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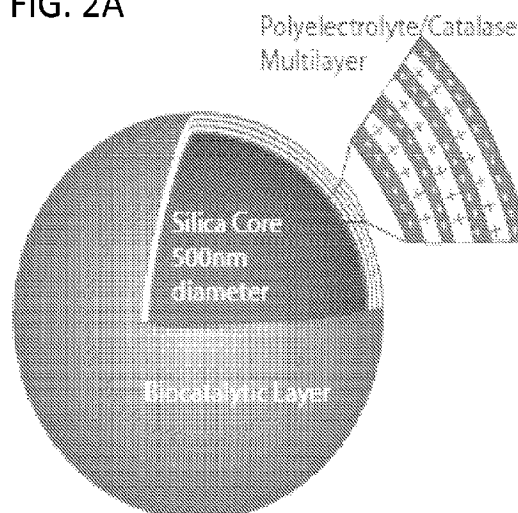
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FIG. 2A



(57) **Abstract:** A method and system are provided for detection of localized hydrogen peroxide within tissue by introducing a catalase-containing material into at location of interest, positioning an ultrasound transducer over the location, and generating an ultrasound image to detect microbubbles, where the presence of microbubbles indicate the presence of localized hydrogen peroxide. The catalase-coated surface may be the inner surface of a microtube, on the surface of or incorporated within a nanosphere or microsphere, or an implantable device.



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METHOD AND SYSTEM FOR *IN VIVO* HYDROGEN PEROXIDE DETECTION WITH ULTRASOUND

RELATED APPLICATIONS

This application claims the benefit of the priority of U.S. Provisional
5 Application No. 61/658,325, filed June 11, 2012, which is incorporated herein by
reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to a method for detecting of biological processes,
and more particularly to the detection of oxygen bubbles produced by hydrogen
10 peroxide using ultrasound imaging.

BACKGROUND OF THE INVENTION

Infection is a common source of morbidity in hospitalized patients, many of
whom have multiple venous lines, catheters, or other implantable devices in place for
extended periods of time. For example, catheter-associated infections represent up to
15 80% of urinary tract infections in hospitalized patients, yet there is currently no means
for detecting whether such an infection exists until the infection has progressed.

Existing practices for management of catheters in a clinical setting rely
primarily on statistically-determined guidelines. For example, guidelines state that
postoperative urinary catheters should be removed 48 hours post operatively.
20 Additional guidelines exist for treatment of select populations with prophylactic
antibiotics. If patient behavior suggests a potential infection, for example, inability to
void, fever, etc., a dipstick and urinary analysis is performed. Similarly, if a central
line infection is suspected, two sets of blood cultures are typically taken for culture.
Urinalysis results can usually be obtained in one to two hours, while urine and blood
25 culture results usually being returned in one to two days. In addition to the
disadvantages of the relatively long turnaround time, these tests have a relatively high
false positive rate and result in many patients being placed on antibiotics unnecessarily.

Hydrogen peroxide (H₂O₂) is a toxic byproduct of many physiologic reactions,
formed either directly or by enzymes to consume the harmful oxygen free radicals
30 produced during oxidative metabolism. Tissues have evolved sophisticated pathways
to control H₂O₂, using it at low levels for intracellular signaling (<20μM), at high levels

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by itself or converted to more harmful oxides for defense ($>50\mu\text{M}$), or catalyze it into O_2 and water by the enzyme catalase to protect themselves. An imbalance results in elevated levels and is seen in oxidative stress, inflammation, and aging. Hydrogen peroxide is also involved in cancer, diabetes, neurodegeneration, acute respiratory distress (ARDS), and cardiovascular disease. As such, it has potential use for localized detection of a wide variety of biochemical processes *in vivo*. However, to date, most methods for detecting H_2O_2 have been confined to in vitro diagnostic use. Prototypes for a few specialized fluorescence, magnetic resonance and genetically encoded probes have been reported, but there is currently no robust injectable probe that can detect H_2O_2 to localize regions with elevated levels of H_2O_2 such as areas of inflammation, etc. in humans.

Existing methods include horseradish peroxidase with artificial substrates, which provides high sensitivity in vitro; ferrous oxidation in the presence of xylenol orange; genetically encoded probes such as those incorporating a substrate of SNAP-tag; roGFP or Hyper; MR contrast agents capable of detecting H_2O_2 ; enzyme electrodes (such as silica nanowire sensors); $(13)\text{C}$ -Benzoylformic acid detection using specialized C-13 hyperpolarized MR sequences; and chemiluminescent nanoparticles.

Currently, detection in collected or voided fluids is compromised by autoxidation when exposed to atmospheric pO_2 , causing artificial increases in H_2O_2 levels and decreased accuracy. Fluorescence-based assays, such as Amplex Red or ferrous oxidation of xylenol orange (FOX), are susceptible to contamination by other urine or plasma constituents and are not routinely available in clinical laboratories. Electrochemical and optical-based probes have been developed but are expensive and more difficult to incorporate into routine devices or standard clinical protocols to be practical.

Results have been reported on detection of implant-associated neutrophil responses using a nanoprobe targeting the formyl peptide receptor, however, this is an optical reporter and its clinical use is limited by overlying tissue thickness.

Another technology makes use of increased turbidity detectable in a discharge fluid from an infected catheter tube. The approach also relies on optical imaging and is likely to generate anomalous results in bloody fluids. In other developments, sensors

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have been designed to monitor the pH of biofilm-producing organisms such as *Proteus* bacilli, however, these are specific to a single organism.

In view of the foregoing, the need remains for a simple and low cost sensor for monitoring oxidative stress that can be incorporated into existing indwelling devices
5 such as Foley or central venous catheters as a means for detecting H_2O_2 in real time using conventional clinical instrumentation.

SUMMARY OF THE INVENTION

Recent advances in chemically-powered synthetic nanomotors have made these devices promising tools for addressing many biomedical challenges. These catalytic
10 nanoconverters have been used to isolate molecular oxygen that either dissolves or takes the form of microbubbles at sufficiently high (0.2%) fuel concentration. Ultrasound is a widely used clinical imaging modality with high sensitivity to gas-liquid interfaces such as microbubbles. The detection of these microbubbles relies on their non-linear oscillations distinguishing them from background tissue and provides
15 the basis of contrast-enhanced ultrasonography.

The present invention employs an ultrasound (US) molecular imaging approach involving the productions of microbubbles (MBs) in situ. Catalytic nanoconverting materials are used to produce microbubbles that can be detected at lower concentrations of H_2O_2 using ultrasound. Neutrophils are the hallmark of acute inflammation. Since
20 H_2O_2 is produced in 60-100 μM quantities by activated neutrophils in inflammatory tissues, the MBs may be used to detect infections or other diseases associated with elevated level of H_2O_2 such as cancer or ischemic injury, etc.. Additional applications of the inventive method include visualization of metabolites or other reactive oxygen species that can be converted to hydrogen peroxide, and fueling oxygen requirements
25 for physiologic reactions.

In one aspect of the invention, a method is provided for detection of localized hydrogen peroxide, including introducing a catalase material into a location of interest; positioning an ultrasound transducer over the location of interest; and generating and detecting an ultrasound signal to generate an image therefrom, wherein the presence of
30 microbubbles within the image indicates the presence of localized H_2O_2 . In one embodiment, the location of interest may be a fluid extracted from a patient such as

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urine, pus, or liquid removed from a mass. In another embodiment, the location of interest is tissues, where the catalase-containing material may be introduced into the tissue percutaneously, by inserting the device through a body orifice, such as the urethra, or by intravenous injection or infusion of a suspension of catalase-containing particles. In all cases, the ultrasound transducer is positioned over the fluid or tissue or location of interest and used to detect a signal generated by microbubbles. This signal can be used to generate an ultrasound image to visualize the generated microbubbles, where the presence of microbubbles indicates the presence of localized H_2O_2 . In one embodiment, the catalase-containing material is a coating on an inner surface of a microtube. In another embodiment, the catalase-containing material is one or more layer of catalase coated onto a nanosphere or microsphere, which may be a silica particle. The layers may be alternating layers of catalase and polystyrene sulfonate. In still another embodiment, the catalase-containing material may be an implantable device with a catalase coating, where the device may be a catheter, central venous line, dialysis catheter, temporary IVC filter, surgical implant, peripheral vascular graft material, orthopedic implant, or hernia mesh. In yet another embodiment the catalase-catalase-containing material may be particles that can be administered as a suspension intravenously. If the catalase is contained within the particle, the particle shell is permeable to water and H_2O_2 . Such material can be silica or hydrogels or the like.

In yet another aspect of the invention, a method is provided to visualize the presence and distribution of low concentrations of hydrogen peroxide (H_2O_2) *in vivo* using ultrasound imaging, by introducing a reacting agent(s) or a catalytic material(s) *in vivo* to convert H_2O_2 into water and oxygen (O_2) bubbles. The bubbles can then be visualized using ultrasound imaging devices. The catalytic materials may be packaged in small sizes ranging from 10nm to 100 microns, depending on the application, or they may be incorporated into a packaging material, either directly into a packaging material having a porous and/or hollow structure, coated onto the surface of the packaging material, or coated onto a surface of an implantable medical device such as a catheter. The surface of the catalytic device, i.e., the packaging material, can itself be formed from catalytic and/or biocatalytic materials. The surface may also be configured to

protect the catalytic materials from the environment and/or to control the size of the microbubbles produced.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A – 1F illustrate ultrasound-based visualization of oxygen microbubbles where FIG. 1A is a diagrammatic view of a first embodiment of a catalytic converter showing production of oxygen; FIG. 1B is an image of microbubble generation produced using light microscopy; FIG. 1C is an image of microbubble generation produced using ultrasound; FIG. 1D is a diagram of the basic experimental set-up; FIG. 1E is a series of ultrasound images generated at different hydrogen peroxide concentrations; FIG 1F is a plot of hydrogen peroxide concentration versus ultrasound signal intensity.

