than or equal to 30 μm , greater than or equal to 40 μm , greater than or equal to 50 μm , greater than or equal to 60 μm , greater than or equal to 70 μm , greater than or equal to 80 μm , greater than or equal to 90 μm) and less than or equal to 100 μm (e.g. less than or equal to 100 μm , less than or equal to 90 μm , less than or equal to 80 μm , less than or equal to 70 μm , less than or equal to 60 μm , less than or equal to 50 μm , less than or equal to 40 μm , less than or equal to 30 μm , less than or equal to 20 μm , less than or equal to 10 μm , less than or equal to 5 μm , less than or equal to 1 μm). Combinations of the above-referenced ranges are also possible (e.g., greater than or equal to 1 micron and less than or equal to 50 microns, greater than or equal to 1 micron and less than or equal to 100 microns). Other ranges are also possible.

In some embodiments, (nano)diamond particles may form relatively large aggregate structures with other (nano)diamond particles (e.g., at a location internal to the subject). For example, in some embodiments, the aggregate of (nano)diamond particles has a largest cross-sectional dimension of greater than or equal to 100 microns, greater than or equal to 200 microns, greater than or equal to 500 microns, greater than or equal to 1000 microns, greater than or equal to 2000 microns, greater than or equal to 5000 microns, or greater than or equal to 7500 microns. In some embodiments, the aggregate of (nano)diamond particles has a largest cross-sectional dimension of less than or equal to 10000 microns, less than or equal to 7500 microns, less than or equal to 5000 microns, less than or equal to 2000 microns, less than or equal to 1000 microns, less than or equal

2026202273 24 Mar 2026

to 500 microns, or less than or equal to 200 microns. Combinations of the above-referenced ranges are also possible (e.g., greater than or equal to 1 micron and less than or equal to 10000 microns, greater than or equal to 100 microns and less than or equal to 10000 microns, greater than or equal to 500 microns and less than or equal to 5000 microns, greater than or equal to 1000 microns and less than or equal to 10000 microns). Other ranges are also possible.

In some embodiments, the (nano)diamond particles may emit electromagnetic radiation. In some embodiments, the emission is a fluorescent emission. In certain embodiments, the wavelength of the emission is greater than or equal to 250 nm, greater than or equal to 300 nm, greater than or equal to 350 nm, greater than or equal to 400 nm, greater than or equal to 450 nm, greater than or equal to 500 nm, greater than or equal to 550 nm, greater than or equal to 600 nm, or greater than or equal to 650 nm. In certain embodiments, the wavelength of the emission is less than or equal to 700 nm, less than or equal to 650 nm, less than or equal to 600 nm, less than or equal to 550 nm, less than or equal to 500 nm, less than or equal to 450 nm, less than or equal to 400 nm, less than or equal to 350 nm, or less than or equal to 300 nm. Combinations of the above-referenced ranges are also possible (e.g., greater than or equal to 250 nm and less than or equal to 700 nm). Other ranges are also possible.

In certain embodiments, the emission is a near infrared emission. In some embodiments, the wavelength of the emission is greater than 700 nm, greater than or equal to 750 nm, greater than or equal to 800 nm, greater than or equal to 850 nm, greater than or equal to 900 nm, or greater than or equal to 950 nm. In certain embodiments, the wavelength of the emission is less than or equal to 1000 nm, less than or equal to 950 nm, less than or equal to 900 nm, less than or equal to 850 nm, less than or equal to 800 nm, or less than or equal to 750 nm. Combinations of the above-referenced ranges are also possible (e.g., greater than 700 nm and less than or equal to 1000 nm). Other ranges are also possible.

In an exemplary embodiment, the (nano)diamond particles have a near infrared emission (e.g., greater than or equal to 650 nm and less than or equal to 750 nm) and an average largest cross-sectional dimension of about 700-900 nm. Other combinations of emissions and cross-sectional dimensions are also possible.

In some embodiments, the (nano)diamond particle may emit a fluorescent and/or near infrared emission upon excitation by electromagnetic radiation having a particular

wavelength. For example, in some embodiments, the (nano)diamond particle may be exposed to electromagnetic radiation having a wavelength of greater than or equal to 250 nm, greater than or equal to 300 nm, greater than or equal to 350 nm, greater than or equal to 400 nm, greater than or equal to 450 nm, greater than or equal to 500 nm, greater than or equal to 550 nm, greater than or equal to 600 nm, greater than or equal to 650 nm, greater than or equal to 700 nm, greater than or equal to 750 nm, greater than or equal to 800 nm, greater than or equal to 850 nm, greater than or equal to 900 nm, or greater than or equal to 950 nm (e.g., such that the (nano)diamond particle emits a fluorescent emission and/or near infrared emission in one of the above-referenced ranges). In certain embodiments, the (nano)diamond particle may be exposed to electromagnetic radiation having a wavelength of less than or equal to 1000 nm, less than or equal to 950 nm, less than or equal to 900 nm, less than or equal to 850 nm, less than or equal to 800 nm, or less than or equal to 750 nm, less than or equal to 700 nm, less than or equal to 650 nm, less than or equal to 600 nm, less than or equal to 550 nm, less than or equal to 500 nm, less than or equal to 450 nm, less than or equal to 400 nm, less than or equal to 350 nm, or less than or equal to 300 nm. Combinations of the above-referenced ranges are also possible (e.g., greater than or equal to 250 nm and less than or equal to 1000 nm, greater than or equal to 550 nm and less than or equal to 650 nm). Other ranges are also possible.

Without wishing to be bound by theory, in some cases, the (nano)diamond particles described herein may be auto-fluorescent (e.g., the (nano)diamond particles emit fluorescent light e.g., after absorption of electromagnetic radiation). In some cases, the (nano)diamond particles may comprise one or more atomistic-type defects (e.g., a point defect such as a nitrogen-vacancy (NV) center, a point defect such as a nitrogen-vacancy-nitrogen (NVN) defect, combinations thereof) which result in near-infrared fluorescence and/or photoluminescence that may be detected and/or quantified. Other defects are also possible (e.g., Gadolinium, Europium, iron, Si-vacancy defects). In certain embodiments, the (nano)diamond particles fluoresce in response to an applied electromagnetic radiation.

For example, in some embodiments, the (nano)diamond particle may be excited (e.g., by applying electromagnetic radiation having a first wavelength) such that the (nano)diamond particle emits a detectable emission (e.g., an electromagnetic radiation having a second wavelength, different than the first wavelength). In a particular set of

– 17 –

embodiments, if an analyte is present in a sample, the analyte binds to the (nano)diamond particle (e.g., binds to a species bound to the (nano)diamond particle) such that an emission from the (nano)diamond particle may be detected and/or quantified. In some cases, detection of an emission of (nano)diamond particles in a subject may indicate that the (nano)diamond particles are bound to the suspected analyte. In some such cases, the emission may be quantified (e.g., to determine the relative amount of analyte present in the subject).

As described herein, certain embodiments comprise a therapeutic agent bound to (nano)diamond particles. According to some embodiments, the therapeutic agent may be one or a combination of therapeutic, diagnostic, and/or enhancement agents, such as drugs, nutrients, microorganisms, in vivo sensors, and tracers. In some embodiments, the therapeutic agent is a nutraceutical, prophylactic or diagnostic agent. While much of the specification describes the use of therapeutic agents, other agents listed herein are also possible.

Agents can include, but are not limited to, any synthetic or naturally-occurring biologically active compound or composition of matter which, when administered to a subject (e.g., a human or nonhuman animal), induces a desired pharmacologic, immunogenic, and/or physiologic effect by local and/or systemic action. For example, useful or potentially useful within the context of certain embodiments are compounds or chemicals traditionally regarded as drugs, vaccines, and biopharmaceuticals. Certain such agents may include molecules such as proteins, peptides, hormones, nucleic acids, gene constructs, etc., for use in therapeutic, diagnostic, and/or enhancement areas, including, but not limited to medical or veterinary treatment, prevention, diagnosis, and/or mitigation of disease or illness (e.g., HMG co-A reductase inhibitors (statins) like rosuvastatin, nonsteroidal anti-inflammatory drugs like meloxicam, selective serotonin reuptake inhibitors like escitalopram, blood thinning agents like clopidogrel, steroids like prednisone, antipsychotics like aripiprazole and risperidone, analgesics like buprenorphine, antagonists like naloxone, montelukast, and memantine, cardiac glycosides like digoxin, alpha blockers like tamsulosin, cholesterol absorption inhibitors like ezetimibe, metabolites like colchicine, antihistamines like loratadine and cetirizine, opioids like loperamide, proton-pump inhibitors like omeprazole, anti(retro)viral agents like entecavir, dolutegravir, rilpivirine, and cabotegravir, antibiotics like doxycycline, ciprofloxacin, and azithromycin, anti-malarial agents, and synthroid/levothyroxine);

substance abuse treatment (*e.g.*, methadone and varenicline); family planning (*e.g.*, hormonal contraception); performance enhancement (*e.g.*, stimulants like caffeine); and nutrition and supplements (*e.g.*, protein, folic acid, calcium, iodine, iron, zinc, thiamine, niacin, vitamin C, vitamin D, and other vitamin or mineral supplements).

5 In certain embodiments, the therapeutic agent is one or more specific therapeutic agents. As used herein, the term “therapeutic agent” or also referred to as a “drug” refers to an agent that is administered to a subject to treat a disease, disorder, or other clinically recognized condition, or for prophylactic purposes, and has a clinically significant effect on the body of the subject to ameliorate, treat and/or prevent the disease, disorder, or
10 condition. Listings of examples of known therapeutic agents can be found, for example, in the United States Pharmacopeia (USP), Goodman and Gilman’s The Pharmacological Basis of Therapeutics, 10th Ed., McGraw Hill, 2001; Katzung, B. (ed.) Basic and Clinical Pharmacology, McGraw-Hill/Appleton & Lange; 8th edition (September 21, 2000); Physician’s Desk Reference (Thomson Publishing), and/or The Merck Manual of
15 Diagnosis and Therapy, 17th ed. (1999), or the 18th ed (2006) following its publication, Mark H. Beers and Robert Berkow (eds.), Merck Publishing Group, or, in the case of animals, The Merck Veterinary Manual, 9th ed., Kahn, C.A. (ed.), Merck Publishing Group, 2005; and “Approved Drug Products with Therapeutic Equivalence and Evaluations,” published by the United States Food and Drug Administration (F.D.A.)
20 (the “Orange Book”). Examples of drugs approved for human use are listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361, and 440 through 460, incorporated herein by reference; drugs for veterinary use are listed by the FDA under 21 C.F.R. §§ 500 through 589, incorporated herein by reference. In certain embodiments, the therapeutic agent is a small molecule. Exemplary classes of therapeutic agents include, but are not limited to,
25 analgesics, anti-analgesics, anti-inflammatory drugs, antipyretics, antidepressants, anti-epileptics, antipsychotic agents, neuroprotective agents, anti-proliferatives, such as anti-cancer agents, antihistamines, antimigraine drugs, hormones, prostaglandins, antimicrobials (including antibiotics, antifungals, antivirals, anti-parasitics), antimuscarinics, anxiolytics, bacteriostatics, immunosuppressant agents, sedatives,
30 hypnotics, antipsychotics, bronchodilators, anti-asthma drugs, cardiovascular drugs, anesthetics, anti-coagulants, inhibitors of an enzyme, steroidal agents, steroidal or non-steroidal anti-inflammatory agents, corticosteroids, dopaminergics, electrolytes, gastrointestinal drugs, muscle relaxants, nutritional agents, vitamins, parasympathomimetics,

stimulants, anorectics and anti-narcoleptics. Nutraceuticals can also be incorporated into the drug delivery device. These may be vitamins, supplements such as calcium or biotin, or natural ingredients such as plant extracts or phytohormones.

In some embodiments, the therapeutic agent is one or more anticancer drugs (e.g., chemotherapy drugs).

Non-limiting examples of suitable anticancer therapeutic agents include alkylating agents (e.g., Cyclophosph, Busulfan, cisplatin), antimetabolic compounds (e.g., folic acid analogs- methotrexate), purine analogs (e.g., mercaptopurine, Pentostatin), pyrimidine analogs (e.g., 5-fluor uracil), vinca alkaloids, camptothecins, proteasome inhibitors (e.g., Gefitinib), hormones (e.g., steroids), biological adjuvants treatments (e.g., antibodies, Herceptin), adjuvant treatments (e.g., BRAF, Melanoma), dabrafenib/Tafinlar; Trametinib/Mekinist), biospecific antibodies, blinatumomab/Blincyto, chemolabeled antibodies, and Brentuximab.

In another embodiment, the therapeutic agent is an immunosuppressive agent. Exemplary immunosuppressive agents include glucocorticoids, cytostatics (such as alkylating agents, antimetabolites, and cytotoxic antibodies), antibodies (such as those directed against T-cell receptors or IL-2 receptors), drugs acting on immunophilins (such as cyclosporine, tacrolimus, and sirolimus) and other drugs (such as interferons, opioids, TNF binding proteins, mycophenolate, and other small molecules such as fingolimod).

In certain embodiments, the therapeutic agent is a hormone or derivative thereof. Non-limiting examples of hormones include insulin, growth hormone (e.g., human growth hormone), vasopressin, melatonin, thyroxine, thyrotropin-releasing hormone, glycoprotein hormones (e.g., luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, TSH), eicosanoids, estrogen, progestin, testosterone, estradiol, cortisol, adrenaline, and other steroids.

In some embodiments, the therapeutic agent is a small molecule drug having molecular weight less than about 2500 Daltons, less than about 2000 Daltons, less than about 1500 Daltons, less than about 1000 Daltons, less than about 750 Daltons, less than about 500 Daltons, less or than about 400 Daltons. In some cases, the therapeutic agent is a small molecule drug having molecular weight between 200 Daltons and 400 Daltons, between 400 Daltons and 1000 Daltons, or between 500 Daltons and 2500 Daltons.

In some embodiments, the therapeutic agent is selected from the group consisting of active pharmaceutical agents such as nucleic acids, peptides, bacteriophage, DNA,

– 20 –

mRNA, aptamers, human growth hormone, monoclonal antibodies, adalimumab, epinephrine, GLP-1 Receptor agonists, semaglutide, liraglutide, dulaglutide, exenatide, factor VIII, small molecule drugs, progestin, vaccines, subunit vaccines, recombinant vaccines, polysaccharide vaccines, and conjugate vaccines, toxoid vaccines, influenza vaccine, shingles vaccine, prevnar pneumonia vaccine, mmr vaccine, tetanus vaccine, hepatitis vaccine, HIV vaccine Ad4-env Clade C, HIV vaccine Ad4-mGag, DNA vaccines, RNA vaccines, etanercept, infliximab, filgastrim, glatiramer acetate, rituximab, bevacizumab, any molecule encapsulated in a nanoparticle, epinephrine, lysozyme, glucose-6-phosphate dehydrogenase, other enzymes, certolizumab pegol, ustekinumab, ixekizumab, golimumab, brodalumab, guselimumab, secikinumab, omalizumab, tnfr-alpha inhibitors, interleukin inhibitors, vedolizumab, octreotide, teriperatide, CRISPR cas9, oligonucleotides, and ondansetron.

While much of the description herein is in the context of (fluorescent) (nano)diamond particles, those of ordinary skill in the art would understand, based upon the teachings of this specification, that other particles are also possible. For example, in some embodiments, the device may comprise a particle such as a nanoparticle (e.g., a silica nanoparticle, a sapphire nanoparticle, a garnet nanoparticle, a ruby nanoparticle, a quantum dot, a quantum dot-polymer composite) having an emission in one of the above referenced ranges associated with a species (e.g., a species capable of binding to one or more target analytes). In some cases, the particle may be auto-fluorescent. In other cases, the particle may be functionalized with (e.g., associated with) a fluorescent molecule.

In an illustrative embodiment, fluorescent (nano)diamond particles administered to a subject gain access to the liver cells (e.g., hepatocytes, kupfer cells), as well as other cells (e.g., endothelium) where the deposition of (nano)diamond particles in the liver is substantially immediate (upon (nano)diamond particles injections). In some such embodiments, the presence of the (nano)diamond particles in the liver is prolonged e.g., a single injections could provide a sustained presence of particles at least over 12 weeks. In some embodiments, (nano)diamond particles present in the liver do not convey adverse effects on the normal liver cells (e.g., measured at least after 3 months). (nano)diamond particles and/or an associated species (e.g., a chemical and/or organic additive functionalized on the (nano)diamond particle) may, in some cases, find facilitated entrance and increased accumulation within cancer cells (over the normal liver

2026202273 24 Mar 2026

– 21 –

cells). In some embodiments, therapeutic agents having anti-cancer properties, when tagged onto the fluorescent (nano)diamond particles, may arrest cancer cells growth (e.g., diminishing the metastatic scale and its progression). In some such embodiments, the (nano)diamond particles (e.g., bound to therapeutic agents) may advantageously afford longer “progression free disease” periods and reduced mortality. In some embodiments, without wishing to be bound by theory, diminishing the metastatic burden in the liver, may advantageously contribute to betterment of liver function (a severe cause of morbidity on its own).

EXAMPLES

The following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

EXAMPLE 1

Rodent POC (efficacy) studies raised an important caveat, i.e. anatomical considerations suggest that recording NIR from locales within the body pose challenges associated with opacity and auto-fluorescence in an organ- and tissue-dependent manner. To optimize NIR signal captured from within the body in a location such as the carotid artery (in humans, 15-20mm from skin surface), large nanodiamond particles were selected: FNDP-(NV)-Z-average~800nm (fluorescent nanodiamond particles having a nitrogen-vacancy and an average largest cross-sectional dimension of about 800 nm), a strain of particles that possess superior NIR emission (650~720nm) over smaller particles of the same strain as well as over the NVN “color center” strain. The particles ‘homed’ to the target (intra-vascular blood clots) shortly after infusion (envisioning the targeted clinical procedure) and considering the rapid clearance of FNDP-(NV) from the circulation (50% clearance from serum within 4 minutes), likely via rapid uptake by the reticuloendothelial system of the liver, the loading dose of the FDNP-(NV) was explored to afford target imaging.

Administration of particles of the size chosen (Z-average~800nm) directly into the systemic circulation may result in prolonged, if not indefinite, particles residency within organs due to unlikely excretion routes (urinary system or the hepatobiliary system). Such concerns are supported by in vitro studies where extended residency of

– 22 –

other similarly sized particles in cells (in culture) suggested interference with biological functions and viability.

The examples herein, designed to explore FNDP-(NV) distribution in rat organs upon both short and long-term exposure demonstrated principle deposition of particles in the liver with secondary deposition to the spleen while other organs shared only a minor fraction. Interestingly, the large deposit of FNDP-(NV) in the liver 5 days after exposure remained unchanged in the 14-days and 12-weeks post-exposure studies.

The present investigation on the intra-hepatic topological distribution of FNDP-(NV) was generally carried out by conventional fluorescent microscopy (FM) and confocal fluorescent microscopy (CFM) of liver slices (5-50 μ m). Furthermore, an in vitro investigation on the kinetics of FNDP-(NV) uptake into cells, such as human hepatic carcinoma cells (HepG2) and human umbilical vein endothelial cells (HUVEC) commonly used as proxies for hepatocytes and vascular endothelium, respectively, was performed. The in-vitro results demonstrated the capacity of liver cells to incorporate FNDP-(NV) as well the subcellular distribution of engulfed particles.

Material and Methods

FNDP-(NV)-Z-average~800nm: source and functionalization

FNDP-(NV)-Z-average~800nm functionalized with carboxyl moieties were purchased from ADAMAS Nanotechnologies (Raleigh, NC, USA). The physical properties of the FNDP-(NV) were determined by dynamic light scattering on a Zetasizer Nano (Malvern) as having an average diameter of 858 $\pm$ 47 nm and Z-potential of -56 mV, as reported previously. Sterile and BSA blocked FNDP-(NV) were used in the cell based studies.

Liver specimens were obtained as follows: Briefly, Sprague-Dawley rats were injected into the femoral vein at 60mg/Kg of FNDP-(NV) suspension in 2mL PBS over 2-3 minutes. After 12 weeks, the animals were sacrificed by exsanguination while under deep (5% isoflurane) anesthesia, perfused with 10mL sterile saline to minimize residual blood in the organs vasculature and further by perfusion of 4% paraformaldehyde in saline for organ preservation. Organs were carefully dissected, suspended in excess of 10% neutral buffered formaldehyde (10% NBF). Liver specimens were then processed and embedded in paraffin for sectioning into 5 or 50 μ m slices for analysis by, fluorescence microscopy (FM) or confocal fluorescent microscopy (SCM), respectively.

The liver specimens evaluated in this study were discrete and holistic lobes dissected after whole organ imaging by IVIS. For histopathology examination 5µm sections of liver specimens were stained with Hematoxylin and Eosin (H&E) and Masson's trichrome by independent histopathology evaluation.