FIGS. 2A-2L illustrate detection of H_2O_2 using catalase-coated silica nanospheres converters (NSCs), where FIG. 2A is a diagrammatic view of an exemplary nanosphere; FIG. 2B is a SEM image of the nanospheres; FIG. 2C is a plot number of particles per batch versus number of layers; FIG. 2D is a plot of catalase activity per batch versus number of layers; FIG. 2E is a plot of catalase per particle versus concentration versus number of layers; FIG. 2F is chart showing detection threshold versus number of layers in saline and plasma; FIG. 2G is a plot of intensity versus H_2O_2 concentration; FIG. 2H is a chart showing the range of H_2O_2 concentration versus detection threshold; FIG. 2I is a plot of intensity versus H_2O_2 concentration for 3 layers with and without PSS; FIG. 2J is a chart showing catalase concentration versus detection threshold; FIG. 2K is a plot of the number of particles versus intensity; and FIG. 2L is a chart showing particle number versus detection threshold.

FIGS. 3A-3E illustrate detection of H_2O_2 in neutrophil suspensions, where FIG. 3A is a diagram of the test set-up; FIG. 3B shows images of exemplary neutrophils taken at 40x magnification after Wright staining; FIG. 3C is a scatterplot showing presence of H_2O_2 in neutrophils versus intensity; FIG. 3D is a set of photographic images showing microbubbles only in tubes containing activated neutrophils and NSCs; and FIG. 3E is a chart showing normalized intensity with and without catalase.

FIGS. 4A-4B are contrast-mode ultrasound images pre- and post-injection of nanospheres into abscesses in a model animal, where FIG. 4A shows NSCs and

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microbubbles post-injection and FIG. 4B shows control nanospheres with no catalase and no post-injection microbubbles.

FIG. 5 is a diagrammatic image of a system for in vivo detection of infection using ultrasound imaging.

5 FIG. 6 is a series of photographs comparing microbubble production on a plain metal surface and catalase-coated surface at different concentrations of H_2O_2 .

DETAILED DESCRIPTION

According to the present invention, oxygen microbubbles formed by nanoconverters can be visualized with ultrasound. FIG. 1D illustrates and exemplary
10 test layout in which a transfer pipette 20 was modified to contain a port 22 through which hydrogen peroxide could be dripped. The pipette 20 was placed in a water bath 10 with transducer 30, which was connected to the ultrasound unit. Microbubbles were imaged using a General Electric LOGIQ® E9 ultrasound system.

The following detailed description uses a variety of terms to describe the
15 particles having a material adapted to catalyze H_2O_2 into water and oxygen microbubbles. Such terms include “catalytic converters”, “nanoconverters”, “nanorockets”, “micromotors” “micromotor converters”, “MMCs”, and “NSCs”. It will be readily apparent to those in the art that each of these alternative terms refers to a micro- or nano-scale particle configured to induce the production of microbubbles in
20 the presence of H_2O_2 .

FIGs. 1A-1F illustrate a first evaluation of the inventive method using a catalytic converter referred to as “nanorockets” or “micromotor converters” (“MMCs”). FIG. 1A diagrammatically illustrates the MMCs, which are tubular (truncated conical) nanostructures lined with platinum or catalase over a gold surface, acting as a catalytic
25 surface causing breakdown of H_2O_2 .

Micromotors were synthesized as described by W. Gao, et al., “Polymer-based tubular microbots: role of composition and preparation”, *Nanoscale*, 2012 Apr. 7; 4(7):2447-53. Briefly, tubular micromotors were prepared using a common template-directed electrodeposition protocol. A CYCLOPORE™ polycarbonate membrane,
30 containing 2 μm diameter conical-shaped micropores (Catalog No 7060-2511; Whatman, Ltd., Maidstone, U. K.), was employed as the template. A 75nm gold film

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was first sputtered on one side of the porous membrane to serve as working electrode using the Denton DISCOVERY™ 18 (Denton Vacuum, LLC, Moorestown, NJ). The sputter was performed at room temperature under vacuum of 5×10^{-6} Torr, DC power 200 W and flow Ar to 3.1 mT. Rotation speed was 65 and sputter time was 90s. A
5 platinum wire and an Ag/AgCl electrode with 3M KCl were ultrasounded as counter and reference electrodes, respectively. The membrane was then assembled in a plating cell with an aluminum foil serving as a contact. Poly(3,4-ethylenedioxythiophene) (PEDOT) microtubes were electropolymerized at +0.80 V for a charge of 0.06 C from a plating solution containing 15 mM EDOT, 7.5 mM KNO₃ and 100 mM sodium dodecyl
10 sulfate (SDS); subsequently, the inner Pt tube was deposited galvanostatically at -2 mA for 1800 sec from a commercial platinum plating solution (Platinum RTP; Technic Inc, Anaheim, CA). The sputtered gold layer was completely removed by hand polishing with 3-4 μ m alumina slurry. The membrane was then dissolved in methylene chloride for 10 min to completely release the microtubes. The latter were collected by
15 centrifugation at 6000 rpm for 3 min and washed repeatedly with methylene chloride, followed by ethanol and ultrapure water (18.2 M Ω cm), three times of each, with a 3 min centrifugation following each wash. All microtubes were stored in ultrapure water at room temperature when not in use.

Catalase lined micromotors were synthesized as described by Gao, et al., *supra*.
20 Briefly, PEDOT microtubes were electropolymerized at +0.80 V for a charge of 0.06 C from a plating solution containing 15 mM EDOT, 7.5 mM KNO₃ and 100 mM sodium dodecyl sulfate (SDS); subsequently, the inner gold layer is plated at -0.9 V for 1 C from a commercial gold plating solution (OROTEMP® 24 RTU RACK; Technic, Inc., Cranston, RI, U.S.). The inner Au layer from the bilayer microtubes was
25 functionalized with a mixed MUA/MCH monolayer. A solution of 2.5 mM MUA and 7.5 mM MCH was prepared in ethanol. The micromotors were incubated in the solution overnight. After rinsing the tubes with ethanol for 5 min, they were transferred to an Eppendorf vial containing a 200 μ L PBS buffer (pH 5.5) solution with the coupling agents 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS) at 0.4 M and 0.1 M respectively, and the
30 enzyme catalase (2 mg mL⁻¹). This incubation was carried out 7 hours at 37°C and

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thereafter rinsed with PBS with a pH of 7.4 and SDS 0.05 wt% for 15 min at each step. The micromotors were washed repeatedly by centrifugation at 6000 rpm for 3 min with water for three times to remove extra catalase in solution before testing.

Enzyme activity was determined spectrophotometrically based on the decrease
5 in absorbance of hydrogen peroxide at $\lambda = 240$ nm, according to an adapted method from the Sigma Enzymatic Assay of Catalase (Sigma, protocol EC 1.11.1.6.) Briefly, 100 μ l of particle solution was washed by centrifugation at 8000 rpm for three minutes and re-suspended in 27 μ l of PBS pH 7.0 before being added to 773 μ l of 11 mM H₂O₂ solution prepared in 50 mM PBS. After shaking for 10 seconds, the solution was
10 placed in the 800 μ l spectrophotometer cuvette and the decrease in absorbance at 240 nm with time was recorded immediately at 20° C for 2 min. One unit of catalase is defined as decomposing 1 μ mol of H₂O₂ per minute at pH 7.0 and 20° C.

FIG. 1B is a photomicrograph of microbubbles visible at 0.2% (v/v), or 65.3mM, H₂O₂ using light microscopy. Microbubbles were first detected by ultrasound
15 when MMCs were exposed to 2-5mM H₂O₂. FIG. 1C provides a parallel image to that of FIG. 1B, but generated at a much lower hydrogen peroxide concentration (0.8mM) using ultrasound. Note that this image has been cropped, rotated and magnified for comparison. Single microbubbles observed at low H₂O₂ concentrations became trails of microbubbles as the hydrogen peroxide concentration increased. At
20 the highest concentrations, echogenic MMCs could be seen traveling in a direction opposite the bubble trail. The microbubbles were visible using both standard ultrasound imaging as well as non-linear imaging, a microbubble-only imaging technique that suppresses all linear tissue signals. The best results were obtained when the highest MMC concentration (27000 MMCs/mL) was exposed to 0.13% (v/v)(42.2
25 mM) H₂O₂. The threshold was lowered further (0.8mM) when the inner surface of the MMC was coated by catalase. However, because these motors were dense, they settled quickly to the bottom of the container.

The gross effect of increasing H₂O₂ concentration is demonstrated in FIG. 1E. As shown in the upper left image, few microbubbles are visible at 0.0125% (4.1mM).
30 These were produced by a few MMCs that had settled to the bottom of the container. More MMCs become active and the number of microbubbles increases with increasing

H₂O₂, as shown by the upper right (0.03%) and lower left (0.06%) images, until a cloud of microbubbles is formed at 0.13% (42.4 mM) H₂O₂, as shown in the lower right image. Ovoid regions of interest were drawn and quantitated in FIG. 1F, demonstrating a logarithmic relationship between H₂O₂ and ultrasound signal intensity. There is a dose dependence with increasing MMCs as well as significant improvement when the inner surface of the MMC is coated with catalase instead of platinum.

To decrease background echogenicity from hollow motors, decrease the overall size of the nanoconverters and eliminate the metal surface in anticipation of future preclinical testing, stationary H₂O₂ nanoconverters (NSCs) were formed by layering concentric shells of catalase and polystyrene sulfonate (sodium polystyrene sulfonate) (PSS) over a 400-500nm silica sphere, diagrammatically shown in FIG. 2A. This compact spherical design theoretically allows for smaller, less dense particles with biodegradable components that are more suitable for *in vivo* use.

NSCs were produced using the following process: 1μl of negatively-charged silicon particles (0.51 μm, Catalog Code SS03N, Sigma-Aldrich) was washed twice by centrifugation for 3 minutes at 8000rpm, first with B&W&B, then with DI water. Layers of catalase (C3155-50, Sigma-Aldrich) diluted daily into 0.05M PBS, pH5.0 and a PSS/saline solution (1mg/mL PSS diluted into 1 mL 0.05M PBS, pH 5.0, containing 29 mg NaCl). Between incubations, particles were washed with 100μl of DI water by centrifuging at 8000rpm for three minutes. These steps were repeated to produce the desired number of layers. Particles were re-suspended in either PBS (pH7, Gibco) or HBSS (Gibco) prior to experiments.

Scanning electron microscopy (SEM) confirms the presence of several 400-500nm particles with uniform spherical shape (FIG. 2B). For all experiments, SEM images were obtained with a Philips XL30 ESEM instrument, using an acceleration potential of 20 kV. Optical images were captured using an inverted optical microscope (Nikon Instrument, Inc. Ti-S/L100) coupled with a 20x objective, a Hamamatsu digital camera C11440 using the NIS-Elements AR 3.2 software.

Increasing the number of layers (shells) in the bioactive multilayer decreases the threshold concentration of H₂O₂ at which microbubbles are first detected as hydrogen is added slowly to the top of the tube. FIGs. 2C-2E illustrate the moderate decrease in

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particle number as more layers are added to the bioactive multilayer, possibly due to washing during the manufacturing process. Error bars show standard deviation. As shown by the curve in FIG. 2D, catalase activity associated with the nanospheres increases until three layers are added, then begins to drop off with additional layers.

5 Increasing the number of catalase layers on the outer surface decreases the threshold concentration of H_2O_2 at which microbubbles are first detected by ultrasound with minimal improvement seen between 3 and 5 layers. Measurements were repeated three times for three distinct batches of particles. The mean $\pm$ standard error is shown. The number of spheres is held constant at 1.3×10^7 ($4.3 \times 10^9/\text{L.}$) FIG. 2E confirms that
10 the catalase per particle increases as expected with increasing layers.

FIGs. 2F and 2G demonstrate that performing the assay in plasma decreases the detection limit compared with buffered saline and sodium hydrate cholate. For all particle geometries, using plasma to suspend the particles instead of a mixture of buffered saline (PBS) and sodium cholate decreased the detection limit by another 10-
15 to 100-fold, possibly due to the presence of lipids and proteins. In FIG. 2F, the black rectangles indicate the range of H_2O_2 concentration when microbubbles were first detected by two blinded observers ($n=6-8$) when H_2O_2 is increased incrementally by factors of 10. White lines within the black rectangles indicate the average H_2O_2 concentration at which bubbles are first detected for each experiment. The effect of
20 increasing H_2O_2 concentration on image intensity is shown in FIG. 2G. Experiments were done in triplicate, and error bars represent standard error of the mean.

FIGs. 2H and 2I demonstrate that adding an outer layer of polyelectrolyte (PSS) does not significantly change sensitivity to H_2O_2 , indicating that diffusion through PSS is not rate limiting. The black rectangles and white lines in FIG. 2H correspond to the
25 same features described above relative to FIG. 2F. The effect of increasing H_2O_2 concentration on image intensity is shown in FIG. 2I. Experiments were done in triplicate, and error bars represent standard error of the mean.

Incorporation of catalase on the NSC surface was more efficient for microbubble formation than was free catalase in solution, since the latter required
30 $>5000\text{U/mL}$ to produce detectable microbubbles. This suggests that sensitivity to H_2O_2 may also be affected by other factors in addition to geometry, possibly because the

catalase on the surface of the NSCs had a much higher effective concentration, irregularities on the PSS surface eased nucleation, and/or the NSCs themselves acted as nucleation sites upon insonation of the surrounding oxygen saturated fluid. FIG. 2J is a plot of H_2O_2 concentration when microbubbles were first detected as a function of free catalase in solution. Experiments were done in triplicate. The black rectangles represent the range of detection thresholds observed; the white lines indicate the average detection threshold.

NSC concentration was determined by diluting the NSCs 100-fold into PBS and injecting the solution into a hemocytometer. The number of particles in a $100\mu m^3$ was counted manually under light microscopy.

Using the experimental set-up shown in FIG. 1D, NSCs at the indicated concentration were placed into a transfer pipette that had been modified to include a port that could be pinned to the back of the water bath for stability. 3mL phosphate-buffered saline (PBS, Gibco) and 0.04 M sodium hydrate cholate (NaCH, Sigma-Aldrich) were added to the NSCs through the port, and samples were allowed to sit for approximately five minutes. Under ultrasound operating in contrast mode (GE LOGIQ[®] E9, 6-15MHz linear transducer, MI<.20, 14 frames per second), the concentration of hydrogen peroxide was increased incrementally by factors of ten (e.g., $8\mu M$, $80\mu M$, $800\mu M$,...) delivered in low volumes of $3\mu L$ or $30\mu L$ so as to avoid excessive dilution. NSCs were tested side by side with control spheres that did not contain catalase. Detection limits were obtained on the fly by two independent observers blinded to the identity of the tubes. The detection limit was defined as the first point at which characteristic rising bubbles were observed and was recorded at the time of the experiment. All experiments were performed in triplicate.

Stacks of images were analyzed using ImageJ, a public domain, JAVA-based image processing program developed at the National Institutes of Health. An ovoid region of interest was drawn encapsulating the largest area of the tube possible while excluding obvious imaging artifact from the sides of the tube when present. This region was averaged both pre- (3-10 frames) and post- (5-20 frames) administration of H_2O_2 . Total intensity was defined as the average of the pre-frames subtracted from the average of the post-frames.

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As shown in FIGs. 2K and 2L, increasing the number of NSCs increases ultrasound image intensity from formed microbubbles. FIG. 2K shows image intensity as a function of particle number in suspension when 9.7 mM of H₂O₂ is added. Experiments were done in triplicate. Error bars represent the standard error of the mean. FIG. 2L shows the range of H₂O₂ concentrations are which microbubbles were detected subjectively when added to four different particle concentrations in saline. The detection threshold could be further decreased by increasing NSC concentration, leading to a final detection threshold as low as 10µm at a NSC concentration of 4.3x10⁶/mL. At the lowest NSC concentration, microbubbles were detected in only one of the three samples when exposed to 9.7 mM H₂O₂.

The three-catalase-layered NSC particles were used for detection of H₂O₂ produced during cytotoxic responses in neutrophils triggered by phorbol myristate acetate (PMA). Two methods were used to isolate the neutrophils. For the first method, ammonium chloride preparation, 30 mL of rabbit blood was drawn and spun down at 3000rcf x15m. The plasma was removed and frozen. The hematocrit and the buffy coat were incubated in isotonic ammonium chloride buffer (15:1 by volume, 8.32g/L NH₄Cl, 0.84g/L NaHCO₃) for 15-20 minutes. The samples were then spun at 300rcf for 15 minutes and rinsed twice with HBSS for 10 minutes. Cells were counted with a hemocytometer. The second method used was dextran sedimentation preparation. In this method, 30 mL of rabbit blood was drawn and added to an equal amount of 3% solution of dextran-500 diluted in normal saline. This was allowed incubate at room temperature for approximately 20 minutes, until the hematocrit had settled. Plasma was then removed and centrifuges at 250 x g. To reduce the number of red cells present, neutrophils were bathed in 20mL of ice cold 0.2 saline for exactly 20 seconds. 1.6% saline was then added and the cells were spun for 250rcf x 10 minutes. This was repeated once. A Ficoll gradient was not performed due to time constraints. By Wright stain, these preparations were estimated to be about 50% pure, which is consistent with published studies. Neutrophil activation was accomplished by addition of PMA at 15ng/mL for one hour.

H₂O₂ concentration was assayed using Amplex Red reagent (Life Sciences) using a protocol supplied by the manufacturer. Samples were diluted by 50x prior to

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use. Samples used for the calibration curve were spiked with non-activated neutrophils to control for the spectral properties of lysed red blood cells contained in the mixture.