5 Rat liver specimens were embedded in paraffin and sectioned at 5 or 50µm thickness as described previously. In brief, slides were de-paraffinized by three consecutive rinses (5 min each) with xylene followed by two consecutive rinses (10 min each) of 100%, 95%, 70% and 50% ethanol and two final washes with deionized water. Cellular actin filaments were stained with FITC-phalloidin. Briefly, slides were
10 permeabilized by incubation with 0.4% Triton X-100 in PBS on ice for 10 min. The slides were then washed 3 times with PBS at room temperature and immersed in FITC-phalloidin (6µM in PBS) for 1 hour. The slides were washed three times with PBS and mounted with mounting buffer containing DAPI to stain nuclei. The 5µm thick slices were analyzed in a fluorescence microscope using 10x and 40x (oil immersion)
15 objectives. The green fluorescence filter set was used to detect the FITC-phalloidin stained microfilaments, the red fluorescent filter to was used detect FNDP-(NV) and the blue fluorescent filters to detect DAPI stained cell nuclei.

Total panoramic views of sagittal sections of the liver were constructed by 'stitching' 4x images using an FSX100 microscope. 50µm sections were stained with
20 FITC-phalloidin for visualization of actin filaments imaged in the green channel, and sections were imaged in the red channel for visualization of FNDP-(NV). Images were collected digitally and further processed with ImageJ 1.51e (NIH, Bethesda MD, USA). In order to improve visualization of FNDP-(NV), which were only a few pixels in size at the ultra-low magnification, particles were magnified by thresholding the red channel
25 using the Maximum Entropy method and dilating the result three times.

FNDP-(NV) presence in cells after image thresholding, but not dilating, was also quantified using the Analyze Particles function in ImageJ. Groups of FNDP-(NV), detected as a single continuous mass (agglomerate) at 4x, were counted and sized. The size distribution by number histogram was constructed to demonstrate the distribution of
30 FNDP-(NV) agglomeration sizes detected within the micrographs, where line height corresponds to the portion of particles detected by diameter. As large numbers of small agglomerations can account for a small number of total particle mass, size distribution by number can be considered biased to magnify the prevalence of small particle sizes. To

– 24 –

reduce this bias, a second histogram of the size distribution by cross-section area was also constructed where line height correlates with portion of total NIR fluorescing area.

Confocal images of liver slices (10-50µm) were taken using an FV1000 scanning confocal microscope and imaged in Fluoview software (v4.2.2.9 Olympus) using a 60x oil immersion objective. For 3D reconstruction, confocal stacks were taken with an image every 0.5µm through the thickness of the tissue. Nuclei were visualized by DAPI staining with a 405nm excitation and 425-460nm emission; the actin cytoskeleton was visualized by FITC-phalloidin with a 488nm excitation and 400-500nm emission. The NIR fluorescence emitted from FNDP-(NV) was visualized with an excitation of 543nm and an emission of 655-755nm. 2D maximum intensity projection and cross-sectional views were prepared in Fluoview. Three dimensional views were reconstructed in ImageJ via the Volume Viewer plugin.

The HepG-2 (human liver hepatocellular carcinoma) cell line was purchased from American Type Culture Collection (ATTC) (Manassas, VA, USA) and cultured in Eagle's Minimum Essential Medium (EMEM, ThermoFisher Scientific) containing 10% fetal bovine serum (FBS). Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and cultured in EGM-2 MV media. HUVEC were used for experiments in passages 5-8. Uptake of FNDP-(NV) by either cell line was performed according to previously published protocols with some modifications, as illustrated in FIG. 1. Briefly, cells were seeded into 2 96-well plates (2 x 10⁴ cells per well), and allowed to grow to 90% confluence. Media was removed from one plate (background control) and 100µL of 4% paraformaldehyde (PFA) in PBS was added to fix the cells. The control plate was then incubated for 20 min at room temperature and washed 3 times with cell culture media. Subsequently, media was removed from each well in both plates (fixed control and live sample), replaced with 100µL of media containing FNDP-(NV) at 0.025, 0.05, and 0.1mg/mL as indicated, and allowed to incubate for 0.5-20hr. Both plates were washed 3 times with Hanks' balanced salt solution (HBSS, ThermoFisher, and Waltham, MA, USA) containing calcium and magnesium to remove excess particles. Cells were then lysed by addition of 100µL of 0.5% Triton X-100 and overnight incubation at room temperature on orbital shaker. Plates were read using spectrophotometer (Infinite M200 Pro, Tecan AG, Männedorf, Switzerland) for FNDP-(NV) associated NIR signal (excitation 570nm, emission

– 25 –

670nm). Fluorescence obtained from FNDP-(NV) attached to the control plate with PFA fixed cells was deducted from fluorescence measured from live (active) cells.

Cells were grown in 8-well chambers slides (ThermoFisher) up to 70% confluence. Cell were treated with FNDP-(NV) at 0.05mg/mL, and incubated for 2 or 20 hrs., and fixed in 4% PFA as described above. Following cell fixation and permeabilization, cells were stained with FITC-Phalloidin as described above. Chambers were removed from the slide, and mounting was completed using buffer containing DAPI (Vectashield) and cover glass affixed by nail polish. Slides were then analyzed on the FM Olympus IX81 at 10x or 40x, using the green, red, and blue filter cubes as described in Fluorescent microscopy of preserved liver slices.

Data are presented as mean $\pm$ SD. Statistical analyses were done by ANOVA (where appropriate) and Student's t-test using SigmaPlot software (SigmaPlot® 12 SPSS; Systat Software Inc., San Jose CA, USA). Statistical significance was established at $P < 0.05$ for the number of independent studies performed.

Results

Fluorescence microscopic (FM) and panoramic analysis of preserved liver slices.

FIGS. 2A illustrates the distribution of FNDP-(NV) within a 5 μ m slice of liver tissue imaged at 160x and 400x magnifications. Two representative regions have been selected; one (FIG. 2A) where vascular elements are present, second (FIG. 2B) an area of parenchyma cells only. The upper panels represent tissue obtained from animals 12 weeks after intravenous (i.v.) administration of FNDP-(NV) and the lower panel from a vehicle (PBS) control animal. FNDP-(NV) (imaged in red, shown in a shade of grey) can be visualized over the DAPI counter stain in the right panel as identified by white arrows. Large agglomerations of 5-10 μ m are clearly noted, as well as particles of very small size. To assess distribution within or between cells, the sections were stained with FITC-phalloidin as shown in the left panels. The corresponding yellow (red-over-green) can be visualized for larger aggregates, indicating possibility of particle endocytosis (left panels (FIGS. 2A and 2B)). In FIG. 2A, red fluorescence of very small aggregates can be spotted in proximity of nuclei that possibly represent portal vein (PV) endothelium but most are distributed in the parenchyma where it is rather difficult to discern venous space from parenchyma cells location.

FIGS. 3A-3H presents an analysis at multiple magnifications of a complete sagittal section from 2 different FNDP-(NV) treated rats. Due to the very low effective magnification of the ‘stitched’ image, the red channel, sensitive to the NIR fluorescence of FNDP-(NV), has been magnified by binary thresholding and dilating as indicated in methods. This allows qualitative visualization of even single pixel FNDP-(NV). FIGS. 3A and 3B depict particles scattered across the complete “panoramic landscape” yet with apparent differential densities in their distribution within the core hepatic lobule unit. For ease of visualization, a select number of hepatic lobules are indicated by “hexagons”. Particles can also be easily spotted in the venous system (yellow boxes). A magnified view, suitable for visualization without enhancement, of a set of four hepatic lobules (region indicated by blue dotted rectangle in FIG. 3B) is presented in FIG. 3C, which illustrates apparent heterogeneity of particle distribution within the hepatic lobule. A higher magnification of a single lobule (yellow hexagon from FIG. 3A) is presented in FIG. 3D. To enhance visualization, a higher magnification of one representative lobule from each animal (as indicated by yellow hexagon in FIG. 3A and B) is presented in FIG. 3E and F. After thresholding and dilating, better illustration of the uneven distribution of particles across the “hexagon” formation of the hepatic lobule is easily noticed. Particle presence appears enriched at the “hexagonal” periphery (for landmarks, red arrows mark central veins), though some particles are clearly present even beside the central vein. FIG. 3G and H depict venous systems (yellow squares in FIG. 3A) with large aggregation of particles (white arrow) that are attached to the wall but protrudes significantly into the vessel lumen (visualized by the yellow-red transition) accounting for 35% and 48% of the vessel cross sectional areas in the 2 examples, respectively. FIG. 3I provides a scheme of the general orientation of the structure of the hepatic lobule including the primary metabolic zones.

The size of FNDP-NV positive regions in the liver “panoramic” view is highly variable as indicated above. To quantify this distribution, a histogram of FNDP-NV positive regions is presented in FIG. 4. The distribution of the regions by number in FIG. 4A demonstrates large numbers of FNDP-NV positive areas from a single pixel, up to an area of 20 μ m in diameter. Although few, hardly visible in FIG. 4A, large agglomerates (FIG. 3G and H) would represent a disproportionate mass of total particles detected in the liver section. To represent the percent of total particle mass, the distribution of the total FNDP-(NV) positive area is presented in FIG. 4B. By area, the modal diameter of

– 27 –

particle agglomeration is roughly 14 μ m. In one animal, large agglomerations 40-100 μ m in diameter can be found in the venous system that account for as much as 20% of the FNDP-(NV) positive area, though agglomerates of this size were not found in the second animal.

Confocal Fluorescent Microscopy (CFM) of preserved liver slices

FIGS. 5A-5D presents four different topographical segments studied by CFM on liver sections (10-50 μ m). In FIG. 5A several peri-nuclear particles agglomerates of about 5-10 μ m are visible (yellow circles and arrows), yet definite intra-cellular location cannot be established. FIG. 5B presents intercellular spaces likely representing portal sinusoid of which some contain large agglomerates of FNDP-(NV) at 10-30 μ m (yellow circles and arrows). The intense red coloring suggests location sufficiently remote from the internal milieu of the parenchyma cells (stained green), though some yellow, indicating potential for at least partial internalization, is present as well. FIGS. 5C and 5D present several non-parenchyma structures (surrounded by parenchyma cells) such as venous, arterial, portal vein and likely a bile duct. In FIGS. 5C and 5D several small particle agglomerates (white circles) are located in the sub-endothelial zone of the vessel intima while some agglomerates residing inside parenchymal cell (yellow circles) are also noted.

FIG. 6 illustrates confocal 3D reconstruction of hepatocytes with differing amount of incorporated FNDP-(NV). Two areas are presented which differ in the mass of particles; the cells in the center panel acquired few while the cells in the right panel appear to have been amassed very large particles agglomerates. The left panel represents the vehicle treated rats; no particles have been identified there. In all of the examples provided, the nucleus and nucleoli of these cells present same and normal phenotype.

Kinetic of FNDP-(NV) uptake into cultured HUVEC and HepG-2 cells

FIGS. 7A-7D depict the kinetics of FNDP-(NV) uptake into HUVEC and HEPG-2 cells under various concentration and time course conditions. FIGS. 7A-7C represents the time course at three different exposure levels of FNDP-(NV). Each of the exposed dose demonstrated same pattern of rapid uptake of particles into the cell body. The rapid uptake phase is attenuated within 1-2 hours reaching a plateau proportional to the amount of FNDP-(NV) exposure. FIG. 7D represents the quantitative accumulation of FNDP-

– 28 –

(NV) monitored by NIR fluorescence for each of the cell lines at the three concentration of FNDP-(NV). The difference in total accumulated FNDP-(NV) is statistically significant between exposure levels, but is similar between cell lines.

FIG. 8A and 8B are FM micrographs of FNDP-(NV) accumulation at an early and late stage of the in vitro experiment. The early phases (up to 2 hour) demonstrate particles largely in the cytoplasm while the terminal time point (20 hours) reveals heavy agglomeration in the form of a peri-nuclear corona. Such a pattern was also documented in the preserved liver slices (12 weeks post exposure), as seen in FIG. 6C.

The images of HUVEC in the early mitosis through the end of cytokinesis are presented in FIG. 9A for cells treated with FNDP-(NV) for 20 hours, and in FIG. 9B for untreated, control cells. All treated cells display heavy peri-nuclear FNDP-(NV) accumulation, including in late stage cytokinesis and cell separation. Similar observation has been made in the control group.

Discussion

The gross distribution of a high dose (60 mg/Kg) of FNDP-(NV) infused to intact rats were characterized and their dispositions were followed acutely, (90 min), sub-acute (5 or 14 days), and long-term (12 weeks) post FNDP-(NV) exposure. Analysis of particle distribution across 6 organs (liver, spleen, lung, kidney, heart and brain) confirmed the liver as the primary repository organ for these particles. Organ histology evaluation did not reveal any FNDP-(NV) related gross or histopathology adverse effects. The lack of adverse effects related to FNDP-(NV) is in accord with reported normal liver function tests.

As described above, the persistence of large numbers of FNDP-(NV) particles in the liver would generally raise concerns about potential negative impacts, especially in the context of long-term residency, possibly indefinite presence. Such prospect might raise regulatory hurdles and potentially impact the GLP (Good Laboratory Practice) pre-clinical development for human use. While the aforementioned certified pathology report confirmed the lack of histopathological findings, the present investigation was aimed at addressing three primary objectives: 1. Comprehensive survey of FNDP-(NV) distribution in the various liver cells, including intra-cellular location in hepatocytes. 2. Localization of FNDP-(NV) in the microvascular system of the hepatic lobule. 3. Explore the kinetics of FNDP-(NV) particle uptake into cultured liver cells and their

intracellular distribution using surrogate (proxy) cell cultures such as HUVEC (endothelium) and HepG-2 (human liver carcinoma) cells.

The primary outcomes of this study include: 1. revealing the unique pattern of spatial distribution of FNDP-(NV) in the hepatic lobules, including parenchymal cells (hepatocytes), non-parenchymal cells (vascular endothelium and adventitia cells) and the venous supply (portal vein) and drainage (central vein) system. 2. Demonstration of intracellular uptake and compartmentalization of FNDP-(NV) in liver cells in vivo and in vitro. 3. Affirmation of the preservation of normal macro and micro morphological phenotypes of liver cells including cells with large coronas of particles in the peri-nuclear space. 4. Preservation of viable cytokinesis processes, from late mitosis to completion of cytokinesis to cell replication including cells with extensive peri-nuclear coronas.

The distribution of FNDP-(NV) across the complete “panoramic” display (FIGS. 3A-3B) revealed a repetitive pattern prevalent in the hepatic “hexagonal” lobules at large (see FIGS. 3D, 3E, and 3F). Particle aggregates were more prevalent at the periphery of the hepatic lobule, surrounding the ‘portal triads’ (PT), yet rather scarce in regions more proximal in the vicinity of the CV. While the mechanism(s) for such distribution are currently not clear, it is hypothesized that this kind of spatial distribution of FNDP-(NV) across the hepatic lobule could be the result of several converging factors.

First, FNDP-(NV) delivery via the PVs often presented aggregated particles at sizes that could barely fit the sinusoid diameter or even exceed it. As shown in FIG. 4A, 30-40% of the detected FNDP-(NV) agglomerates were in excess of 7 μ m, making them, without wishing to be bound by theory, prone to mechanical capture at the more proximal part of the sinusoids. While this does not account for the majority of FNDP-(NV) positive regions by number, these particles account for 75-85% of the total FNDP-(NV) positive area (FIG. 4B), which may account for the strong fluorescence bias within the lobule, despite the significant number of smaller aggregates (individual or limited replicates) which could travel further down the sinusoid, transverse the sinusoids and recycle into the systemic circulation. The presence of small particles at the entry port of the sinusoid into the central vein (CV) supports this possibility (see FIGS. 3D and 3G).

Second, Kupffer cells that serve the scavenging function of the liver (the Reticulo-Endothelial System, RES) are generally abundant in the sinusoids and more so at the proximal zone of the sinusoids exiting from the PV. These macrophage-like cells

– 30 –

rapidly scavenge particles with preferential kinetics for the larger over smaller particles, which in the case of the FNDP-(NV) will augment their deposition more proximal to the PV over the CV zone, as demonstrated by the data.

Third, the terminal zone of the sinusoid/venule is generally more ‘spacious’ than the port of exit of the sinusoid from the PV. Such anatomy could support hemodynamic conditions, which facilitate clearance of particles into the CV, and further down into the systemic circulation, thereby contributing to the relative paucity of particles in vicinity of the CV.

Fourth, the venous microcirculatory system is a critical element in securing the hepatic lobule’s most delicate biochemical functions. The data described herein clearly indicate the presence of large particle aggregates in the PV and possibly CV along with enhanced presence in the outer circumference of the hepatic lobules (peri PT), and scant but notable small particles throughout the lobule (see FIG. 3E). Particles within these spaces could interfere with the delicate balance of blood flow in the sinusoids, causing hemodynamic disturbances (e.g., turbulence flow) and congestions that obstructs the flow. Disruption of flow could bear on oxygen delivery as well as distribution of nutrients to the parenchymal cells, thereby negatively affecting synthetic and catabolic functions of the liver. While micro-hemodynamic disturbances in the sinusoids cannot directly be ruled out, detailed histological analysis (Supplementary Materials) failed to observe areas of blood congestions (due to partial blood flow blockage), thrombosis (due to stasis), or ischemic consequences at a microscopic level.

Nevertheless, the topographical inhomogeneity of FNDP-(NV) distribution could still carry physiological implications by virtue of particles mass or size, intra-cellular location localization and micro-hemodynamic factors not yet matured (at the time of the study termination) to manifest aberrant consequences on the anatomy and physiology of the hepatic unit at large. The peripheral zone of the hepatic lobules, where larger aggregates of particles were most prevalent (see zone 1 in FIG. 3H), is the locale for many important and critical biochemical and cell survival functions in the liver (e.g., fatty acids oxidation, gluconeogenesis, bile production, xenobiotics metabolism and regenerative cells replenishment).

Support for the likely preservation of liver morphology at the micro-environment is presented in FIGS. 6A and 6B. The topological survey across the panoramic field of the whole liver surface suggests that percent of particles and the area that they occupy

24 Mar 2026

2026202273

– 31 –

are only a small (or moderate) fraction of the total. Since the data presented in this manuscript evaluated a situation generated 12 weeks earlier, acute post FNDP-(NV) exposure cannot be rejected.

Lastly, cell culture studies were performed to gain insights on the direct interactions of liver cells with FNDP-(NV) in an isolated system to explore the kinetic of particle internalization, compartmentalization and viability of cytokinesis capability in the presence of particles. The two different cell types, used as surrogate for the respective human hepatocytes and endothelial cells, indicated rapid initial uptake of FNDP-(NV) into the cells in time- and concentration-dependent, manner. This in vitro study supports the in vivo observations of intracellular uptake of FNDP-(NV) into non-scavenging liver cells (hepatocytes).

Summary

In this work, the interactions of FNDP-(NV)-Z-800 nm with liver cells in vitro and in vivo were studied. These studies addressed the scale and extent of FNDP-(NV) deposition in terms of their cellular and sub-cellular resolution, their presence in parenchymal and non-parenchymal cells, as well as in the micro-circulation. In vivo data were complemented by studies conducted in vitro (HUVEC, HepG-2 cells), where direct kinetic studies of particle uptake and assembly in these surrogate liver cells supported the results obtained from whole animal exposure study. Taken together, the data described above strongly suggests liver bio-compatibility of the FNDP-(NV), as no aberrant consequences could be identified in terms of preservation of cellular phenotypes, cytoskeletal, nuclear structure, as well as unabated cytokinesis and cell replication. As such, FNDP-(NV) could potentially be well tolerated by humans exposed FNDP-(NV) by intravenous route of exposure.

EXAMPLE 2

The following example describes cellular and biochemical functions in cultured Human Umbilical Endothelial cells (HUVEC) and human hepatic cancer cell line (HepG-2) exposed to FDP-NV~800 in vitro at exposure levels within the pharmacokinetics (Cmax and the nadir) reported in vivo.

Nanomedicine is a fast-growing medical discipline featuring intense pre-clinical research and emerging clinical exploratory studies as evident by over 25,000 articles listed in PubMed over the past 10 years. Nanomedicine offers a ‘third leg’ of

– 32 –

pharmaceutical technology above and beyond synthetic organic molecules and engineered biologicals. Nanomedicine builds on diverse materials co-junctional to additives that aim to direct biologically active nanoparticles to specific cells, organs, or pathological processes.

5 Of major contemporary interest are particles engineered to emit near infrared (NIR) light in response to an electromagnetic stimulus (excitation light) that generates fluorescence either due to innate properties (e.g., “Color Centers”) or coatings with organic fluorescent additives. The ability to emit in the NIR opens the possibility for imaging of bodily structures *per se* or as adjunct to state-of-the art imaging technologies
10 (e.g., MRI/magnetic resonance imaging, ultrasound) along with targeted delivery of therapeutic agents.