FIG. 3A diagrammatically illustrates the experiment in which neutrophil ($1-3 \times 10^6$ cells/mL), pre- and post-PMA activation, were added to NSCs and control
5 nanospheres (without catalase) suspended in saline using coded containers. Blind observers rendered judgment as to whether microbubbles were visible on the ultrasound unit display. Both dextran sedimentation and ammonium chloride preparations were used for neutrophil separation in separate experiments, with similar results. Wright staining confirmed the presence of neutrophils (FIG. 3B), and an Amplex red assay for
10 H_2O_2 confirmed that the neutrophil-enriched fractions contained 8-60 μM H_2O_2 (see FIG. 3C), which is consistent with published data. Neutrophils were counted with a hemocytometer and added to NSCs, or similar control nanospheres without catalase, suspended in saline for a final cell concentration of $1-3 \times 10^6$ cells/mL. Subjective analysis of the real-time ultrasound was made at the time of the experiment by two
15 blinded observers when the PMA-activated or naïve neutrophils were added to NSCs in each of the three experiments. 25 μL (1.3×10^7) or 250 μL (1.3×10^8) nanospheres were placed into each tube along with 800 μL of saline. Neutrophils were added to a concentration of 1×10^6 cells per mL (typically $\sim 100-200 \mu L$). Images were assessed for bubble formation at the time of the experiment by both observers, and preliminary
20 results were documented while still blinded. The test results are shown in FIG. 3D. Microbubbles were observed only when activated neutrophils were added to NSCs. Both 1.3×10^7 and 1.3×10^8 particles/mL yielded ultrasound-detectable microbubbles, although more microbubbles were seen at the higher concentration.

As in the preceding experiments, images were processed using ImageJ. The
25 quantification of video intensity within a region of interest drawn over the lumen of the container was averaged over 20 frames prior to injection. Depending on the experiment, the first 80-200 frames after the addition of neutrophils were ignored to eliminate the possibility of erroneously-introduced microbubbles. Up to 30 frames were collected and again averaged. Frames in which tube movement caused obvious
30 artifacts to appear in the region of interest were eliminated prior to averaging. Intensities of “pre”-images were subtracted from intensities of “post”-images to provide

net intensity. FIG. 3E shows the results of a representative experiment done in triplicate, confirming the subjective assessment. Similar results were observed in each of four other experiments, each done in triplicate.

NSCs were tested in an *in vivo* model of abscess in three eight to ten month old Sprague Dawley rats (Harlan Laboratories). Abscesses are characterized by the presence of a large number of neutrophils that release H_2O_2 as a response to bacterial invasion. Methicillin-sensitive *Staphylococcus aureus* (MSSA) was grown in LB media (Sigma-Aldrich) at 37°C until reaching mid-log growth phase as determined by serial OD_{600nm} measurements. Bacteria were then pelleted and re-suspended in 400 µL LB. 25µL of the re-suspension (corresponding to 6×10^8 colony-forming units (CFU), determined retrospectively by plating dilutions onto agar plates) was injected into the right lateral thigh of each animal. Animal vital signs and abscess formation were monitored for each rat over the subsequent four days by visual examination and ultrasound. When ultrasound-detectable abscesses had formed, the animals were anesthetized for nanosphere injections using 1% isoflurane in oxygen. Ultrasound imaging was performed using the 15L8 S transducer of a SEQUOIA[®] 512 (Siemens Medical Solutions, Mountain View, CA) operating at 7.0 MHz, MI=0.18, 16 frames/sec using the CPS microbubble-specific imaging mode. Ultrasound images acquired in real-time from before and for several minutes after nanosphere were digitally recorded. First, the control nanospheres (8×10^5 particles suspended in 50 µL PBS) were injected into the abscess margin until ultrasound guidance and the needle withdrawn. Using a different needle, NSCs were injected at the same site 5-10 minutes later. Immediately after injection with NSCs, an echogenic focus formed at the injection site that was best seen on the contrast-specific imaging technique (B-mode (brightness-mode), also known as “2D mode”), shown in FIG. 4A. A corresponding focus was not seen where control nanospheres were injected (FIG. 4B).

Recorded ultrasound frames were analyzed using ImageJ. Four frames taken prior to NSC injection were averaged as the “pre”. Eighty frames taken when movements from the injection subsided were averaged as “post”. The “pre” images were subtracted from the “post” images for both control and catalase-containing NSCs in each of the animals and significance was assessed using a Wilcoxon rank sum test,

with $n=4$, $U=16$, $p=0.02$. The results suggests that the signal seen at the site of the NSC injection was due to production of oxygen microbubbles in the presence of H_2O_2 .

Capsuled catalase combined with ultrasound scanning can be used to detect and image elevated H_2O_2 locations *in vivo* for diagnosis and/or real time monitoring of many pathologies and body's response to external perturbations, such as inflammation, cancer, arthritis; detect and image metabolites that can be converted to hydrogen peroxide; and to fuel oxygen requirements for physiologic reactions, among other applications.

Relative to the existing methods of H_2O_2 detection, the inventive method provides a number of advantages including detection and imaging H_2O_2 in deep tissue (up to 33cm deep) *in vivo*; generation of real-time images with high spatial resolution; relatively non-toxic; and readily detected with standard, commercially available ultrasound imagers.

While the foregoing example of *in vivo* H_2O_2 detection was performed using direct injection of capsulated catalase into the infection site, alternative methods for introducing the catalase include suspending catalase-containing particles (nanospheres or microspheres), such as those previously described, in an appropriate fluid for intravenous injection or infusion. The particles may having one or more catalase layers on their exterior surfaces, or they may be formed from a material having a porous or hollow structure so that the catalase may be incorporated into the body of the particle and/or surrounded by a protective shell. Pores or openings in the particle body or shell permit H_2O_2 to access the catalase while protecting the catalase from the environment. The intravenous approach provides means for identifying an infection/inflammation when the exact site is not known, or if multiple potential sites may be affected.

An important practical application of the inventive technology is for detection of infection at the location of implanted devices such as catheters or prosthetic devices. In this application of the invention, the surface of an implantable device is coated with a high-density catalase prior to implantation. The catalase may be packaged inside thin-walled cellulose tubing, a water-permeable hydrogel, or other biocompatible coating material. Packaging aids in preservation of enzymatic function in a potentially hostile biological environment and protects the patient from potential toxicity. The

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coating should be sufficiently porous to allow the H_2O_2 to access the catalyst while keeping larger, potentially destructive molecules out. Using the example of a catheter, as shown in FIG. 5, catheter 50 with catalase coating 52 is implanted. At some point after implantation, if bacteria 60 appear at the implant site, a respiratory burst from neutrophil 62 is triggered, releasing H_2O_2 64 to attack the bacteria. When the H_2O_2 comes into contact with the catalase 52, oxygen microbubbles 68 are produced. Ultrasound transducer 54 is positioned so that it can generate an image of the catheter 50 and the microbubbles 68 that were produced by catalysis of the H_2O_2 .

FIG. 6 provides photographic images of drops of H_2O_2 at different concentrations, 900mM, 90mM, and 9mM on slides. The upper images show the H_2O_2 on silver slides. The slides in the lower images were coated with concentrated catalase. As is apparent from the images, catalase dried on the slide creates visibly-detectable (large) bubbles when overlaid with relatively low concentrations of H_2O_2 . Ultrasound-detectable microbubbles are detectable from catalase-coated microspheres at H_2O_2 concentrations as low as 50-100 μ M, which is similar to the level of hydrogen peroxide detected clinically in urine from infected patients. This demonstrates the additional application of the invention for *in vitro* detection of localized hydrogen peroxide in a fluid sample extracted from a patient, e.g., urine, pus, or liquid removed from a mass, and may be used as a quick test for the presence of oxidative stress.

The inventive detection strategy can be utilized in conjunction with Foley catheters, temporary and permanent central venous lines, dialysis catheters, temporary IVC filters, surgical implants such as peripheral vascular graft material, orthopedic implants or hernia mesh. In each case, the invention has the potential for avoiding the surgical removal of expensive implants if the infection site is elsewhere.

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CLAIMS

1. A method for detection of localized hydrogen peroxide, comprising:
introducing a catalase material into a location of interest;
positioning an ultrasound transducer over the location of interest; and
5 generating and detecting an ultrasound signal to generate an image therefrom,
wherein the presence of microbubbles within the image indicates the presence of
localized H₂O₂.
2. The method of claim 1, wherein the catalase material is introduced as a
coating on an inner surface of a microtube.
- 10 3. The method of claim 1, wherein the catalase material is incorporated into a
nanosphere or microsphere particle.
4. The method of claim 3, wherein the nanosphere or microsphere particle
comprises a silica particle having one or more catalase layers.
5. The method of claim 4, wherein the one or more catalase layers are
15 alternated with layers of polystyrene sulfonate.
6. The method of claim 1, wherein the catalase is coated onto a surface of an
implantable device.
7. The method of claim 6, wherein the implantable device has a protective
coating thereon.
- 20 8. The method of claim 7, wherein the protective coating is a cellulose tubing.
9. The method of claim 7, wherein the protective coating is a water-permeable
hydrogel.
10. The method of claim 6, wherein the implantable device is selected from the
group consisting of catheters, central venous lines, dialysis catheters, temporary IVC
25 filters, surgical implants, peripheral vascular graft material, orthopedic implants, hernia
mesh, etc.
11. The method of claim 3, wherein the nanosphere or microsphere particle is
formed from a material having the catalase incorporated therein.
12. The method of claim 11, wherein the nanosphere or microsphere particle
30 further comprises a shell adapted to permit the entry of H₂O₂ to interact with the
catalase.

13. The method of claim 1, wherein the step of generating an ultrasound image comprises generating a B-mode image.

14. The method of claim 1, wherein the location of interest is an *in vitro* fluid sample from a patient.

5 15. The method of claim 1, wherein the location of interest is a tissue site in a live subject.

16. The method of claim 15, wherein the step of introducing is selected from the group consisting of percutaneous injection, intravenous injection, intravenous infusion, implantation of a medical device, and insertion of a medical device through a
10 body orifice.