 Of particular interest are diamond particles, such as nanodiamond particles or fluorescent diamond particles, carrying nitrogen-vacancies (FDP-NV-) that enable the particles to become fluorescent upon excitation at 580-620 nm, resulting in near infrared
15 (NIR) emission in the peak range of 720-740 nm. The NIR light emission of such particles displays exceptional stability, negligible interference by biological elements such as water and oxyhemoglobin. Furthermore, surfaces of these particles can be functionalized with a variety of chemical groups (carboxyls, amines, etc.) that provide diverse linkages opportunities, from small organic molecules, to polymers, proteins, and
20 nucleic acids.

 It has been discovered within the context of this disclosure a bioengineered fluorescent diamond particles-NV-Z~800nm (FDP-NV) conjugated with snake venom disintegrin, bitistatin (Bit), and it has been shown (in vitro and ex vivo) that FDP-NV~800nm/Bit binds specifically to the platelet fibrinogen receptor α IIB β 3 integrin.
25 Subsequently, *in vivo* studies have demonstrated the binding of FDP-NV-Bit to acutely generated (iatrogenic) blood clots in rat carotid arteries. Taken together, FDP-NV~800nm/Bit demonstrated targeted homing in vivo and hence showed the potential to serve as a diagnostic tool for high-risk vascular blood clots.

 The studies were followed by 3 safety and biocompatibilities studies, where a
30 high dose (60 mg/Kg, delivered as a single intravenous bolus) of FDP-NV~800 nm (FDP-NV) blocked by BSA was infused to intact rats to establish the pharmacokinetic profile, organ distribution and to assess a comprehensive panel of hematologic, metabolic and biochemical safety biomarkers. In these studies, it was found that within

2026202273 24 Mar 2026

– 33 –

the 5 days to 12 weeks follow up periods, FDP-NV primarily distributed to the liver and spleen, and that virtually none were found in the lung, heart, and kidney. Furthermore, no specific histopathological observations related to the FDP-NV particles were observed. However, no study so far addressed possible acute safety or toxicological consequences in endothelial or hepatic parenchyma cells exposed to FDP-NV-800nm.

In the present example, the search for possible direct FDP-NV-800 nm related toxicological effects were studied using two different cell-types, HUVEC and HepG-2. These cells were chosen since endothelial cells are the first line of exposure to FDP-NV when infused into the systemic circulation (as per the intended clinical indication), while hepatocytes are the primary repository of circulating FDP-NV. FDP-NV exposure levels were selected according to the acute pharmacokinetic levels observed in vivo, including the maximal blood levels and its nadir at 90 minutes post-exposure. Considering that acute biocompatibility studies with FDP-NV-800 nm have yet to be reported in the published literature, the studies presented here provide new information and insights into the acute biocompatibility of FDP-NV in support of the intended clinical development in humans.

The data overall support reasonable biocompatibility of FDP-NV-800nm with respect to short term proliferation at C_{max} exposure in cultured HUVEC. HepG-2 have not been affected at the same exposure and time.

Methods

Diverse cellular and biochemical functions were monitored, which in summation provide insights on the cells' integrity and vital functions. Cell proliferation, migration, and regeneration were assessed by quantitative microscopy. Mitochondrial (oxidative) functions were tested by MTT redox reaction and cytosolic esterase activity studied by calcein AM assay. ER-stress biomarkers were examined by chaperons CHOP and BiP and apoptosis by caspase-3 activation using Western blot (WB). MAPK Erk1/2 signaling was assessed by detection of the phosphorylated form of the protein (P-Erk 1/2) and its translocation into the cell nucleus.

Results

2026202273 24 Mar 2026

– 34 –

Exposure of HUVEC cells to 100 µg/mL FDP-NV (Cmax) suggested potential adverse effects on cell proliferation, cytosolic esterase activity, and oxidative functions. Cell signaling (MAPK Erk1/2) and ER-stress biomarkers remained intact as did the activation of the pro-apoptotic pathway (caspase 3 activation). With a similar exposure and time frame, no aberrant tests have been observed in HepG-2 cells, which demonstrated resilience in some studies at some levels of exposure.

Material and Methods

Preparation of nanoparticles

Carboxyl-functionalized FDP-NV~800nm (FDP-NV) were purchased from ADAMAS Nanotechnologies (Raleigh, NC, USA). FDP-NV were sanitized by suspension in 70% ethanol for 15 min at room temperature (RT) followed by centrifugation for 7 min at 2900 x g at RT to isolate the particles. Passive blocking of potential non-specific protein binding sites on the particles was performed by incubation with PBS (phosphate buffered saline, pH=7.4, ThermoFisher Sci., Waltham, MA, USA), containing 3% BSA (bovine serum albumin, Sigma, St Louis, MO, USA) at 37°C for 1 hour. FDP-NV-BSA were isolated by centrifugation as described above and particles were stored as a stock solution in PBS at 1 mg/mL in 4°C.

Analysis of Z-average and ζ-potential of FDP-NV-800nm in different dispersants

Particles blocked with BSA (FDP-NV-BSA) or 'naïve' (FDP-NV-COOH, pre-BSA blocking), were suspended in deionized (DI) water, PBS, or culture media according to the various protocols used in the cell experiments (vide infra). HepG-2 (human liver hepatocellular carcinoma) cells were cultured in Eagle's Minimum Essential Medium (EMEM, ThermoFisher Sci.), supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Sci.) and penicillin/streptomycin (ThermoFisher Sci.), HUVEC were cultured in EGM-2MV media (Lonza, Basel, Switzerland). Particles were suspended in each culture media as dispersant at a density of 0.5 mg/mL and applied into dual-purpose capillary cuvettes (1 mL total volume). Samples were tested in a Zetasizer Ver. 7.11 (Malvern Panalytical Ltd., Malvern, UK).

Cell counting assay

Cell counting was performed. The HepG-2 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Primary HUVEC were purchased from Lonza and used for experiments between the 5th-8th passages.

– 35 –

Cells maintained (37°C at 5% CO₂ atmosphere) in their respective culture media as described above. HepG-2 and HUVEC were ‘seeded’ in 96-well plates (2 x 10³ per well in 100 µL medium) and treated or not with FDP-NV-BSA for 24 hours. In each experiment, vincristine (50 µg/mL), a cell-cycle proliferation inhibitor, was added as a positive control. At 24 hrs, the medium was removed, the cells were fixed with 4% paraformaldehyde (PFA, ThermoFisher Sci.) and the nuclei were stained using DAPI (4’,6-diamino-2-phenylindole, dihydrochloride, ThermoFisher Sci.). The plates were analyzed in a fluorescence microscope (Olympus IX81, Olympus, Tokyo, Japan) by imaging 7 observation fields for each well using 100x magnification and DAPI (blue filter) for nuclei visualization, and TRITC (red filter) for FNDP-NV-BSA visualization. The number of viable cells in each field was determined by analysis of DAPI stained nuclei using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with a digitally set-up cell counter.

Cells metabolic activity monitored by MTT assay.

The MTT assay was performed as a colorimetric assay using the Cell Proliferation Assay Kit (ThermoFisher Sci.), composed of component A (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) and component B (SDS (sodium dodecyl sulfate)) according to manufacturer’s protocol. Briefly, HepG-2 cells and HUVEC were seeded in 96-well plates at a density of 1 x 10⁴ cells per well in media described above for each cell type. Cells were treated or not with FDP-NV-BSA or vincristine (50 µg/mL) for 24 hours. Media (100 µL) were changed to phenol red-free DMEM (Dulbecco's Modified Eagle Medium) (ThermoFisher Sci.), containing MTT component A. Plates were incubated for 4 hours and cells lysed by adding equal volumes of 10% SDS (kit component B). Plates were incubated overnight and read using an ELISA plate reader ELx800 (BioTek, Winooski, VT, USA) at 562 nm wavelength.

Calcein AM cell cytosolic esterase assay

Seeding and treatment of HepG-2 cells and HUVEC were performed as described above for the MTT assay. Cells were treated with 5 µg/mL calcein AM (ThermoFisher Sci.) in serum-free media and incubated for 30 min in 37 °C. Plates were read using a fluorescence microplate reader FLx800 (BioTek) with 485 nm Excitation and 530 emission wavelengths.

“Wound healing” (WH) in vitro assay

2026202273 24 Mar 2026

– 36 –

HUVEC were seeded in 12-well plates and maintained for 1-2 days until 80-90% confluency and treated or not with FDP-NV-BSA for 24 hrs. Confluent HUVEC cells (monolayer) were subjected to a ‘gentle scrape’ across the plate using a plastic spatula tip, resulting in a gap area (devoid of cells) of approx. 1 mm width. Cells treated with FNDP-NV-BSA were stimulated for 24 hrs migration time by replacing the media to those containing 2% FBS. Control cells (non-exposed to FNDP-NV-BSA) were divided for positive stimulated by 2% FBS, and negative where stimulator of migration was minimalized to 0.1% FBS (HUVEC are sensitive for complete removal of FBS and detach from the surface). Cells were fixed with 4% PFA and stained with DAPI, as described above. Imaging of scratches was performed in a fluorescence microscope (Olympus IX81) at 20x magnification and DAPI (blue filter) for nuclei visualization and TRITC (red filter) for FNDP-NV visualization. Control plates included confluent cells subjected to the same scratch immediately before PFA exposure. The migration index was estimated by measurement of total surface area cell-free region of the images, using ImageJ software.

Cell signaling assay represented by phosphorylation of MAPK Erk1/2

HepG-2 cells and HUVEC were cultured in 6 cm diameter Petri dishes to 90% confluency and treated or not with FDP-NV-BSA (density 0.1 mg/mL), as described above. Cells were serum-starved for 24 hours and then stimulated with 2% FBS for 0, 10, and 20 minutes. Cells were lysed in ice-cold RIPA (Radioimmunoprecipitation assay) buffer (Teknova Inc., Hollister, CA, USA), containing a ‘cocktail’ of protease inhibitors (Sigma Inc.) and the “Halt” phosphatase inhibitor cocktail (ThermoFisher Sci.).

Protein lysate (20 µg) was applied on SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis) using Mini-PROTEAN precast gradient (4-20%) gels (Bio-Rad Inc., Hercules, CA, USA), and transferred into PVDF (Polyvinylidene difluoride) membranes (Sigma Inc.) using a semi-dry blotting system (Bio-Rad Inc.). The presence of phospho- and total-Erk1/2 (after membrane ‘stripping’) was detected using polyclonal antibodies (Cell Signaling Techn., Danvers, MA, USA). Visualization of the protein bands on the membrane was performed using a C-DiGit Blot Scanner (LI-COR Biosci., Lincoln, NE, USA). The intensity of the bands was quantified using UN-Scan-It software (Silk Scientific Corp., Orem, UT, USA) for calculation of the ratio of phosphor-Erk1/2 to total-Erk1/2.

– 37 –

Nuclear translocation of phospho-Erk1/2

Fractionation of cell lysates. HepG-2 cells and HUVEC were grown in 6 cm diameter dishes, treated or not with FDP-NV (0.1 mg/mL), and ‘starved’ under the same conditions as described above for MAPK Erk1/2 cell signaling. Cells were stimulated by exposure to 250 nM TPA (Tetradecanoyl phorbol acetate, Sigma Inc.) for 15 minutes. Fractionation of the cells was performed using Subcellular Protein Fractionation Kit for Cultured Cells (ThermoFisher Sci.) according to the protocol provided by the manufacturer. Cytoplasmic and nuclear extracts of fractions were analyzed by WB using phospho- and total-MAPK Erk1/2 as described above. Verification of cytoplasmic and nuclear fractions was performed by WB analysis using, an anti-Mek polyclonal antibody and an anti-HDAC1 polyclonal antibody (Cell Signaling Techn.), respectively.

Immunocytochemistry for the detection of phospho-MAPK Erk1/2 in cytoplasm and nucleus.

Immunostaining of cells cultured in 8-wells glass chamber slides was performed as described previously. Cells were treated or not with 0.1 mg/mL of FDP-NV-BSA in the presence or absence of TPA (see above). A Polyclonal anti-phospho-Erk1/2 antibody (Cell Sign. Techn.) was used in conjunction with a FITC-tagged goat anti-rabbit IgG (Vector Labs Inc., Burlingame CA, USA). Slides were analyzed in a fluorescence microscope (Olympus IX81) using 400x magnification with an oil objective and FITC (green filter) to detect phospho-MAPK Erk 1/2 and TRITC (red filter) to detect FNDP-NV.

Cell apoptosis assay (caspase-3) and Endoplasmic Reticulum (ER)-stress biomarkers

HepG-2 cells and HUVEC were treated (or not) with 0.1 mg/mL FDP-NV-BSA as described above. Treatment with vincristine (200 µg/mL) was used as a positive control for apoptosis, and with tunicamycin (25 µg/mL) as a positive control for ER-stress. A rabbit polyclonal antibody against caspase 3 (Cell Sign. Techn.), which recognizes both the cleaved and the non-cleaved protein, was used for apoptosis detection. Rabbit mAb (clone C50B12) against BiP and mouse mAb (clone L63F7) against Chop (both from Cell Sign. Techn.) were used for the detection of ER-stress. Equal loading of proteins was verified by membrane stripping and re-probing with an anti-actin mouse monoclonal antibody (Sigma Inc.).

Results

Characteristic of physical properties of FDP-NV~800 nm suspended in various media

The FDP-NV~800 nm was suspended in various dispersants known to modify particle diameters (Z-average), and surface ζ -potential. FIG. 10 presents the changes in diameters (Z-average) of FDP-NV-COOH (native particles without passive absorption of BSA) or FDP-NV-BSA suspended in DI water, PBS (pH = 7.4), or media used in each of the cell cultures. A substantial and statistically significant increase in the Z-average was observed when FDP-NV-COOH were suspended in PBS; The particle size increased from 778 nm (DI water suspension) to 1488 nm (PBS), 1215 nm (HUVEC media), and 1403 nm (HepG-2 cell media), respectively. Passive absorption of BSA on the surface of the particles (FDP-NV-BSA) neutralized the increases observed in the respective solutions. The ζ -potential was substantially impacted trending to more positive charges to isoelectric (e.g., FIG. 10B). The ζ -potential of FDP-NV~800nm-COOH dispersed in DI water was -47.9 mV, which increased to -21.9 mV when the particles were immersed in PBS, -9.9 mV for HUVEC media and -10.9 mV for HepG-2 cell media, respectively. However, unlike the impact on Z-average, passive coating of FDP-NV with BSA had minimal impact on the ζ -potential of FDP-NV-COOH. It is noteworthy that the media used for HUVEC or HepG-2 did not differ in their impact on either the Z average or the ζ -potential.

Effect of FDP-NV on HUVEC or HepG-2 cell proliferation

HepG-2 cell line was not impacted by the presence of FDP-NV-BSA (up to 0.1 mg/mL), as inferred from the increase in cell numbers over 24 hours (FIG. 11A). In contrast, HUVEC exposed to a high concentration of FDP-NV (0.1 mg/mL FDP-NV-BSA) showed a statistically significant reduction in the cell number to approximately 60%. Impact following exposure of HUVEC was not observed to a lower concentration (1/10th) of particles (FIG. 11). As expected, vincristine suppressed proliferation to 50% and 80% of controls in HepG-2 and HUVEC, respectively. Representative images of cells treated for 24 hours with 0.1 mg/mL FDP-NV-BSA confirmed particle accumulation uptake into the cells and their peri-nuclear agglomeration especially in HUVEC (FIG. 11D). Similarly, but less conspicuously, HepG-2 cells also displayed an accumulation of FDP-NV-BSA in cytoplasm and formation of a perinuclear corona (e.g.,

FIG. 11C). This observation is in accord with recently reported studies in both cells' types.²⁰

Effect of FDP-NV-BSA on the redox intensity in cultured HUVEC or HepG-2 cells.

The redox state of cultured HepG-2 cells, as assessed by MTT, was resilient to the presence of FDP-NV-BSA at some concentrations including C_{max} (0.1 mg/mL, FIG. 12A). In contrast, HUVEC demonstrated a diminished oxidative capacity at exposure levels within the C_{max} and nadir (0.01 mg/mL) of the pharmacokinetic blood levels. However, at lower tested concentration of FDP-NV-BSA, (0.001 mg/mL), MTT activity as indistinguishable from that of the untreated controls (FIG. 12B). The positive control, vincristine, decreased redox activity to ~ 25-30% of normal controls for both cell types.

Effect of FDP-NV-BSA on HUVEC or HepG-2 cell cytosolic esterase activity

The calcein AM assay provided information on non-specific esterase activities in the cytosol. FIG. 13 shows no deviation of this test in HepG-2 cells (FIG. 13A), while HUVEC (FIG. 13A) showed a ~ 30% reduction at a concentration of 0.1 mg/mL FDP-NV and no interference at the nadir level of exposure (0.01 mg/mL).

Effect of FDP-NV-BSA on HUVEC migration stimulated by FBS in a “scratch” injury model in vitro

The effects of FDP-NV-BSA on cell migration were investigated in an in vitro model of ‘wound healing’ (“scratch assay”, FIG. 14). This assay was applied only for HUVEC since the pattern of growth of HepG-2 (forming clusters of colonies) was not suitable for this test. Quantification of cell migration across an artificially generated cell-free region (area of scratch) revealed no difference between control, untreated cells and cells exposed to FDP-NV-BSA. HUVEC treated with 2% FBS migrated readily even when exposed to a high concentration of the particles (0.1 mg/mL, FIG. 14A). Interestingly, the fluorescence microscopic images revealed a visually similar particle burden of internalized FDP-NV-BSA (overlapping blue and red colors, shown in different shades of gray) in the active cells (migrating into the “scratch zone”) and in “stationary” cells located outside the scratch zone (FIG. 14B).

Effect of FDP-NV-BSA on the activation of MAPK Erk1/2 in HUVEC and HepG-2 cells

FIG. 15 shows no significant difference in the FBS-induced activation of MAPK Erk1/2 between HUVEC and HepG-2 cells exposed or not to 0.1 mg/ μ L FDP-NV-BSA

– 40 –

at two time points (10- and 20-min) post stimulation. Interestingly, HepG-2 cells reached the plateau of FBS stimulation in 10 min (FIG. 15A), whereas HUVEC reached maximal phosphorylation of MAPK Erk 1/2 in 20 min (FIG. 15B).

Translocation of proteins from cytosol to nucleus is one of the paradigms that may be affected by intense peri-nuclear accumulation of FNDP-NV. Therefore, translocation of phospho-MAPK Erk 1/2 to nucleus was tested using an applied stimulator of this process, TPA. For this, the cells were fractionated and assessed phospho- and total-MAPK Erk1/2 in the cytoplasmic and nuclear fractions by WB (FIGS. 16A-16B) and by fluorescence microscopy (FIGS. 16C-16D). HepG-2 cells (FIG. 16A) and HUVEC (FIG. 16B) showed no difference between FND-NV-BSA exposed and control (no exposure) cells in the amount of phospho/total MAPK Erk1/2 in their respective nuclei or cytoplasm. It should be noted that exposure to TPA potentiated the internalization and perinuclear accumulation of FDP-NV-BSA, which could be observed in the fluorescence microscopic images as intensive, yellow color (overlap of red and green (FIGS. 16C-16D).

Effect of FDP-NV-BSA on the induction of apoptosis and ER-stress

The internalization of FDP-NV-BSA into the cells' cytoplasm and perinuclear accumulation may suggest a possible interference in essential traffic between the nucleus and cytosol, leading to stress conditions as manifested by activation of apoptosis or ER-stress. Therefore, both HepG-2 cells and HUVEC biomarkers were evaluated for stress conditions, such as caspase 3 activation and expression chaperon proteins, CHOP and BiP, using WB (FIG. 17). Exposure to FDP-NV-BSA (at 0.1 mg/mL) did not yield activation of caspase 3 in either of the cells in contrast to vincristine (positive control, FIG. 17A). Strong perinuclear accumulation of FDP-NV appears to persist without consequences within the experimental time. The expression of two chaperon proteins, CHOP and BiP, was also not impacted by the presence of the FDP-NV. Furthermore, there was no apparent difference between HepG-2 and HUVEC (FIG. 17B). Both types of cells were sensitive to tunicamycin, which served as standard control for ER-stress protein activation.

Discussion

The present set of experiments were aimed at exploring the safety of FDP-NV (800 nm) and constitute part of the pre-clinical evaluation of these particles, before we

– 41 –

commence Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) for phase I clinical studies. The safety and tolerability of FDP-NV-800 nm administered intravenously at a relatively high dose to intact rats, high biocompatibility was seen as inferred from the lack of morbidity and mortality monitored over 5 days, 14 days or 12 weeks post exposure. No aberrant hematological and biochemical functions, including blood cells number and differential, or histopathological observations in liver, kidney and lung were detected in all of these studies. Furthermore, the pharmacokinetics ranges following acute infusion of a high dose of FDP-NV-800 nm, indicated rapid clearance from the systemic circulation and fast up-load into the liver and spleen. It is important to note that particles deposited in the liver and spleen were retained over the 12 weeks follow-up.

The present experiments were designed to address potential adverse effects of FDP-NV-800 nm in an acute in vitro setting to gain deeper insights into potential biochemical consequences that could not be discerned by histological and histochemical biomarkers in vivo. Such studies are justified since no record of public data can be found, investigating the same FDP-NV size (~800 nm). Moreover, acute safety biomarkers might not display in the subacute or chronic dosing studies due to compensatory mechanism following exposure.

Adverse interactions of nanodiamond particles with cellular functions have already been reported albeit using different particle sizes, shapes, and adjuvants. These reports stress the importance of probing the effects of FDP-NV~800 nm on cellular functions, especially of cells that will be exposed to the maximum blood levels (C_{max}) during infusion of the particles and shortly thereafter. Naturally, endothelial cells and circulating blood cells are the prime targets for acute, high dose exposure and as are liver cells, which serve as an instant repository of the FDP-NV-800nm. Indeed, pilot studies with FDP-NV~800nm using HUVEC and HepG-2 cells revealed uptake of particles into each of these cells' cytoplasm (over 1-2 hrs.) with ultimate peri-nuclear zone assembly to form "coronation" over 20-24 h post-exposure. However, cytogenesis and cell division of these target cells were preserved, suggesting that cell cycle and trafficking in and out of the nucleus remained intact.

The present studies extend observations to probe additional key cellular functions and biochemical processes, including cell proliferation, migration, and signal transduction ER-stress, and apoptosis that are cardinal for cell integrity. The present

2026202273 24 Mar 2026

– 42 –

studies followed pharmacokinetic data obtained after high dose (60mg/Kg) infusion in the in vivo (rat) experiments. In the studies described in this manuscript, cultured cells were exposed to C_{max} levels (immediate post-infusion, 0.1mg/mL) or nadir (0.01 ug/mL, 90 minutes post infusion) over 24 hrs.

5 A well-known issue concerning size changes of FDP-NV-COOH upon suspension in solutions containing electrolytes, proteins and various organic additives was addressed. Indeed, monitoring the Z-average and ζ -potential of FDP-NV~800nm-COOH in DI water (the native product provided by the manufacturer) revealed close similarity with the manufacturer's information (778 nm for Z-average) and -48mV for ζ -
10 potential. The marked shifts in Z-average generated by dispersing the particles in PBS or culture medium were abrogated in the presence of 3% BSA, yet had only a mild (or negligible) impact on ζ -potential shifts (FIGS. 10A-10B). The possible impact of persistent ζ -potential changes on the experimental outcomes remains to be explored in detail.

15 The cellular effects of nanodiamond particles (NDP) have intensively been investigated in vitro with a variety of cultured cell types, mainly in terms of cell viability, as reported by the MTT assay. In general, NDP are well tolerated by most cell types, when incubated in complete media. The mitochondria-dependent respiratory chain is not affected by NDP even at extremely relatively high concentrations, 1 mg/mL.
20 Capping exposure at the C_{max} concentration of 0.1 mg/mL, suggested no interference in the redox state of the HepG-2 cell line (FIG. 11), in line with prior reports on other cancer cell lines. By contrast, a significant inhibitory effect was noted in HUVEC (FIG. 11B), in line with previous data using the immortalized HUVEC-ST cell line. Direct cell counting and the calcein AM assay also suggested interference of the particles with
25 cytosolic esterase activity in HUVEC at 0.1 mg/mL, although, in contrast to MTT, no effect was observed for lower (nadir) levels of 0.01 mg/mL. These data suggest that primary cells, in contrast to a cell line, may be more sensitive to FDP-NV in terms of vital biochemical processes and overall cell functions.

30 The pro-proliferative cell signaling pathway MAPK Erk 1/2 was not affected by exposure to FDP-NV at the C_{max} dose in either cell type (FIG. 15). The extensive perinuclear accumulation of particles suggested a potential interference by this "coronation" in the cytosol-nucleus cross trafficking. This possibility was probed by tracking the

2026202273 24 Mar 2026

– 43 –

translocation of phospho Erk 1/2 into the nucleus. FIG. 16 indicates the presence of P~phospho-Erk 1/2 in the nucleus following activation of this signaling pathway by the strong agonist TPA. Likewise, FDP-NV did not activate central pathway of apoptosis (Caspase-3) in either FDP-NV concentration (FIG. 17). Taken together, the data suggest

5 that the physical presence of the FDP-NV-BSA appears to subject HUVEC to some stress conditions at the Cmax, but not at the nadir exposure level. However, the ability of HUVEC to fully migrate (see FIG. 14) even at the highest FDP-NV exposure, suggesting disparities of functional sensitivity to the intra-cellular particle load.

HepG-2 cells generally appear to be resilient across some tests as compared to the

10 HUVEC. In some cases, adverse effects of NDP on HepG-2 cell migration at the same exposure levels used in the HUVEC ‘wound healing’ model (“scratch assay”) were observed. For example, exposure of HepG-2 cells to 50-100 µg/mL FND resulted in 25-50% inhibition of migration, which was further inhibited (90%) at 200 µg/mL over the same time frame (24hrs). It is of interest to note that these exposure levels did not

15 interfere with HepG-2 cell proliferation in line with data reported herein (FIG. 10). Differences between the two sets of data could represent variances in particle size (100nm vs 800 nm) and physical properties of non-functionalized NDP of some existing systems vs. carboxy-functionalized FDP-NV used in the present disclosure.

In vitro studies showed either cell type was exposed to the particles over 24 hrs.;

20 however, the pharmacokinetic data of some existing systems indicate that in vivo endothelial cells are exposed to Cmax levels of FDP-NV for no more than 15-30 minutes, as the fast clearance into the liver depletes blood levels to <10µg/mL within 90 min after infusion of the particles. In this light, this indicated that FNDP-NV~800 nm are observed in the cytosol of hepatocytes within 1-2 hrs. post cell exposure. However,

25 within the relevant in vivo short exposure time the intracellular levels of particles are several folds lower compared to the prolonged (24hrs) in vitro fixed FDP-NV concentration. The resiliency of the HepG-2 line across all conditions of stress lends credibility to our in vivo observation of hepatocytes health following high dose FDP-NV-800nm infusion to rats over 12 weeks follow up.

30 Taken together FDP-NV-800 nm had no adverse effect when infused in vivo to intact rats 18-20, nor were there any adverse consequences in cultured HepG-2 cells line across the 7 ‘stress tests’ these cells were subjected in vitro. In some cases, aberrant consequences related to immune-inflammatory cells or other cells/organs especially

2026202273 24 Mar 2026

– 44 –

those with a high capacity phagocytosis or priming effects that could exacerbate underlining pathological conditions could not be excluded. The results obtained in this study indicate that further development of FDP-NV~800 nm for in vivo imaging, and as vehicle for the delivery of drugs and therapeutics may be warranted.

Summary

The present example demonstrates the biocompatibility of FDP-NV~800 nm with respect to endothelial (HUVEC) and hepatic (HepG-2) cells in vitro. This study appears distinct from existing systems in that it probes biocompatibility within the realm of the pharmacokinetics of the particles in vivo (in a rat model). It can be concluded that HUVEC are more sensitive than HepG-2 cells to FDP-NV~800 nm accumulation; this observation has not been described for any negative response at top exposure level (Cmax). Considering the mild to moderate interferences in certain biochemical functions in HUVEC and in light of the pharmacokinetics profile the particles display in vivo, it is plausible to predict limited aberrant consequences to the endothelium. The resilience of HepG-2 cells in each and all of the biochemical tests under the top dose of FDP-NV~800 nm supports in vivo data on normal liver function in spite of the prolonged retention of the particles in this organ. Overall, the results obtained in this example indicate FDP-NV-800 nm may be useful for in vivo imaging, and/or as vehicle for the delivery of drugs and therapeutics.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are

– 45 –

presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, and/or methods, if such features, systems, articles, materials, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to “A and/or B,” when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one

2026202273 24 Mar 2026

– 46 –

element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

Some embodiments may be embodied as a method, of which various examples have been described. The acts performed as part of the methods may be ordered in any suitable way. Accordingly, embodiments may be constructed in which acts are performed in an order different than illustrated, which may include different (e.g., more or less) acts than those that are described, and/or that may involve performing some acts simultaneously, even though the acts are shown as being performed sequentially in the embodiments specifically described above.

Use of ordinal terms such as “first,” “second,” “third,” etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

2026202273 24 Mar 2026

– 47 –

CLAIMS

What is claimed is:

1. An article configured for administration of a therapeutic agent, comprising:
5 a plurality of fluorescent diamond particles; and
the therapeutic agent bound to at least a portion of the fluorescent diamond
particles,
wherein the article is configured for prolonged residence internal to an organ of a
subject.
10
2. An article for use with a subject, comprising:
an injection component configured to administer a composition to the subject;
and
a reservoir associated with the injection component containing the composition,
15 the composition comprising a plurality of fluorescent diamond particles.
3. An article as in any preceding claim, wherein the plurality of diamond particles
have an average particle diameter of greater than or equal to 100 nm and less than or
equal to 2000 nm.
20
4. An article as in any preceding claim, wherein the plurality of diamond particles
are configured to aggregate within the organ of the subject.
5. An article as in any preceding claim, wherein the plurality of diamond particles
25 are configured to be captured by the organ of the subject.
6. An article as in any preceding claim, wherein an aggregate of diamond particles
has an average diameter of greater than or equal to 1 micron and less than or equal to 100
microns.
30
7. An article as in any preceding claim, wherein the plurality of diamond particles
have a characteristic near infrared emission.

24 Mar 2026
2026202273

– 48 –

8. An article as in any preceding claim, wherein the organ is the liver of the subject.

9. An article as in any preceding claim, wherein prolong residence is greater than or equal to 5 days.

10. A pharmaceutical composition, comprising:
an intravenous carrier fluid; and
a plurality of fluorescent diamond particles suspended in the intravenous carrier fluid.

11. A pharmaceutical composition as in claim 10, comprising a therapeutic agent bound to at least a portion of the fluorescent diamond particles.

12. A pharmaceutical composition as in claim 10, comprising a diagnostic agent bound to at least a portion of the fluorescent diamond particles.

13. A pharmaceutical composition as in any preceding claim, wherein the plurality of diamond particles have an average particle diameter of greater than or equal to 100 nm and less than or equal to 2000 nm.

14. A pharmaceutical composition as in any preceding claim, wherein the plurality of diamond particles are configured to aggregate within the organ of the subject.

15. A pharmaceutical composition as in any preceding claim, wherein the plurality of diamond particles are configured to be captured by the organ of the subject.

16. A pharmaceutical composition as in any preceding claim, wherein an aggregate of diamond particles has an average diameter of greater than or equal to 1 micron and less than or equal to 10000 microns.

17. A pharmaceutical composition as in any preceding claim, wherein the plurality of diamond particles have a characteristic near infrared emission.

2026202273 24 Mar 2026

– 49 –

18. A pharmaceutical composition as in any preceding claim, wherein the organ is the liver of the subject.

19. A pharmaceutical composition as in any preceding claim, wherein prolong residence is greater than or equal to 5 days.

20. A pharmaceutical composition as in any preceding claim, wherein the prolong residence time up to at least 12 weeks.

21. A method of treating a disease, comprising:
administering intravenously, to a subject, a plurality of diamond particles and a therapeutic agent bound to at least a portion of the diamond particles, wherein the plurality of diamond particles is configured for prolonged residence internal to an organ of a subject.

22. A method as in claim 21, wherein the plurality of diamond particles are present in the liver of the subject.

23. A method of monitoring disease progression in a subject suspected of having a disease, comprising:

administering to the subject a plurality of diamond particles;

after the step of administering, obtaining a first image of a location internal to the subject suspected of containing the plurality of diamond particles;

obtaining, after a predetermined period of time, a second image of the location internal to the subject suspected of containing the plurality of diamond particles;

measuring a morphological change of the location internal to the subject, between the first image and the second image, relative to the plurality of diamond particles, wherein the morphological change is associated with progression of the disease.

24. A method as in claim 23, wherein the morphological change is determined by measuring the increase in area of an internal organ of the subject relative to the portion of the internal organ containing the plurality of diamond particles in the second image versus the first image.

2026202273 24 Mar 2026

– 50 –

25. Use of a plurality of fluorescent diamond particles in the manufacture of a medicament for the treatment of liver disease and/or liver cancer.

26. A use as in claim 25, comprising a therapeutic agent bound to the plurality of
5 fluorescent diamond particles.

27. Use of a plurality of fluorescent diamond particles in the manufacture of a medicament for monitoring of disease progression.