17. A system for detecting localized hydrogen peroxide, comprising:
a catalase-containing material adapted for introduction into a location of interest;
an ultrasound transducer; and
15 an ultrasound imaging system for detecting microbubbles at the location of interest.

18. The system of claim 17, wherein the catalase-containing material comprises a coating on an inner surface of a microtube.

19. The system of claim 17, wherein the catalase-containing material is a
20 nanosphere or microsphere having a catalase coating.

20. The system of claim 19, wherein the nanosphere or microsphere comprises a silica particle having a plurality of alternating layers of catalase and polystyrene sulfonate.

21. The system of claim 19, wherein the nanosphere or microsphere is
25 suspended in a fluid.

22. The system of claim 17, wherein the catalase-containing material comprises a catalase coating on an implantable device.

23. The system of claim 22, wherein the implantable device has a protective coating thereon.

30 24. The system of claim 23, wherein the protective coating is a cellulose tubing.

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25. The system of claim 23, wherein the protective coating is a water-permeable hydrogel.

26. The system of claim 22, wherein the implantable device is selected from the group consisting of catheters, central venous lines, dialysis catheters, temporary
5 IVC filters, surgical implants, peripheral vascular graft material, orthopedic implants, and hernia mesh.

27. The system of claim 17, wherein the ultrasound imaging system is operating in B-mode.

28. The system of claim 17, wherein the location of interest is an *in vitro* fluid
10 sample from a patient.

29. The system of claim 17, wherein the location of interest is a tissue site in a live subject.

30. The method of claim 15, wherein the catalase-containing material is adapted for introduction using a method selected from the group consisting of
15 percutaneous injection, intravenous injection, intravenous infusion, implantation of a medical device, and insertion of a medical device through a body orifice.

31. A method for visualizing the presence and distribution of hydrogen peroxide (H_2O_2) using ultrasound imaging, comprising:

introducing a catalytic material into a location of interest, wherein the catalytic
20 material is adapted to convert H_2O_2 into water and oxygen bubbles.

32. A method for detection of localized hydrogen peroxide within a tissue location, comprising:

introducing a catalase-containing particle into a tissue location directly or
intravenously;

25 positioning an ultrasound transducer over the tissue location; and
generating an ultrasound image to detect microbubbles, wherein the presence of microbubbles indicates that H_2O_2 is present.

33. The method of claim 32, wherein the catalase-containing particle comprises a particle shell that is permeable to H_2O_2 .

30 34. The method claim 33, wherein the particle shell is silica or hydrogel.

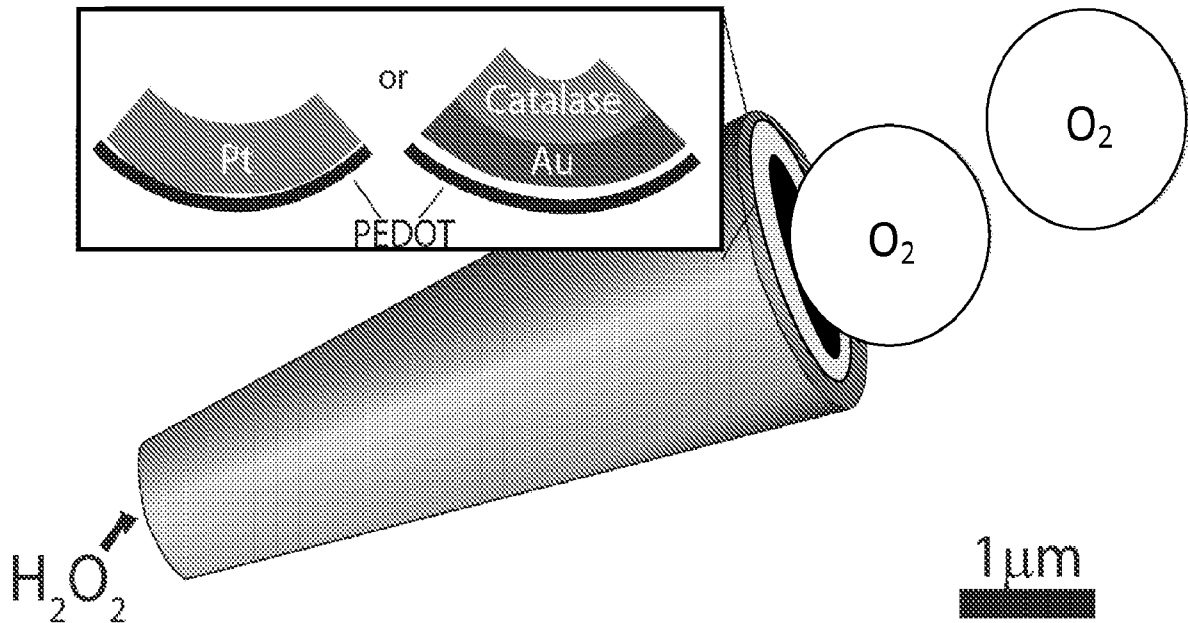


FIG. 1A

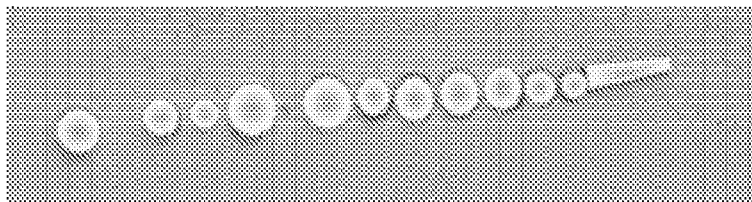


FIG. 1B

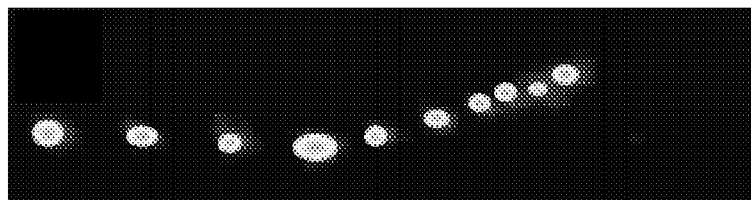


FIG. 1C

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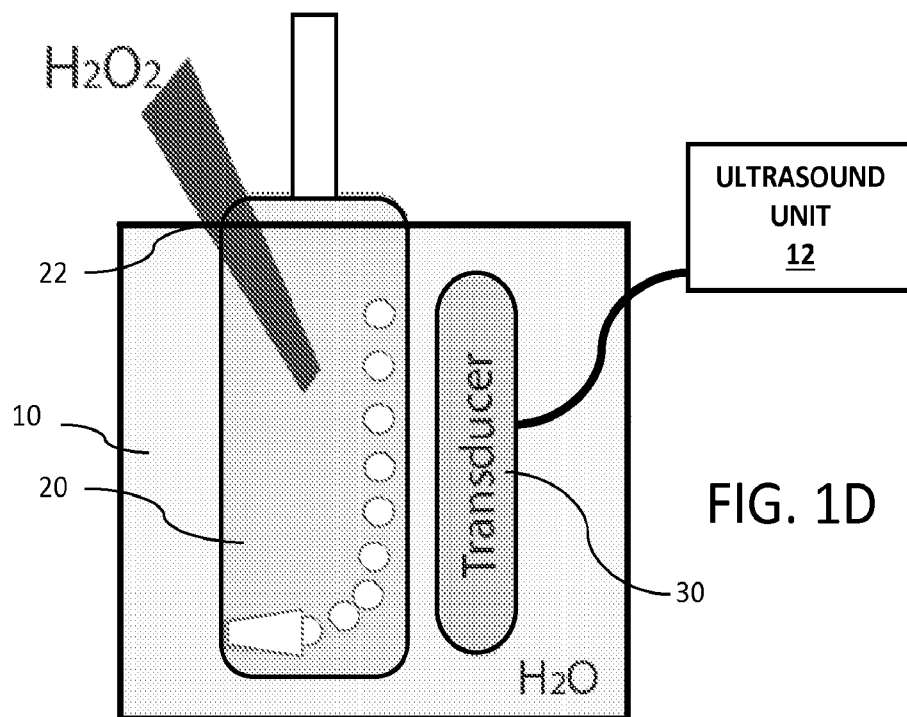