2026202273 24 Mar 2026

2026202273 24 Mar 2026

1/24

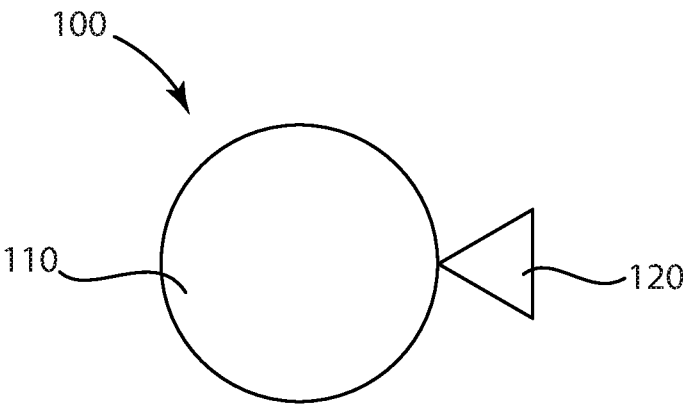


FIG. 1A

Design of cell based FNDP-(NV) uptake protocol

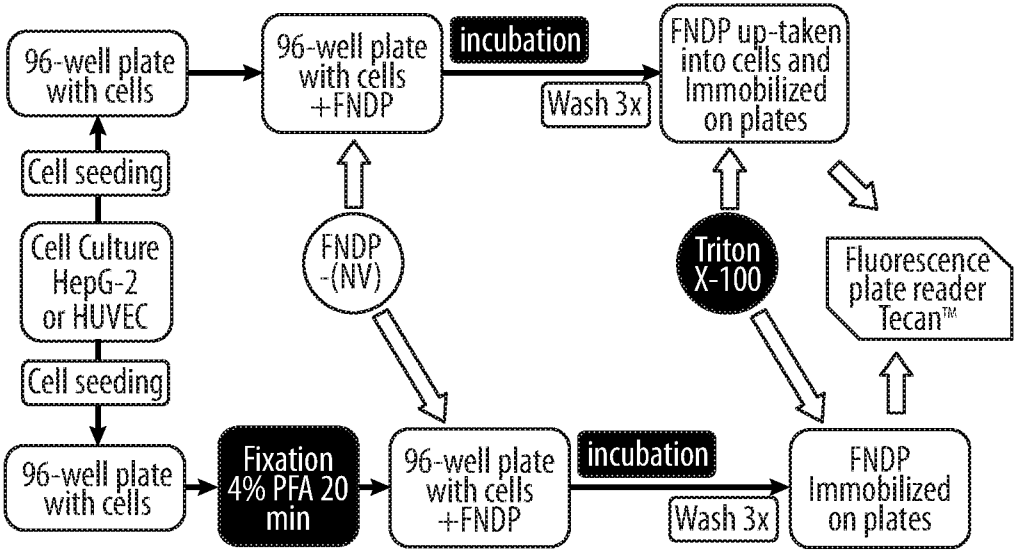


FIG. 1B

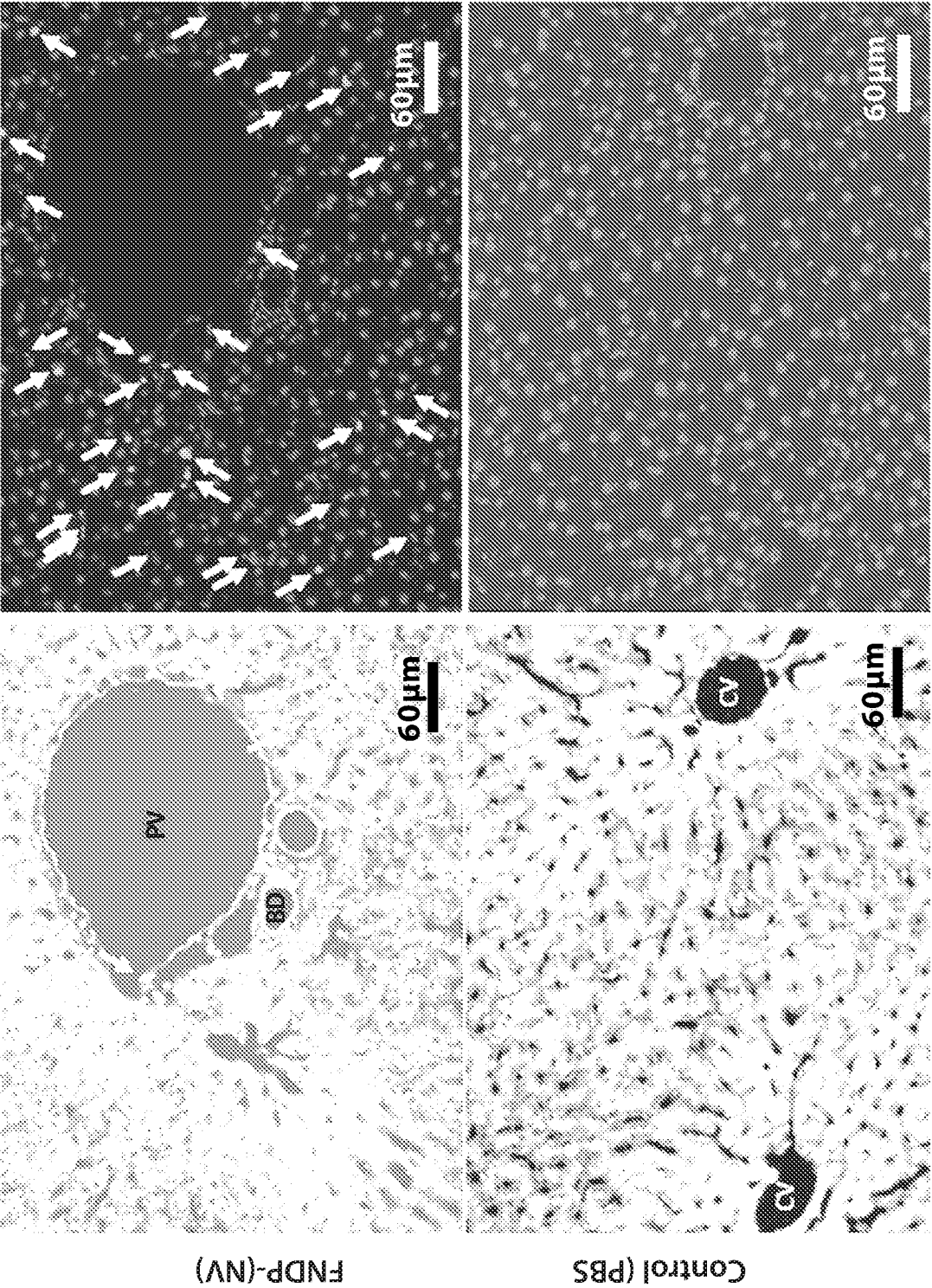


FIG. 2A

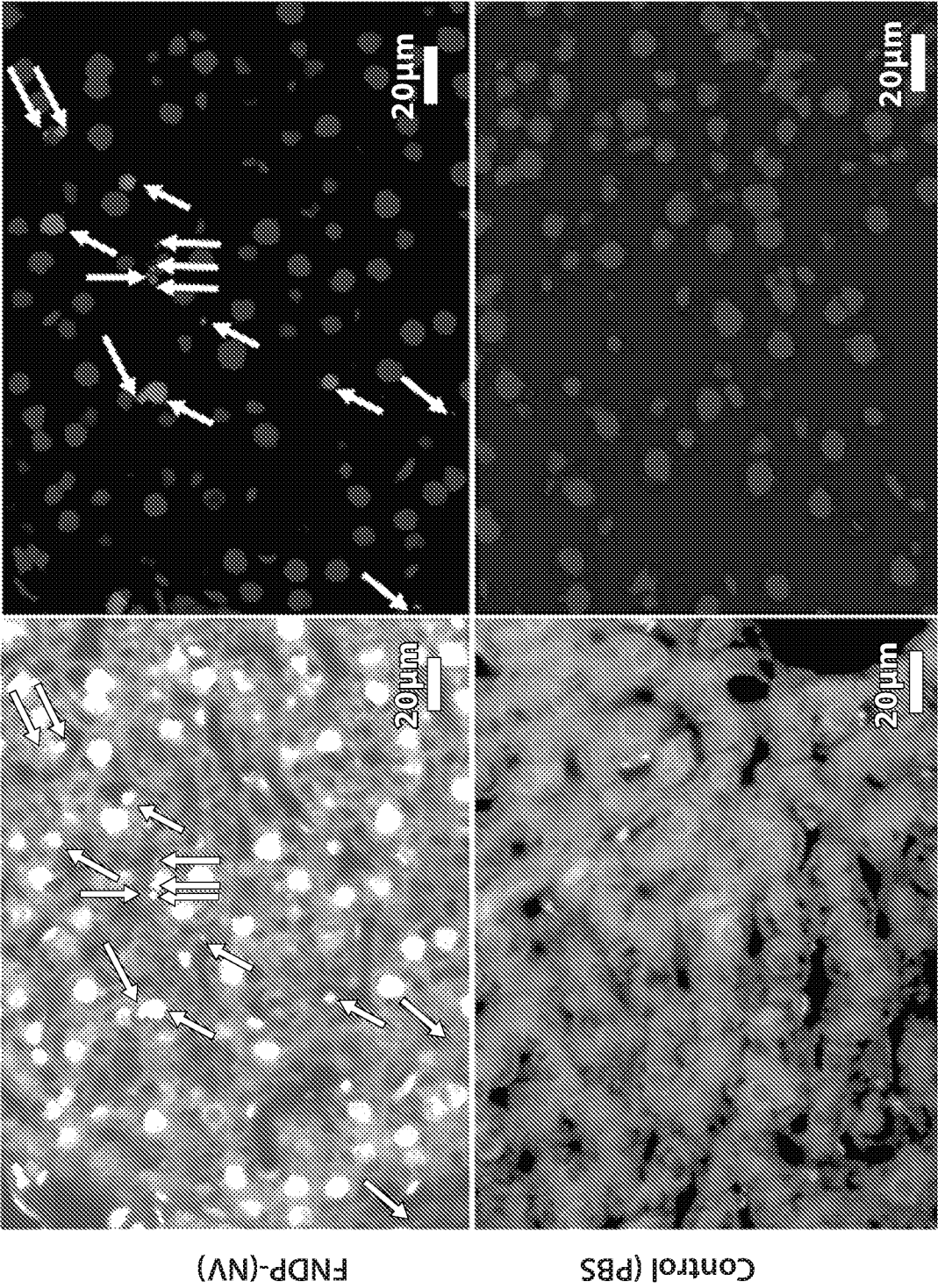


FIG. 2B

4/24

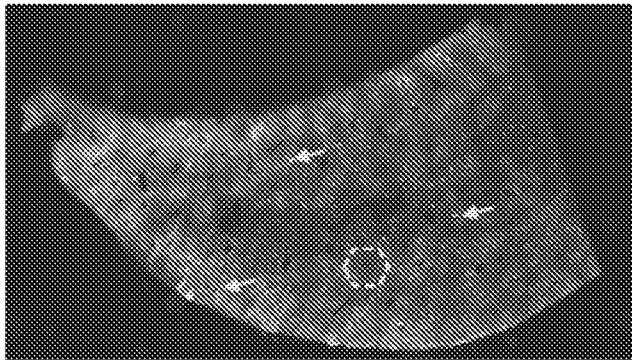


FIG. 3A

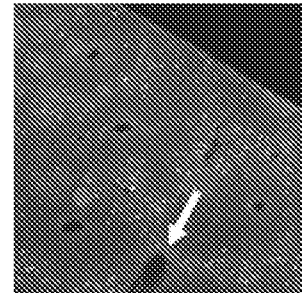


FIG. 3C

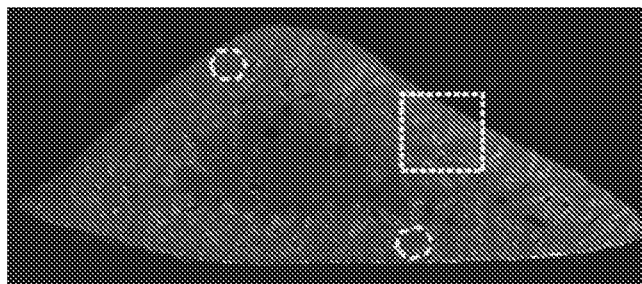


FIG. 3B

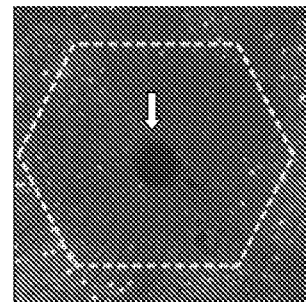


FIG. 3D

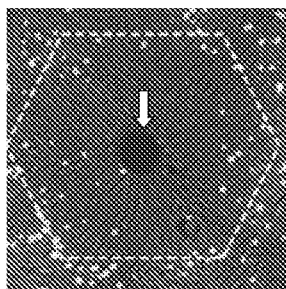


FIG. 3E

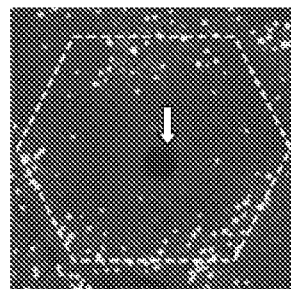


FIG. 3F

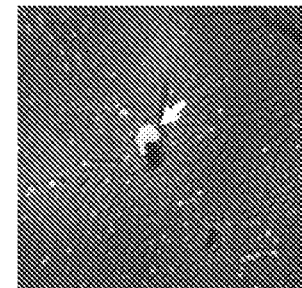


FIG. 3G