FIG. 1D

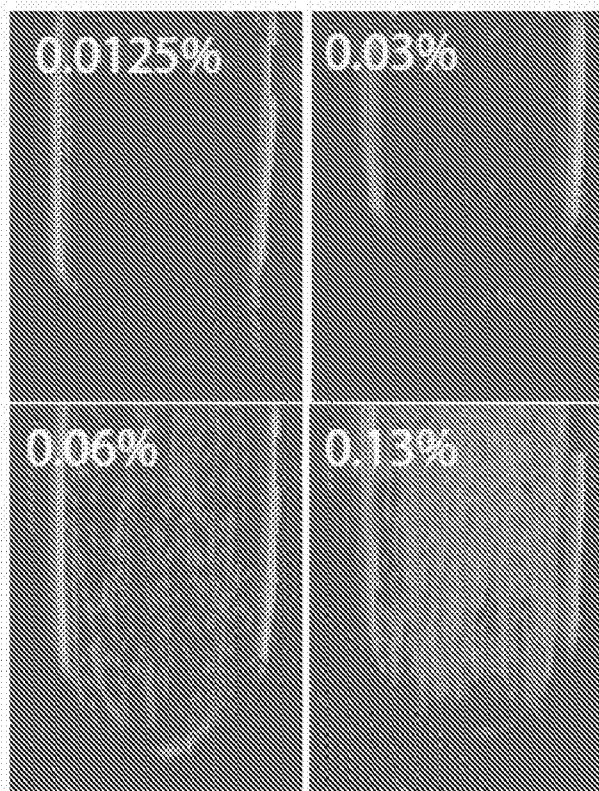


FIG. 1E

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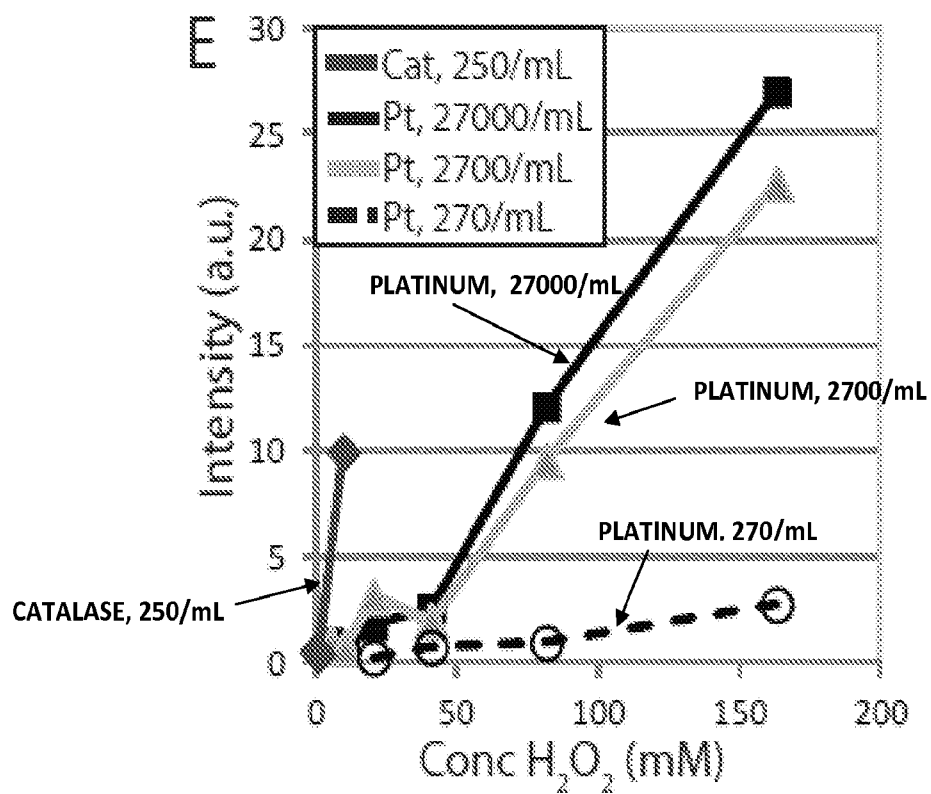


FIG. 1F

FIG. 2A

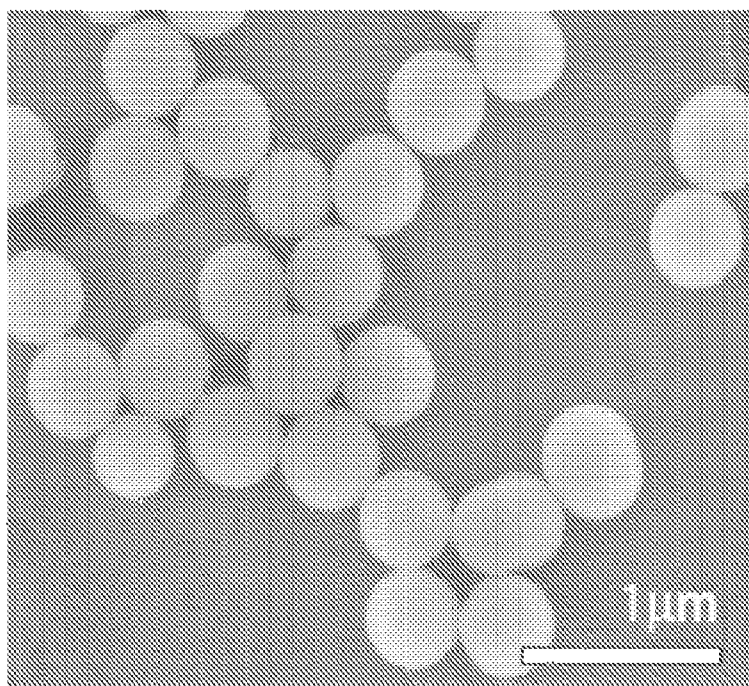
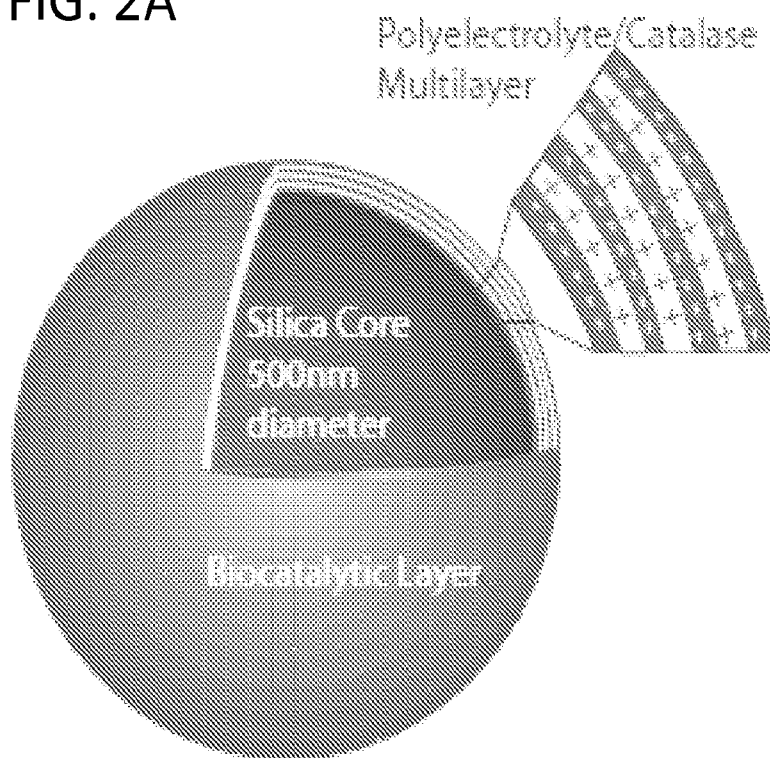


FIG. 2B

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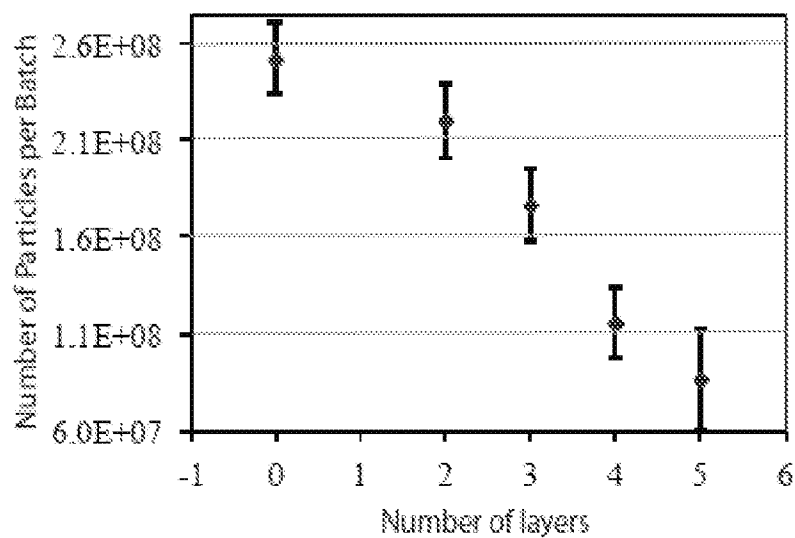


FIG. 2C

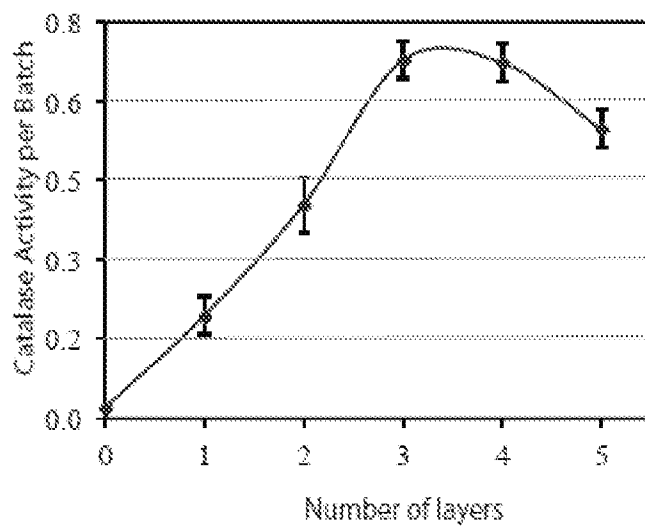


FIG. 2D

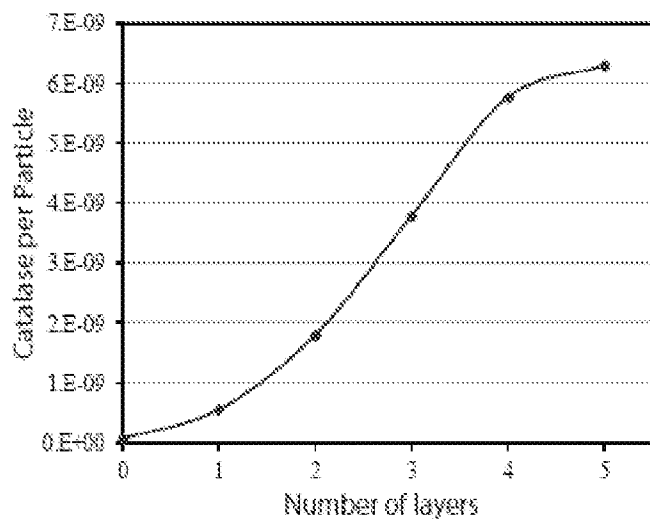


FIG. 2E

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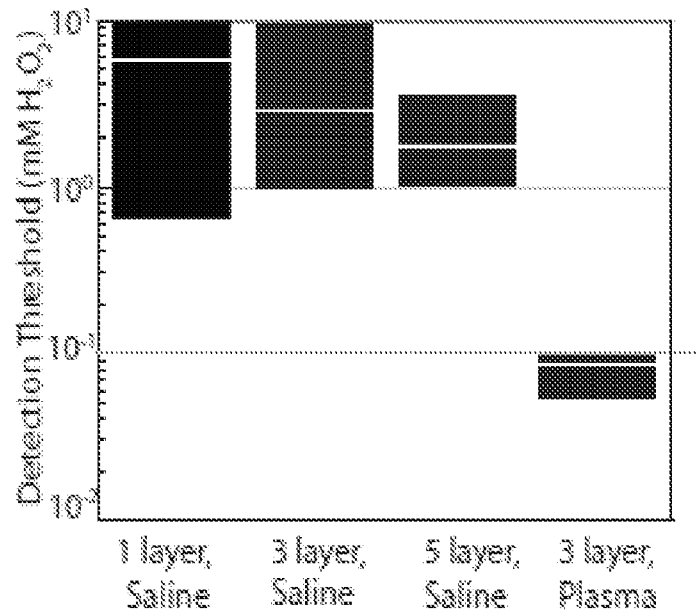


FIG. 2F

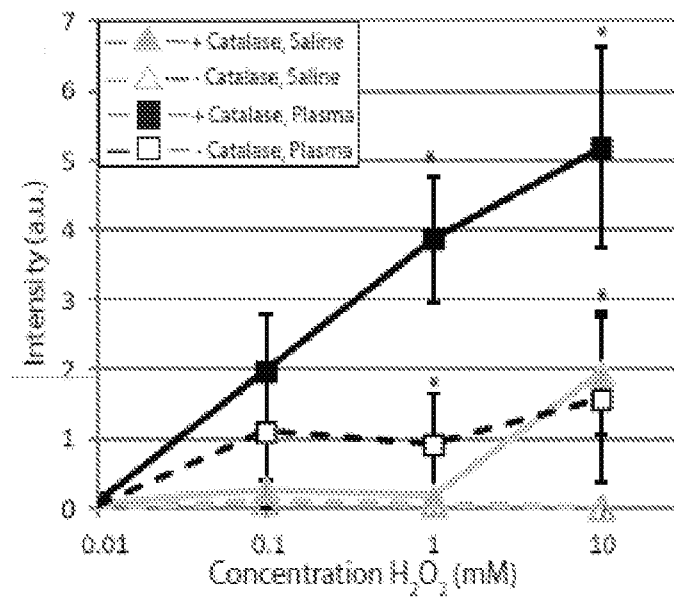


FIG. 2G

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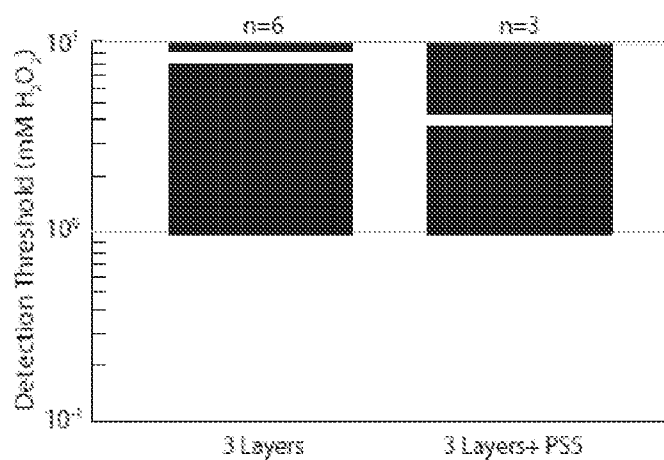


FIG. 2H

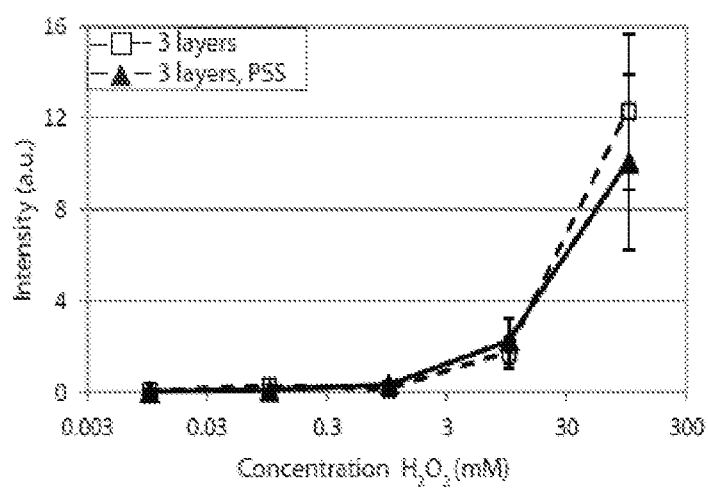


FIG. 2I

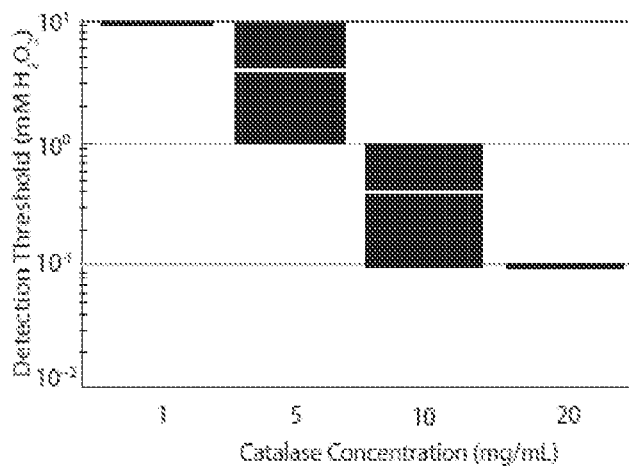


FIG. 2J

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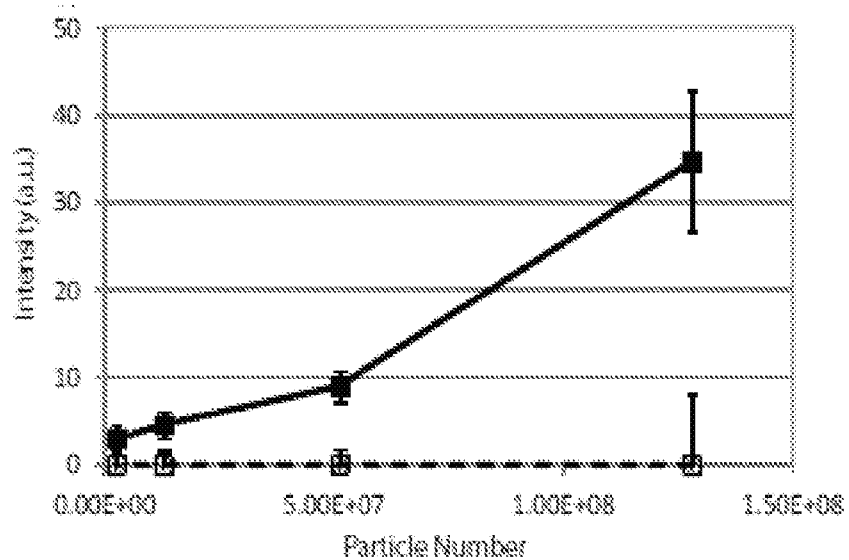