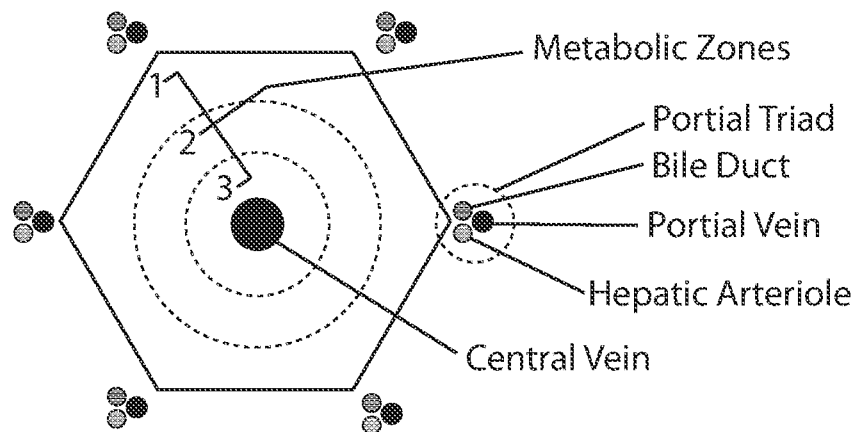


FIG. 3H

2026202273 24 Mar 2026

5/24

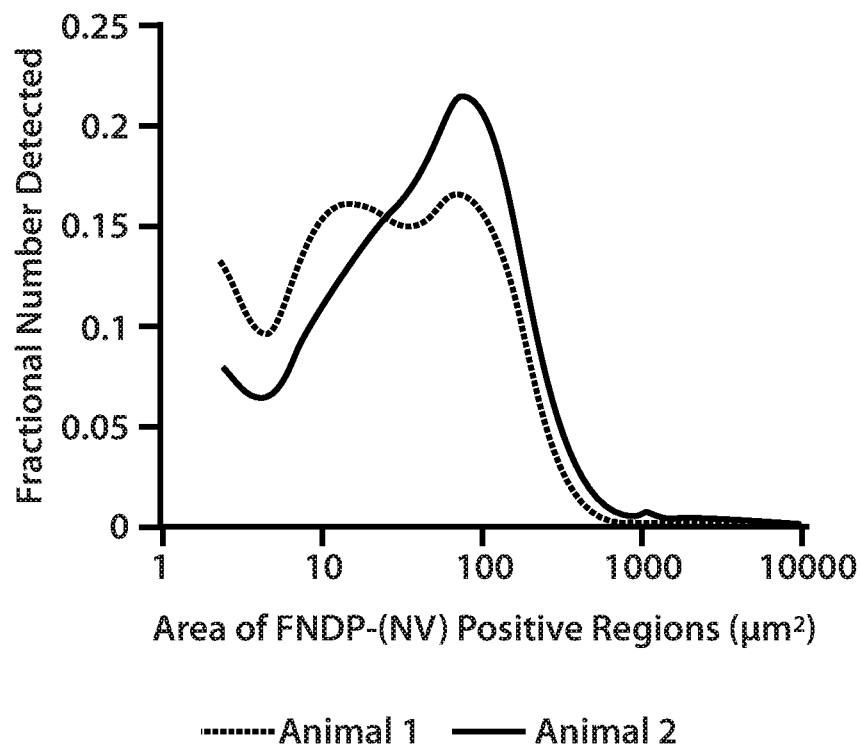


FIG. 4A

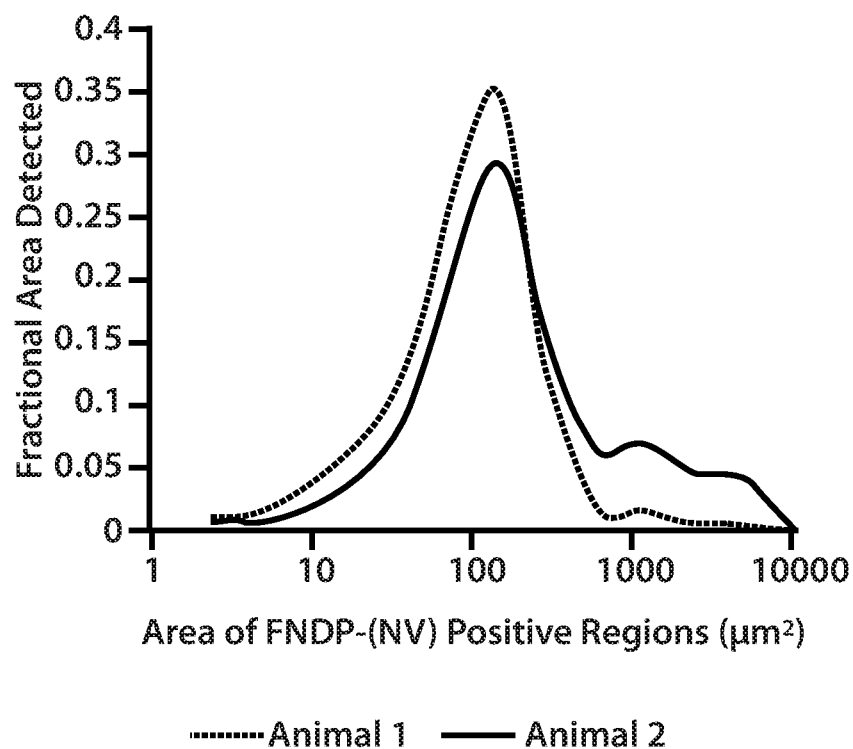


FIG. 4B

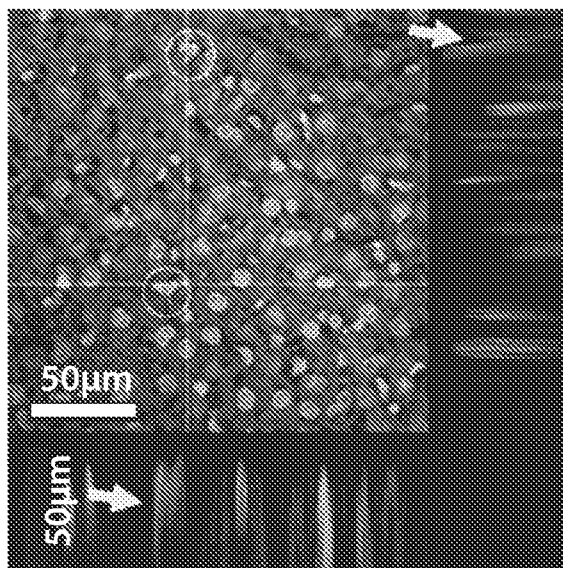


FIG. 5A

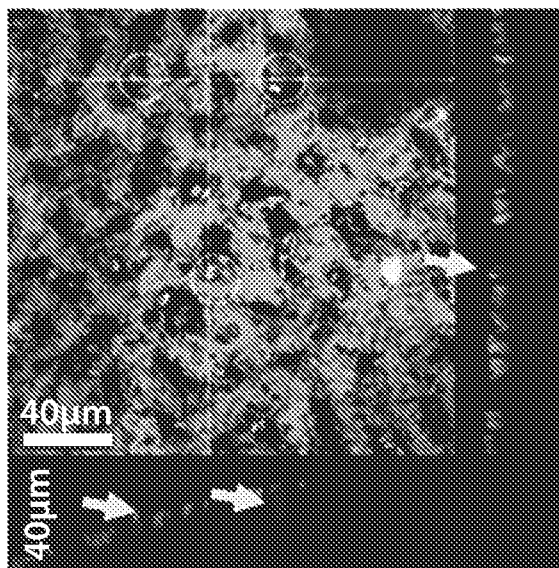


FIG. 5B

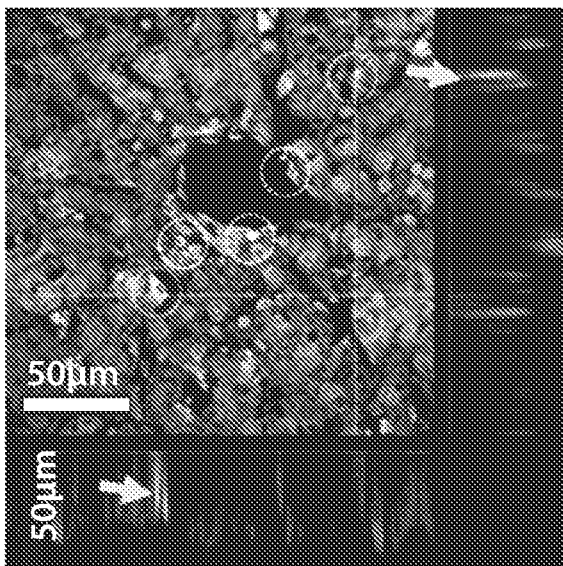


FIG. 5C

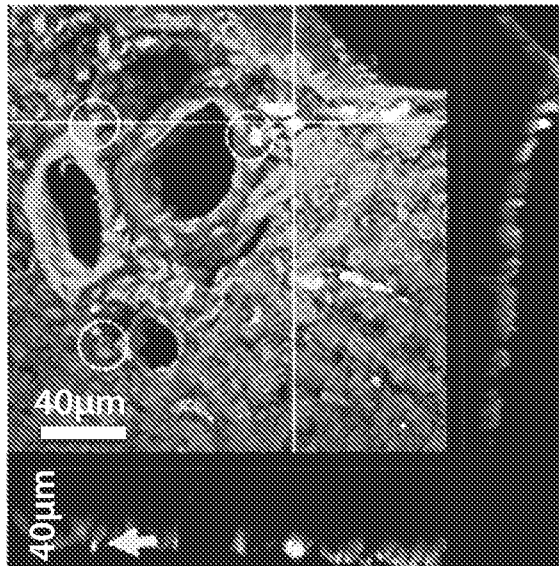


FIG. 5D

7/24

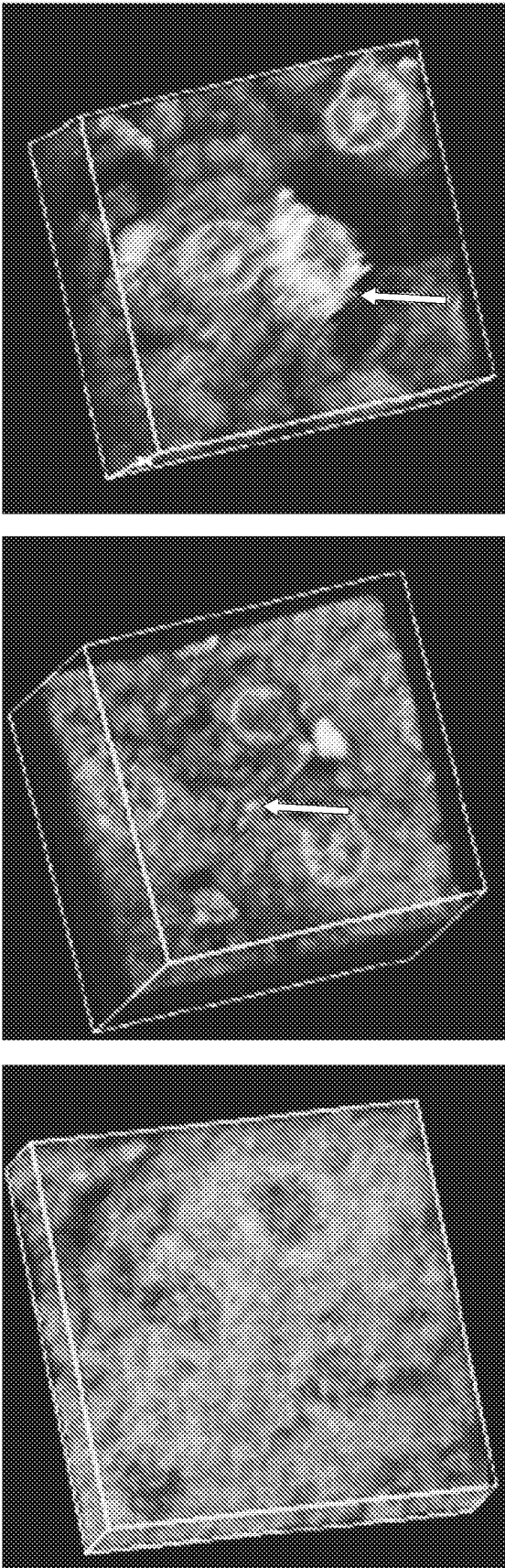


FIG. 6

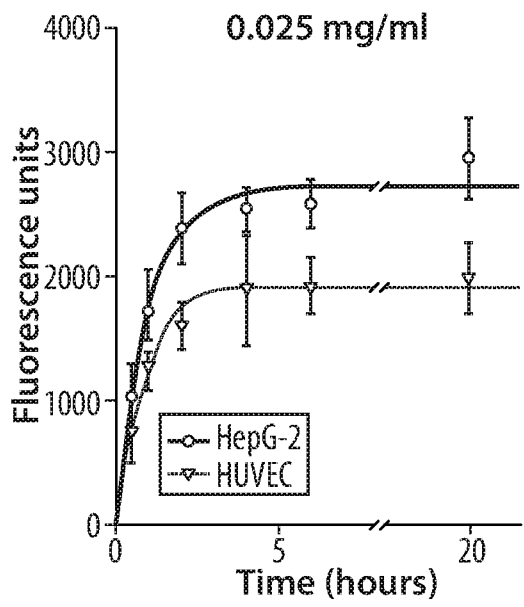


FIG. 7A

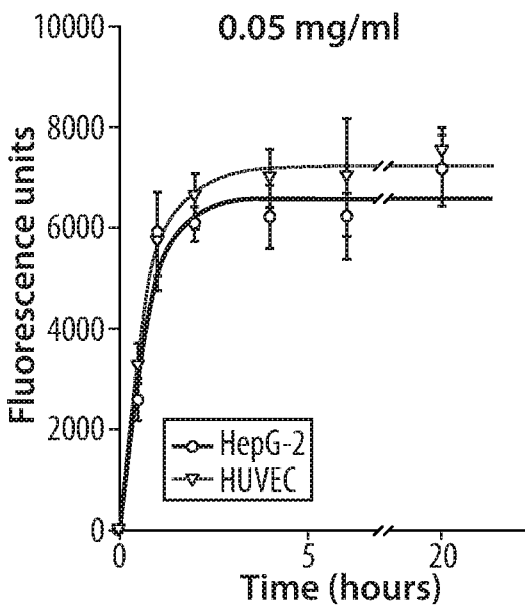


FIG. 7B

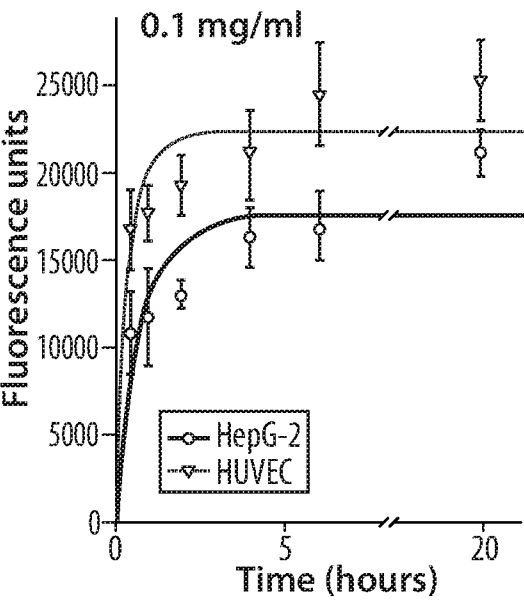


FIG. 7C

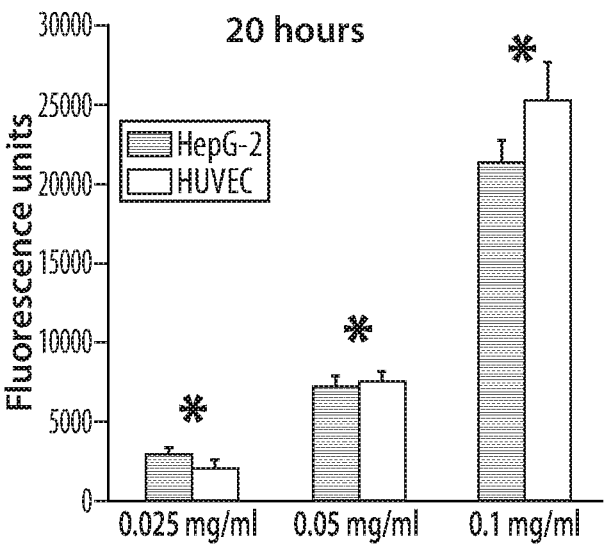


FIG. 7D

9/24

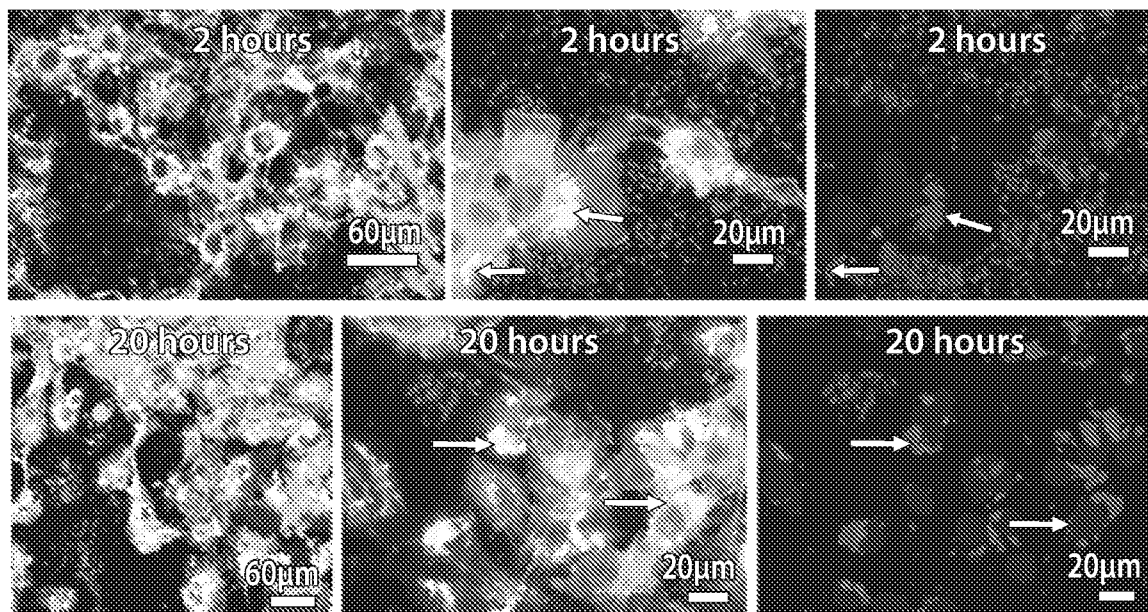


FIG. 8A

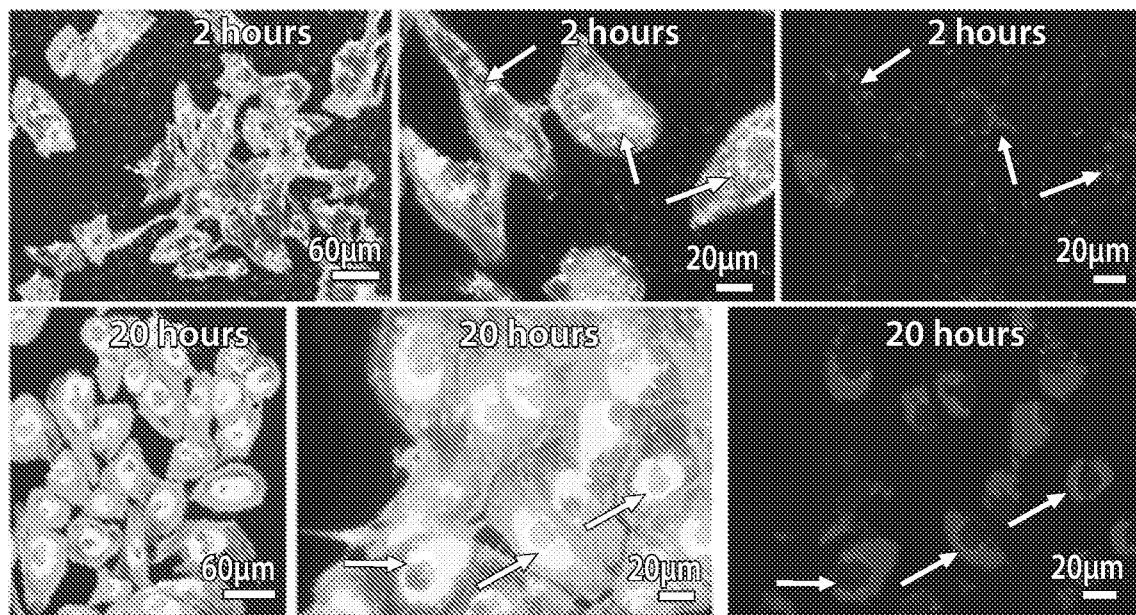


FIG. 8B

10/24

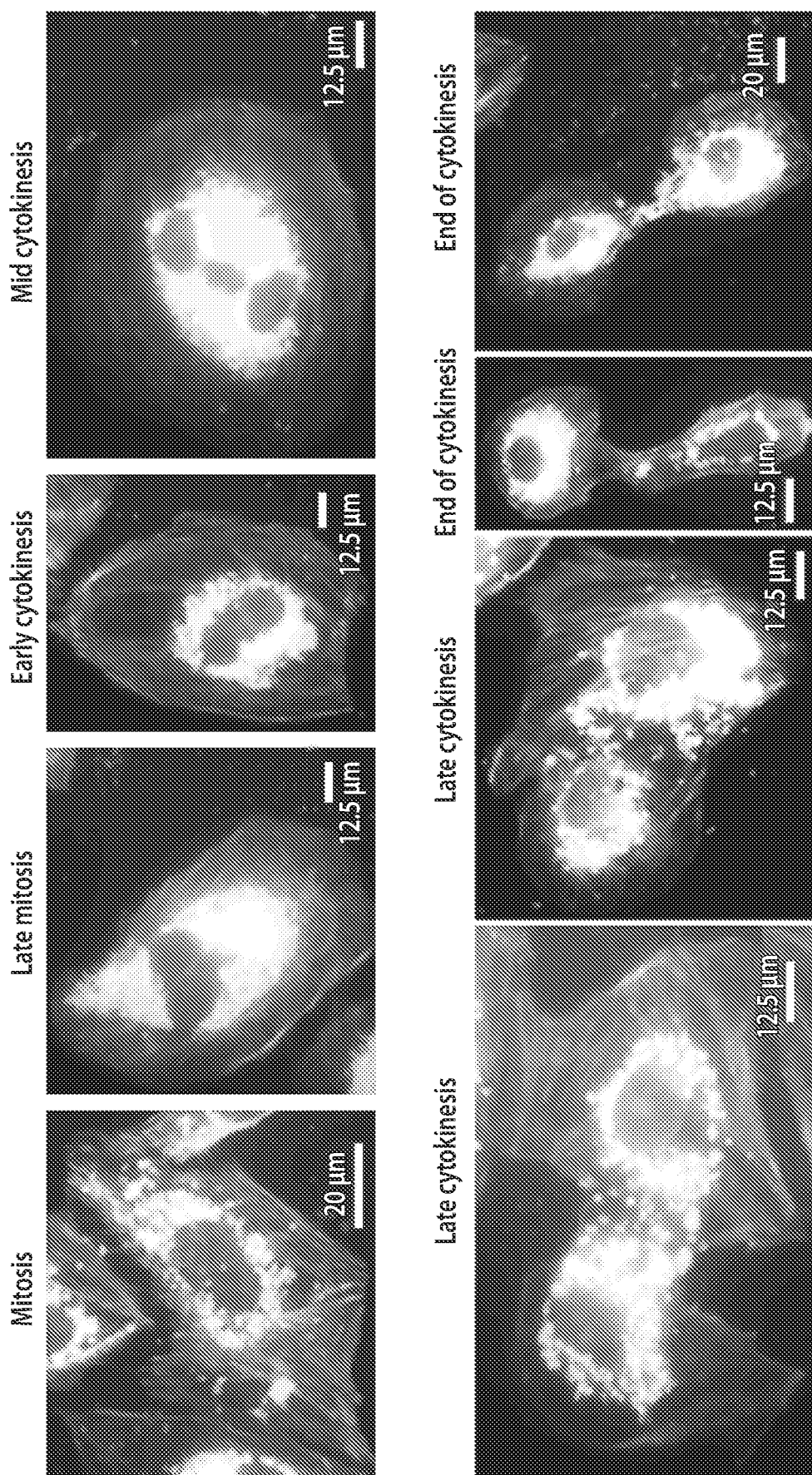


FIG. 9

2026202273 24 Mar 2026

11/24

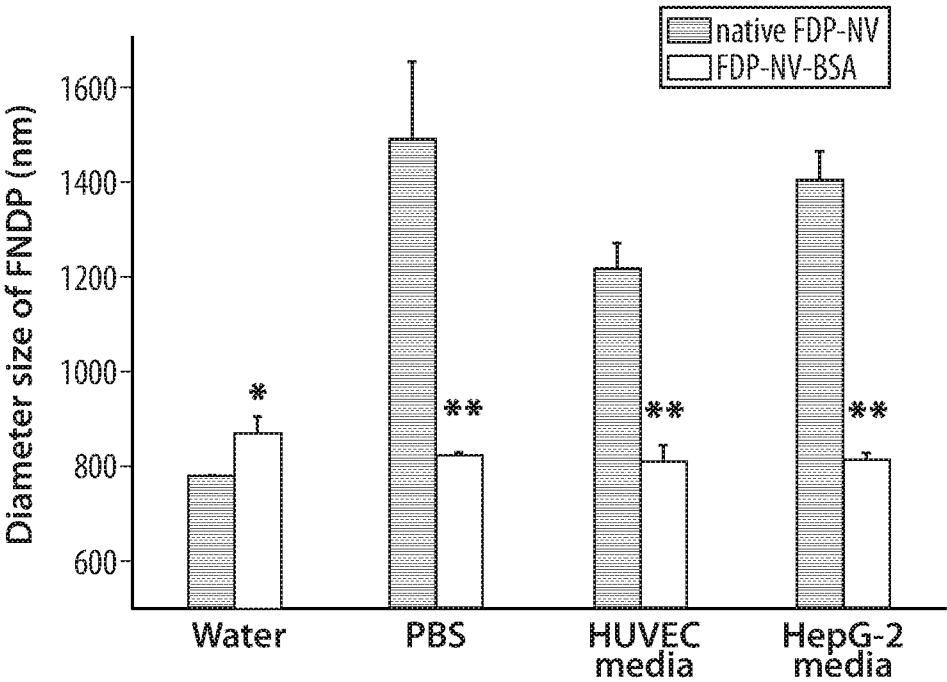


FIG. 10A

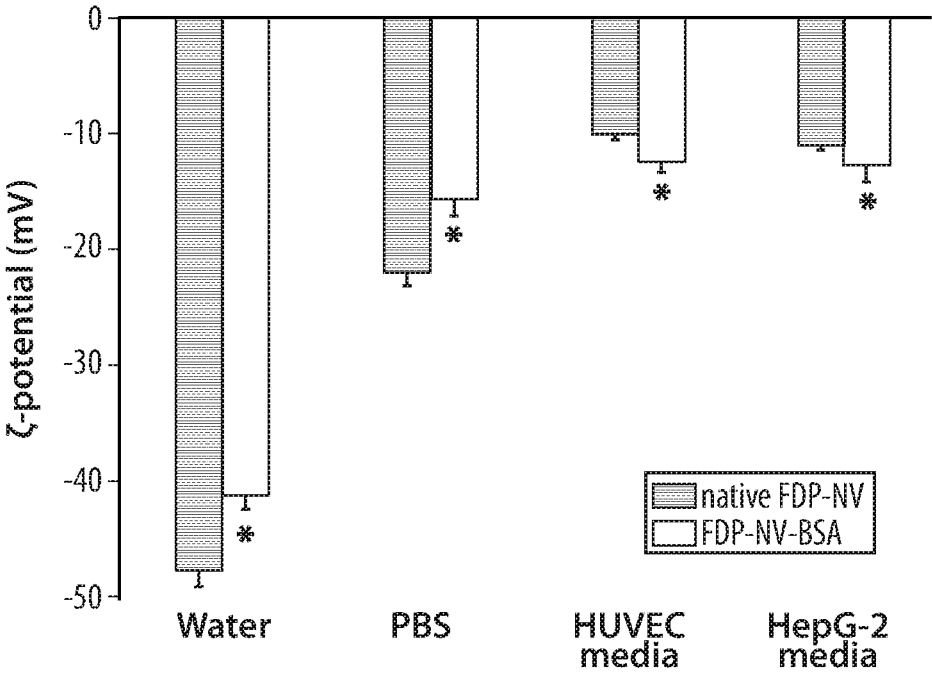


FIG. 10B

2026202273 24 Mar 2026

12/24

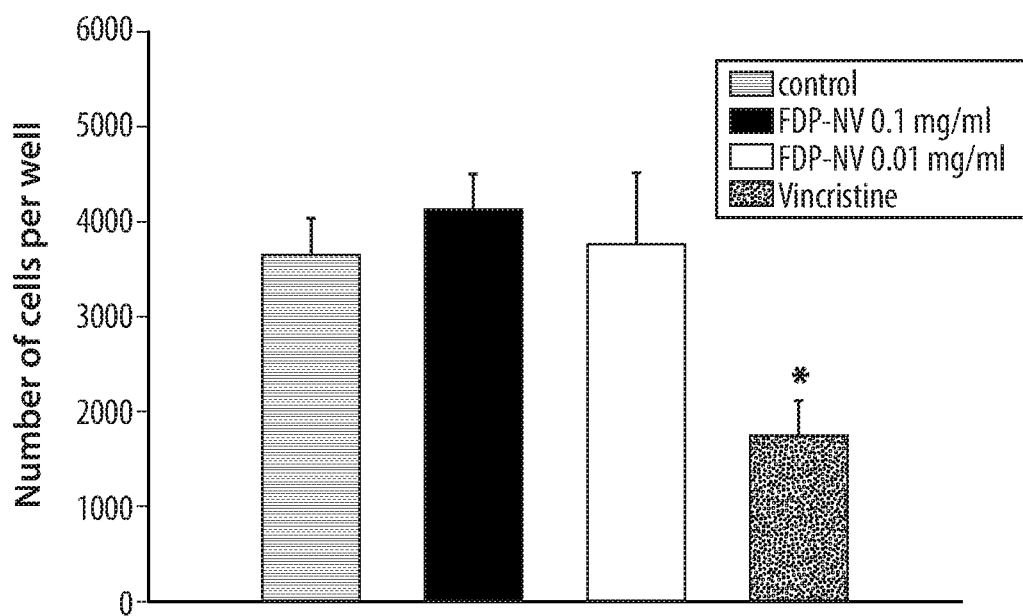


FIG. 11A

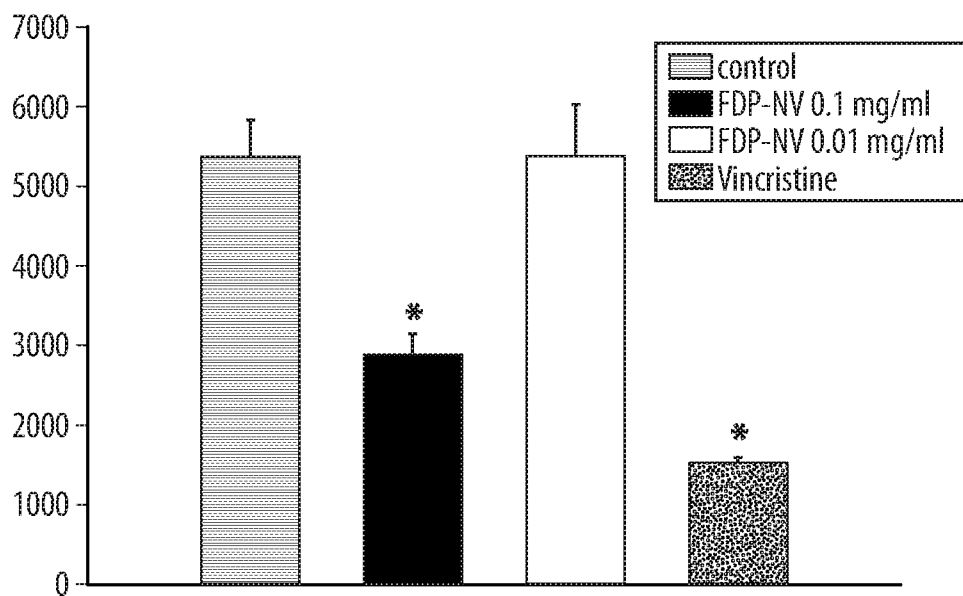


FIG. 11B

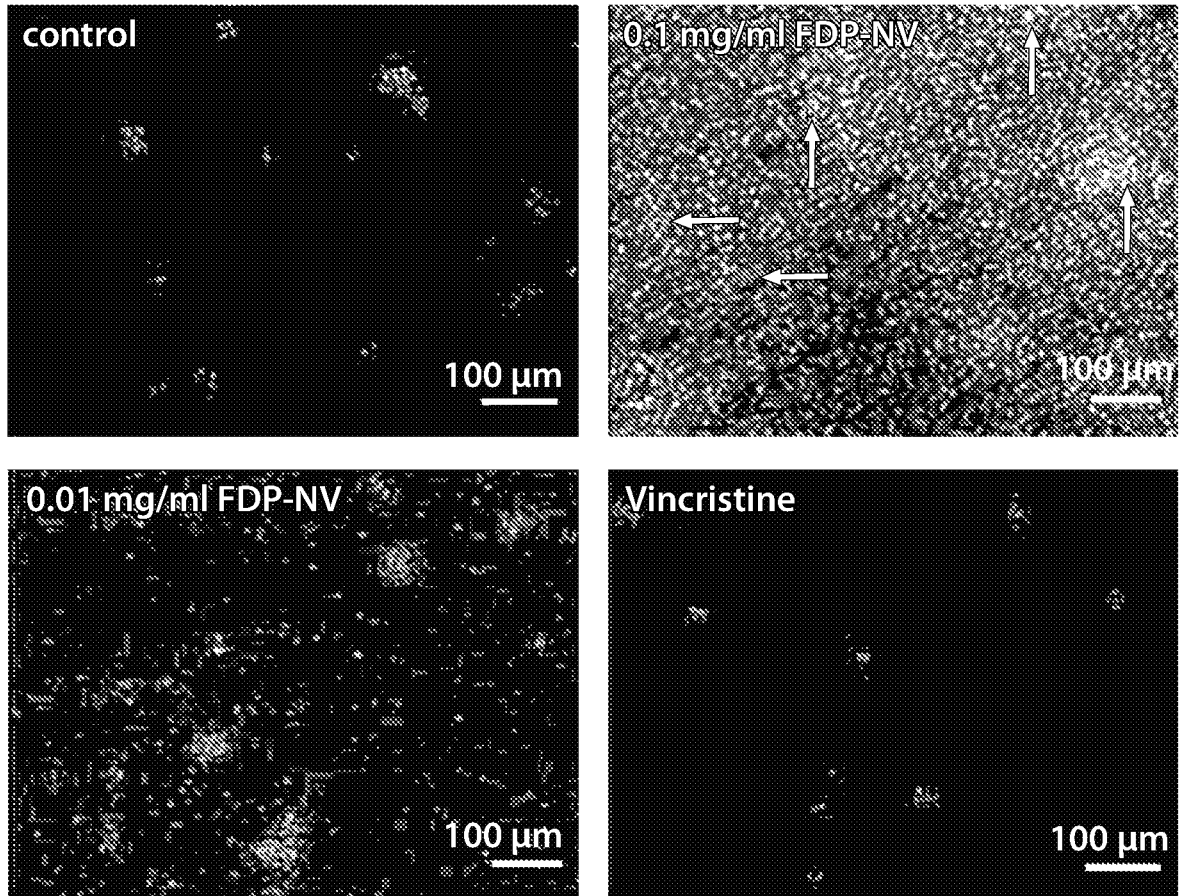


FIG. 11C

14/24

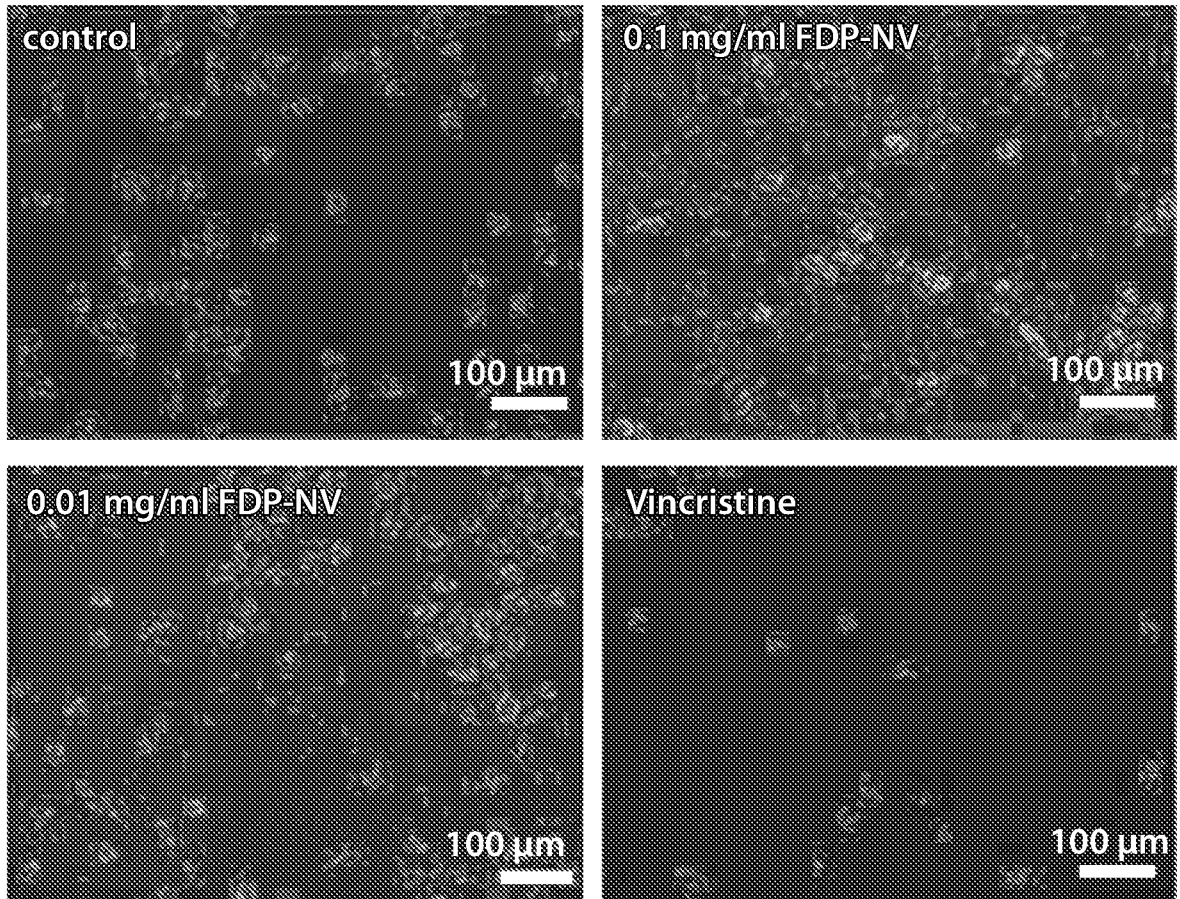


FIG. 11D

2026202273 24 Mar 2026

15/24

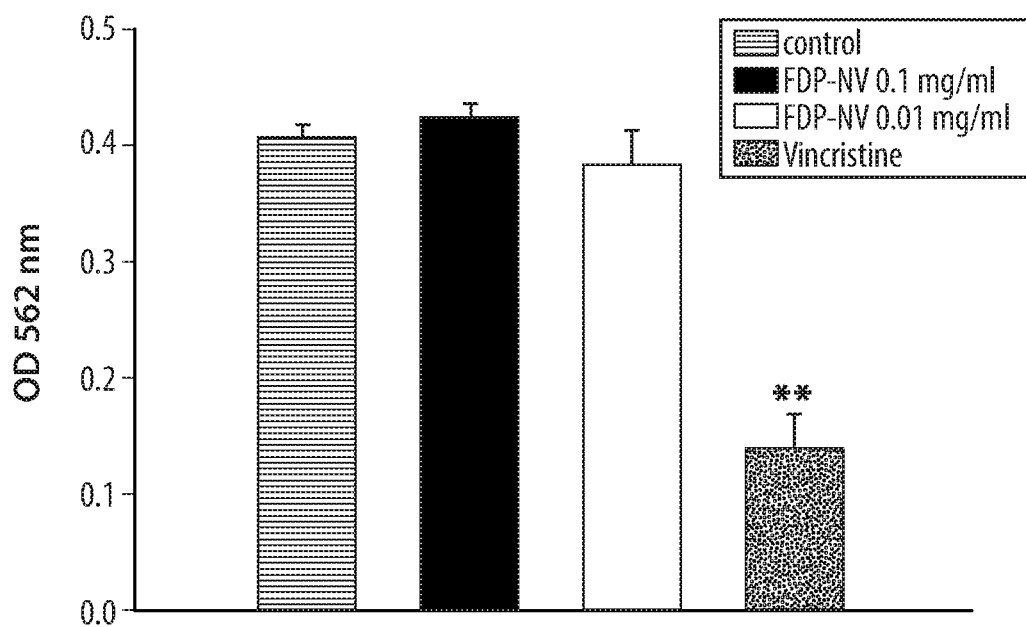


FIG. 12A

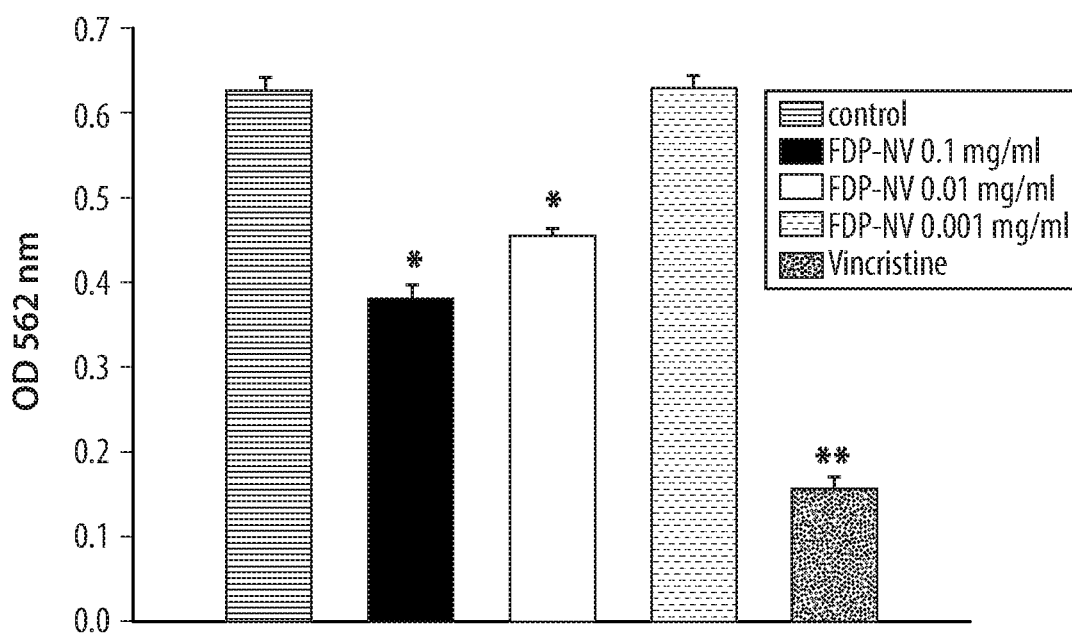


FIG. 12B

2026202273 24 Mar 2026

16/24

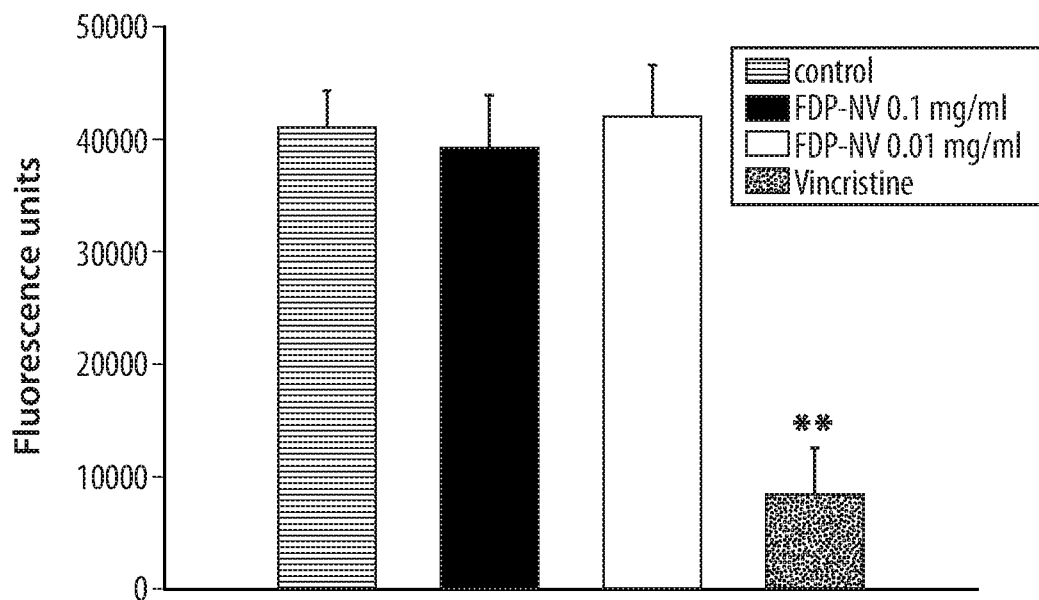


FIG. 13A

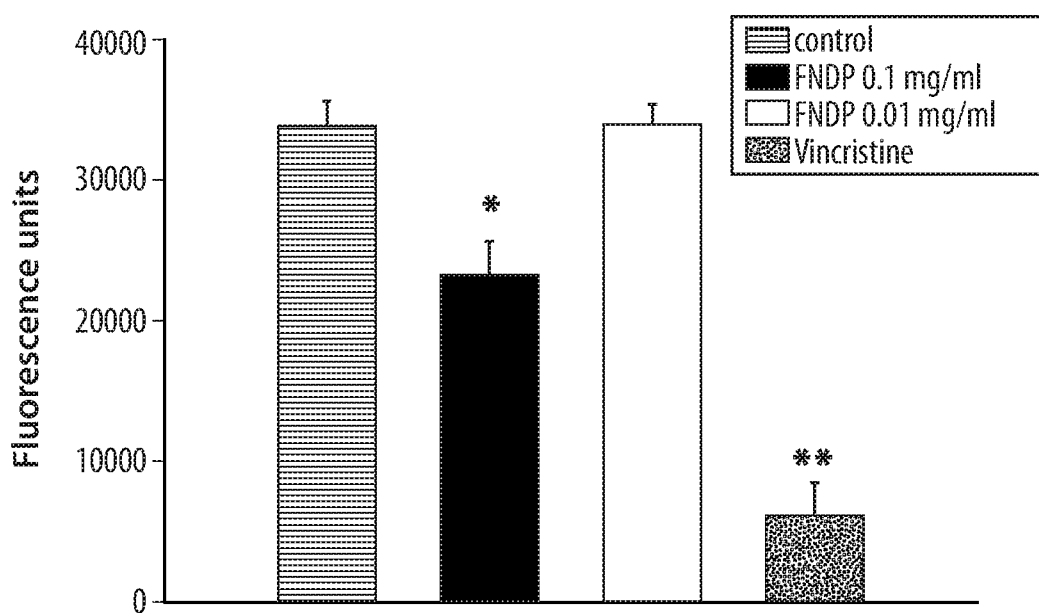


FIG. 13B

17/24

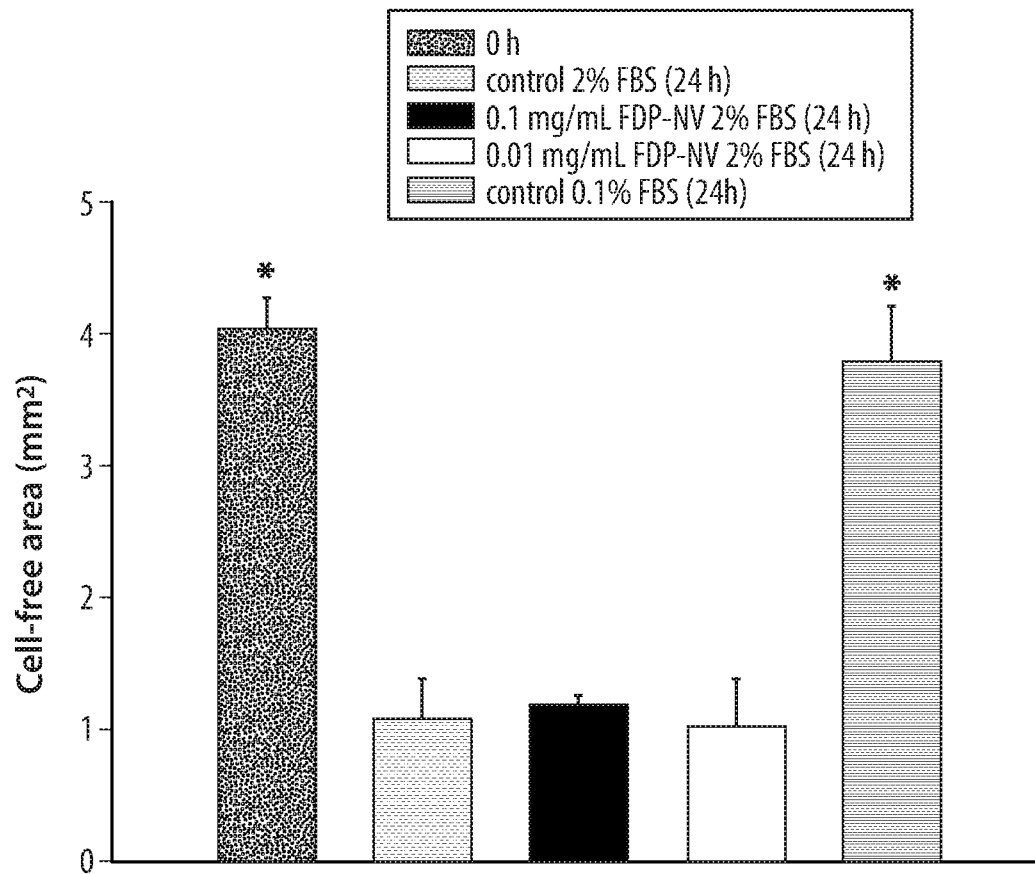


FIG. 14A

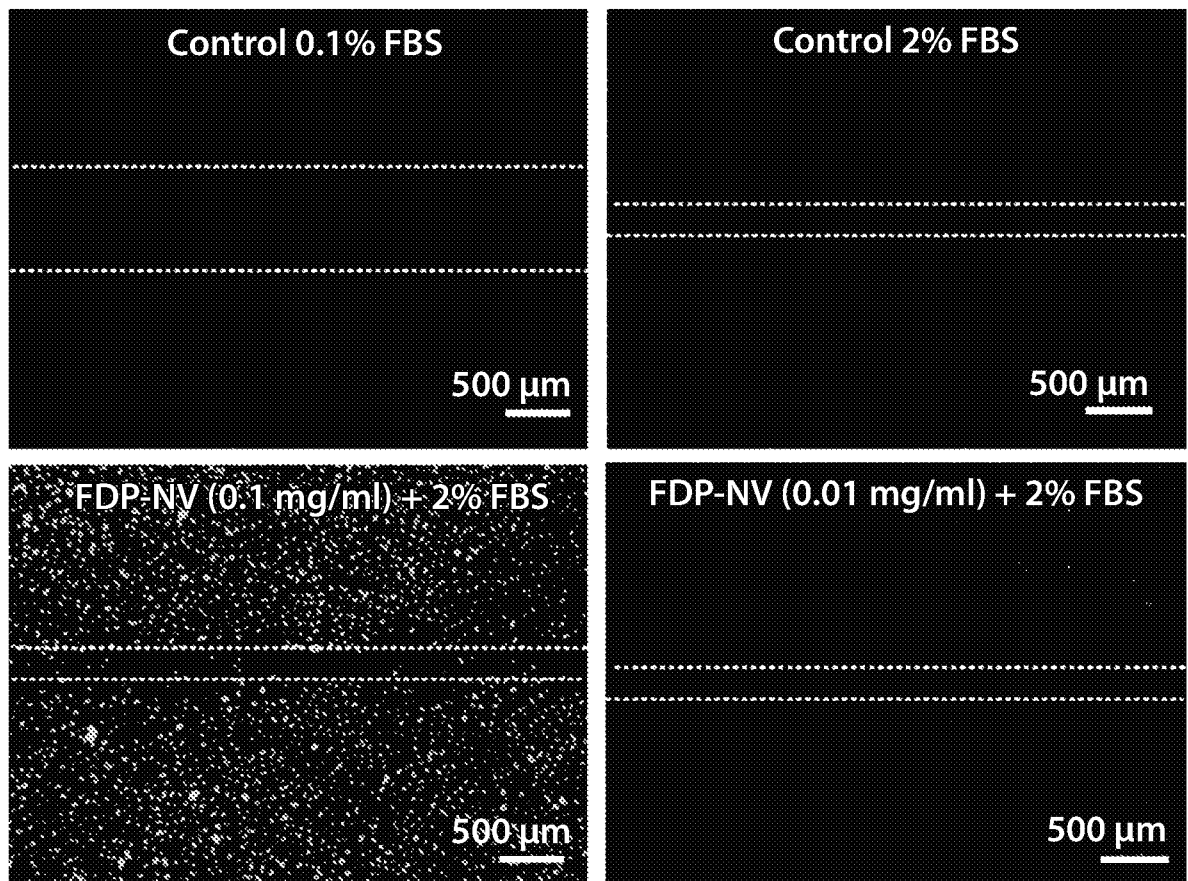


FIG. 14B

2026202273 24 Mar 2026

19/24

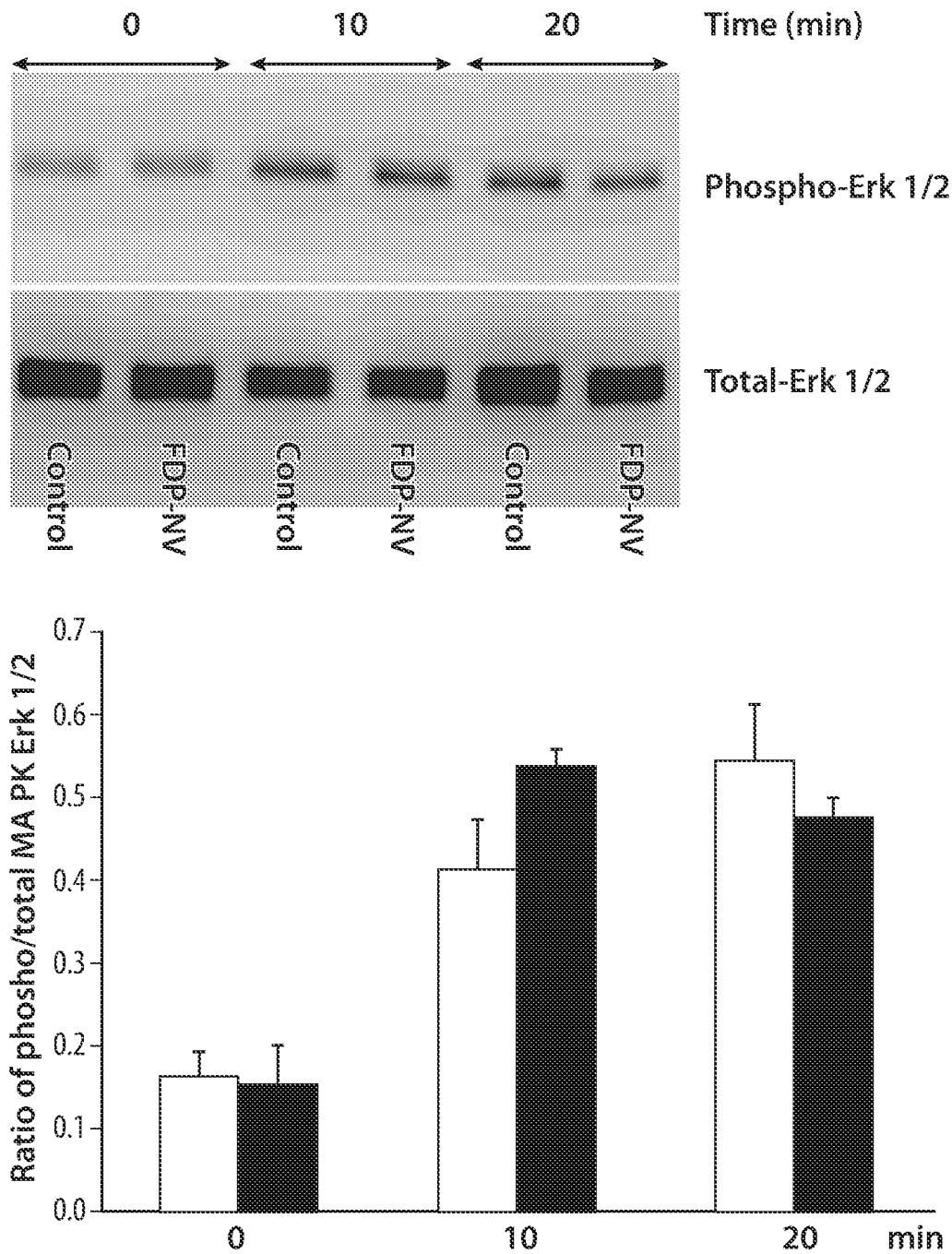


FIG. 15A

2026202273 24 Mar 2026

20/24

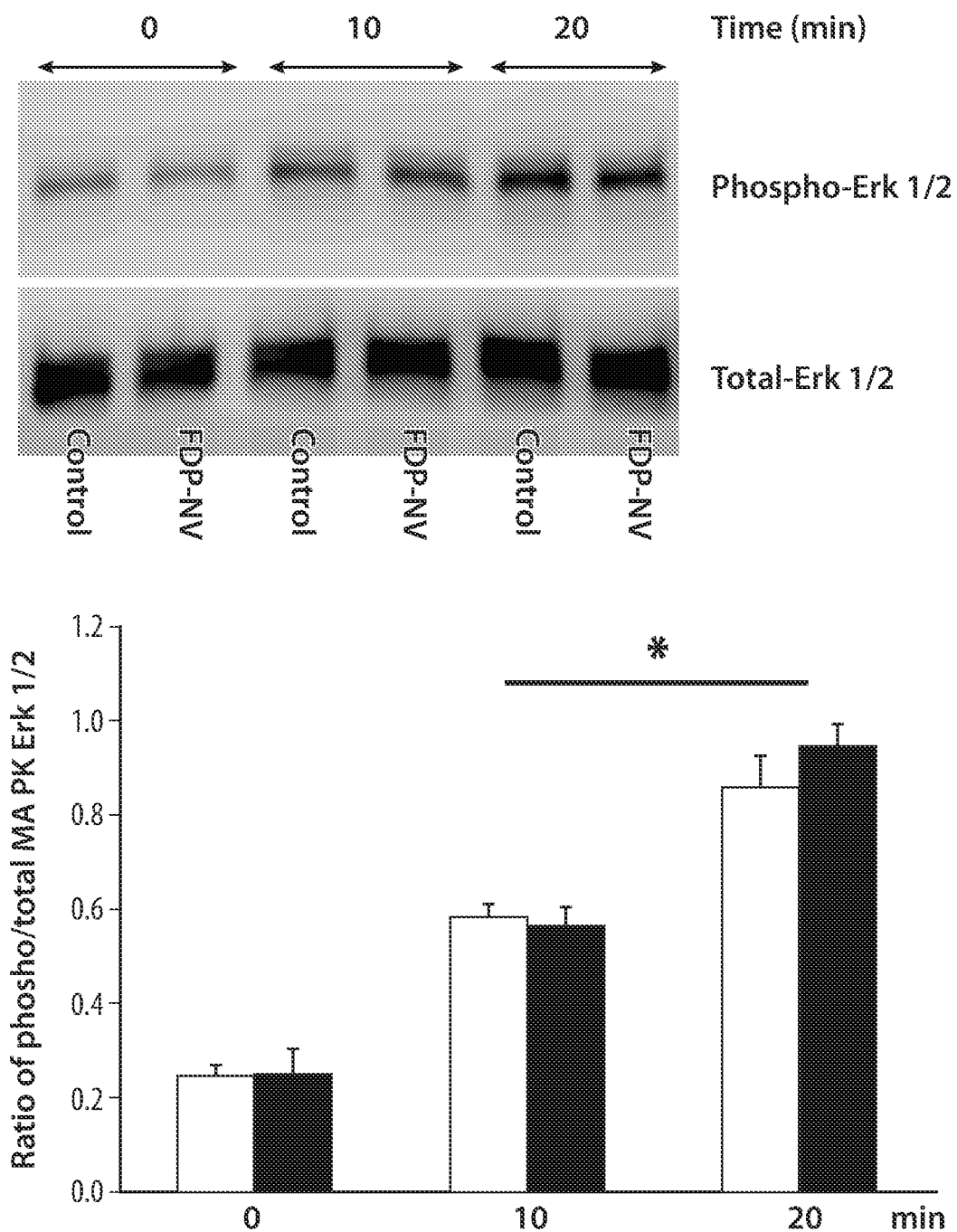


FIG. 15B

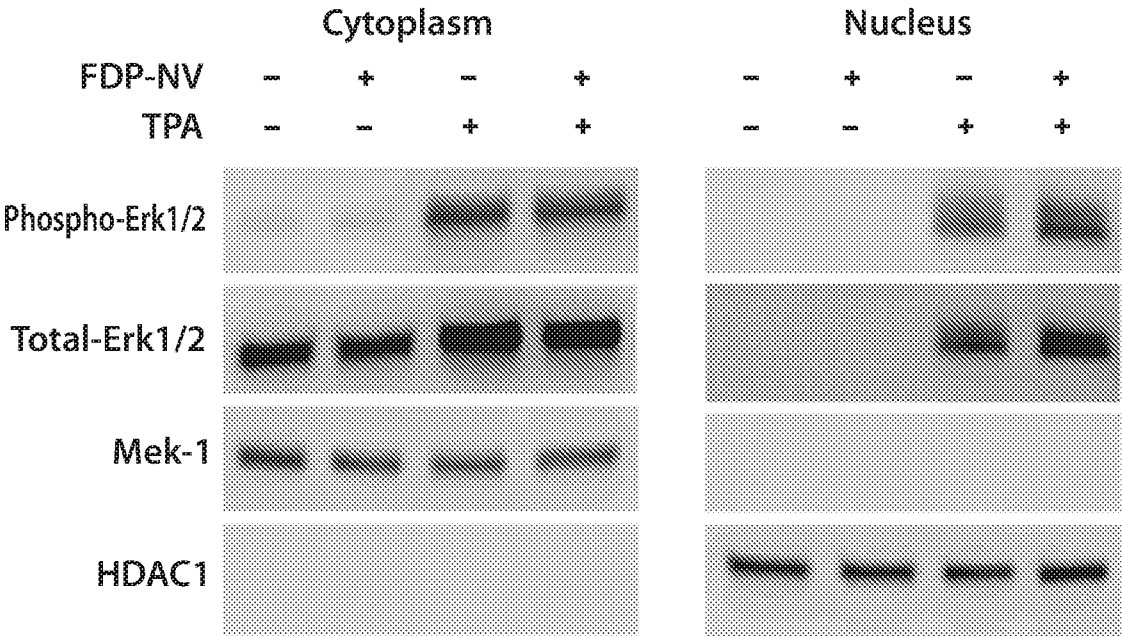


FIG. 16A

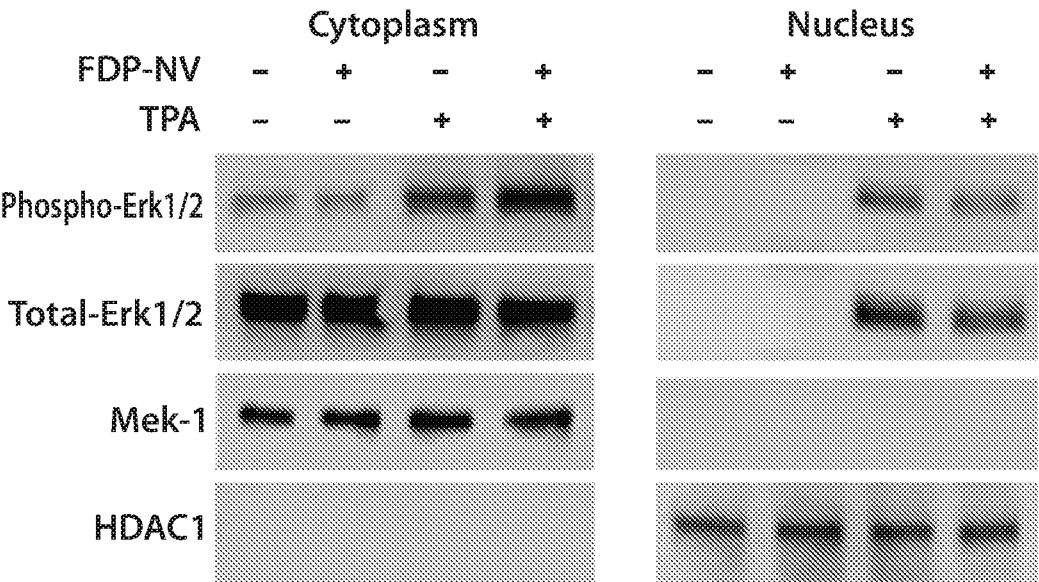


FIG. 16B

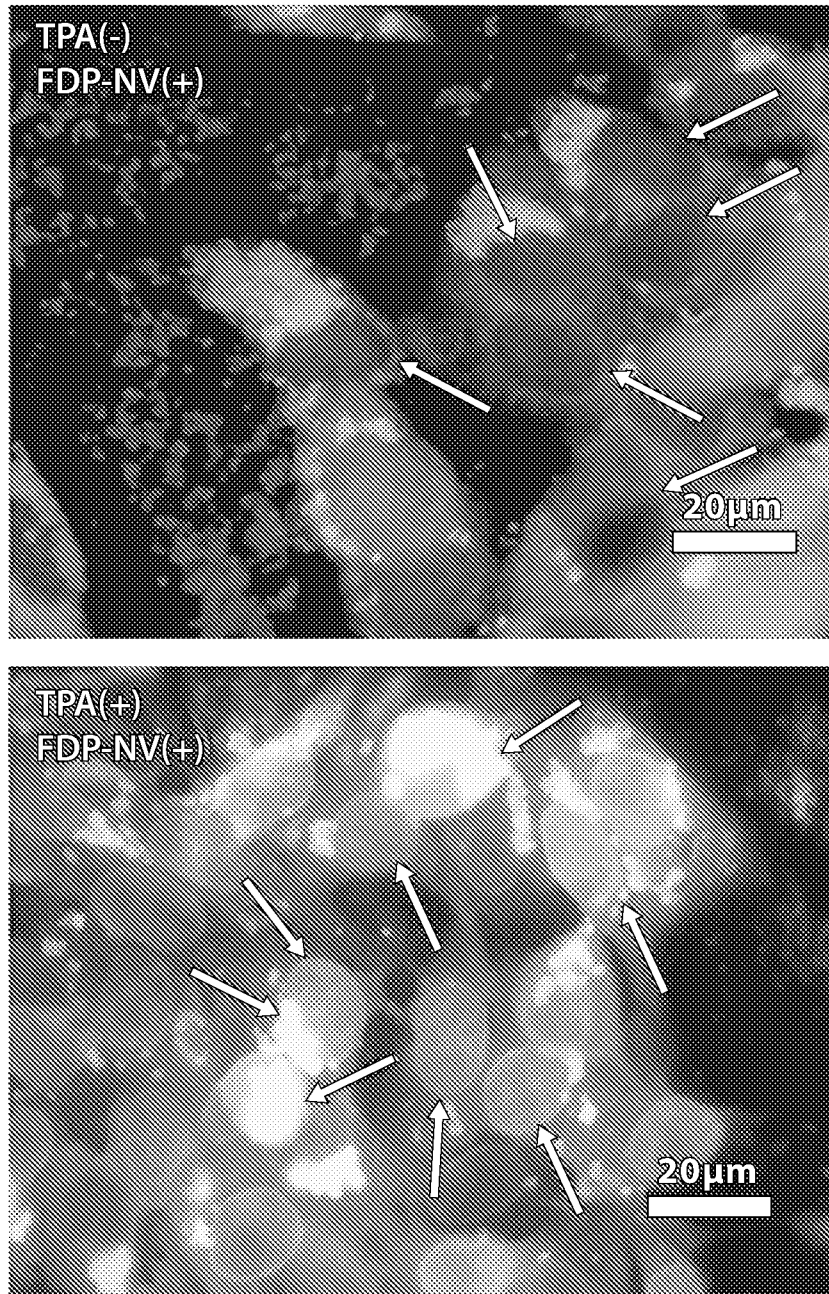


FIG. 16C

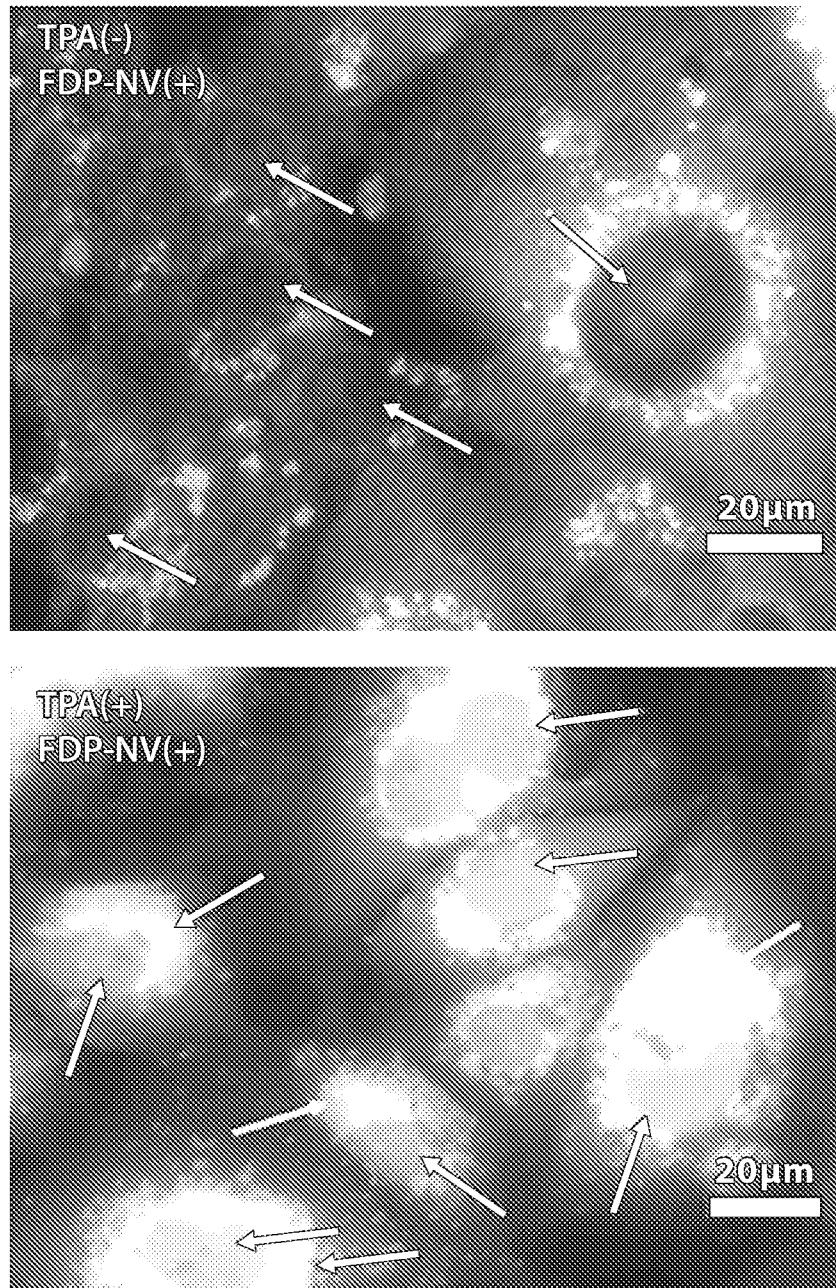


FIG. 16D

2026202273 24 Mar 2026

24/24

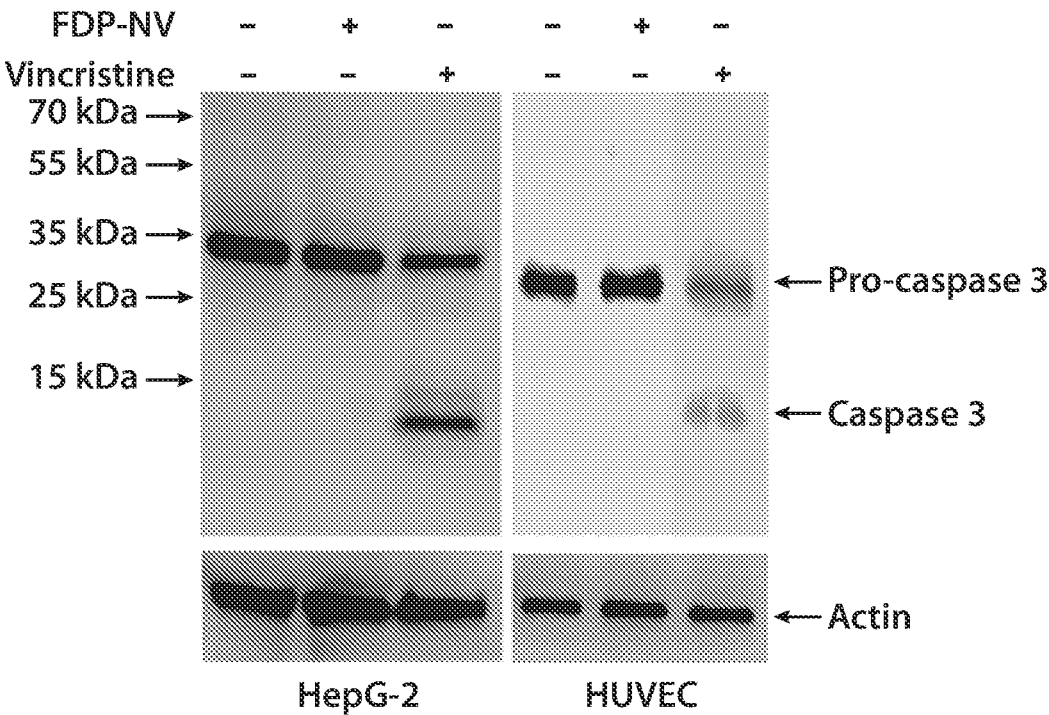


FIG. 17A

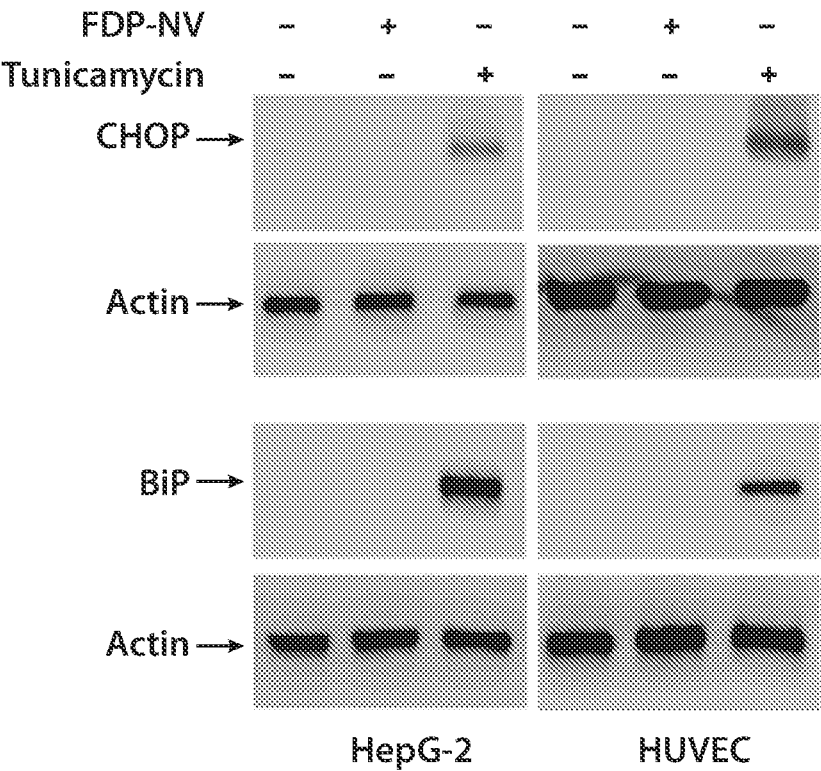


FIG. 17B