FIG. 2K

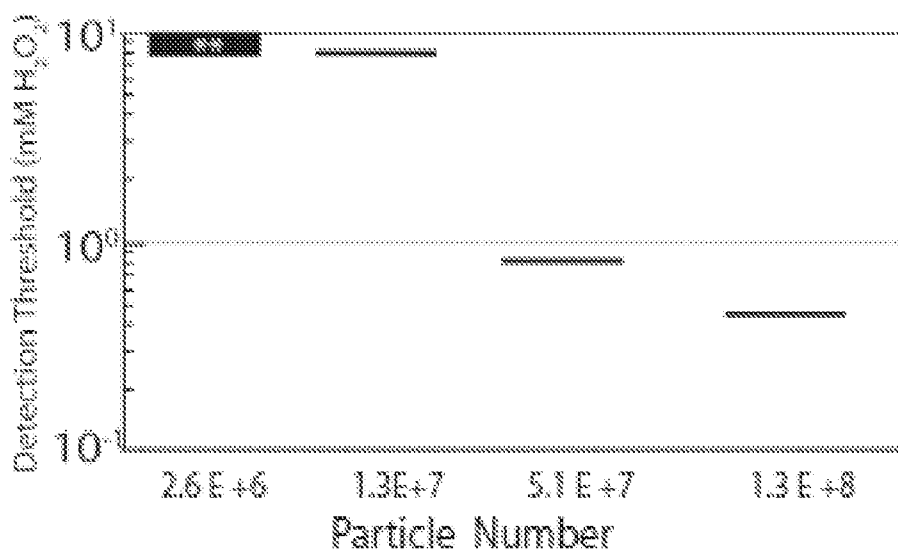


FIG. 2L

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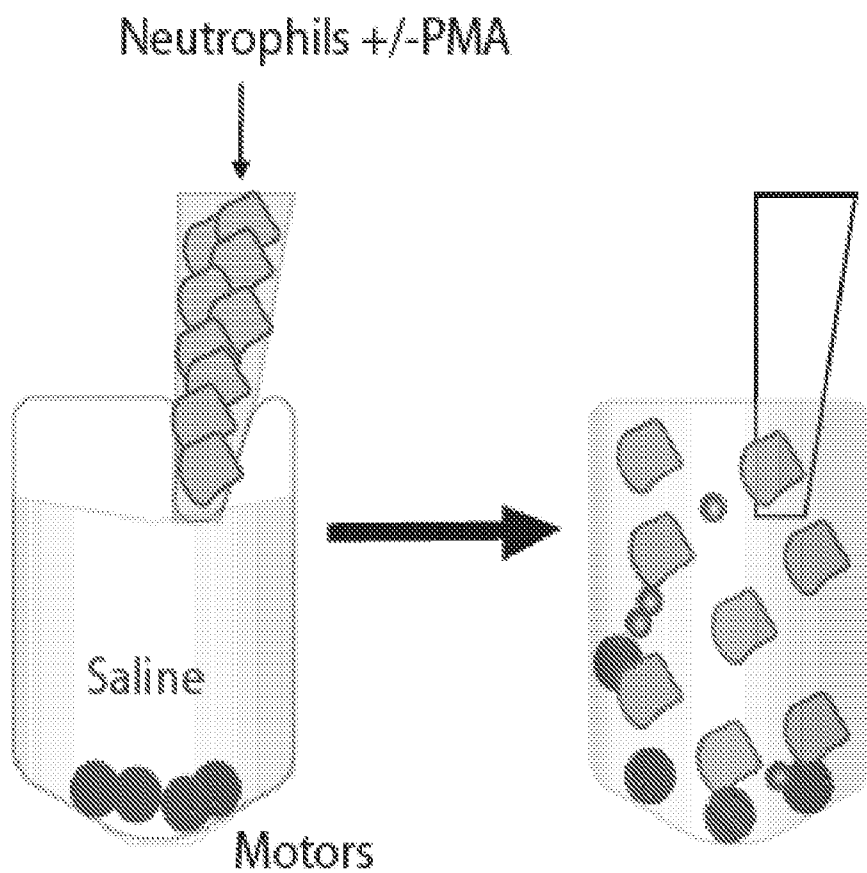


FIG. 3A

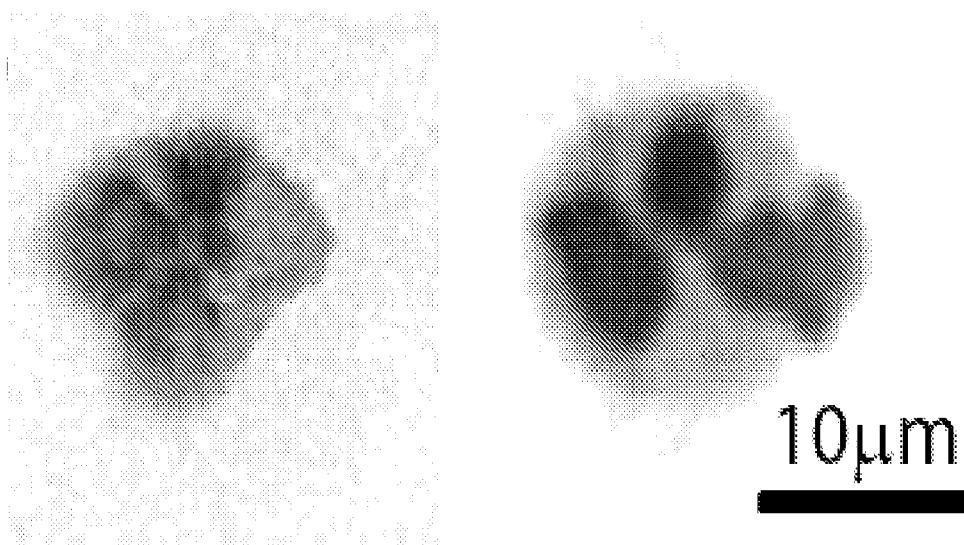


FIG. 3B

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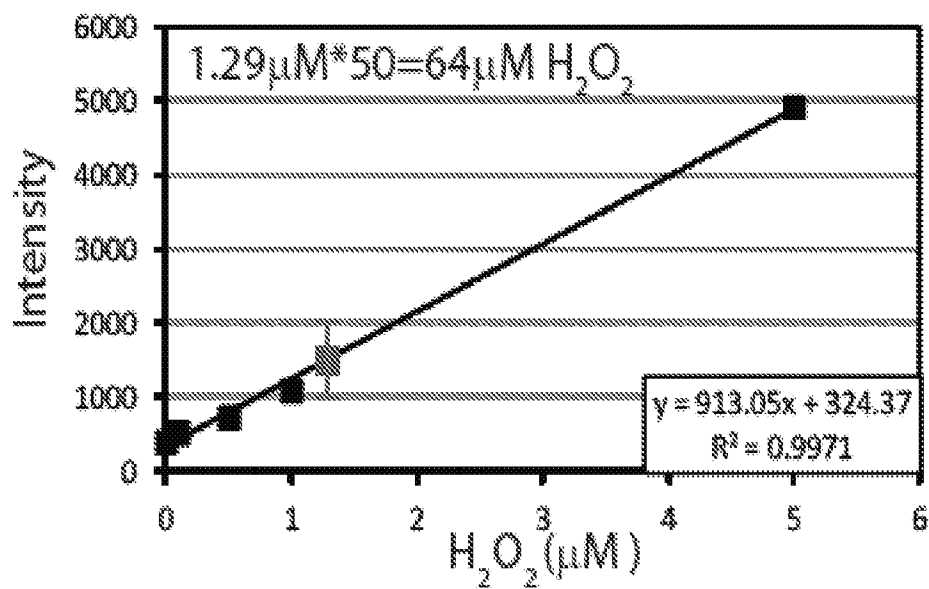


FIG. 3C

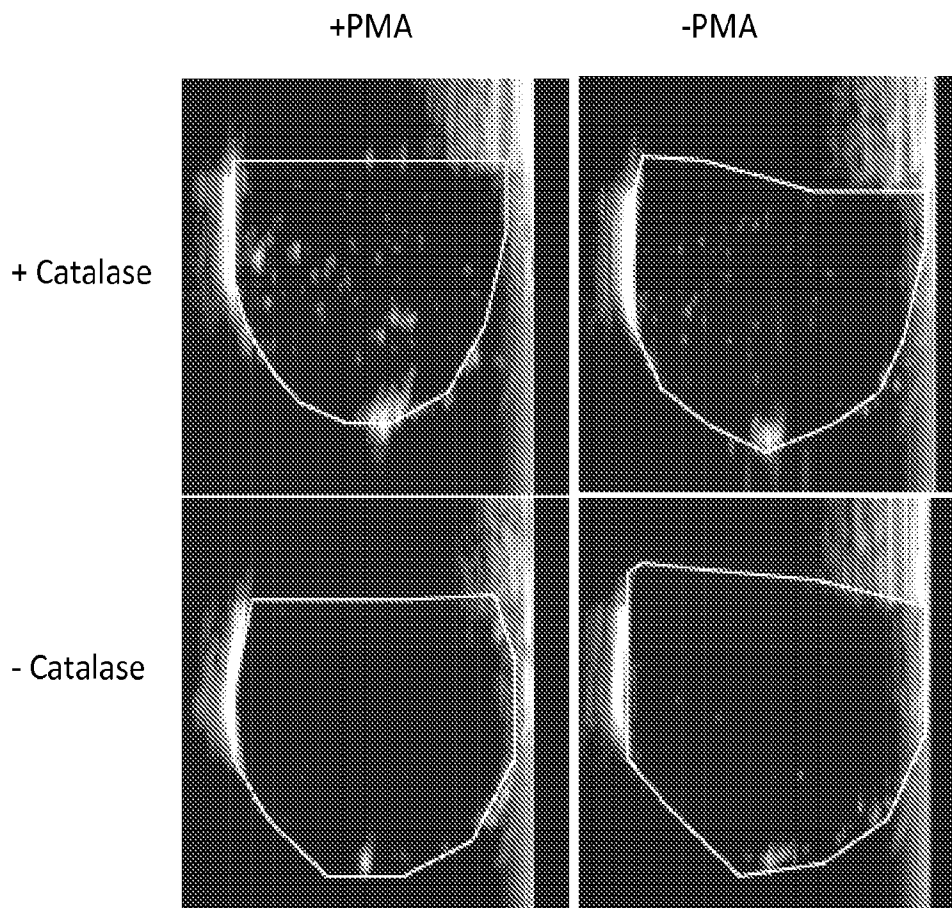


FIG. 3D

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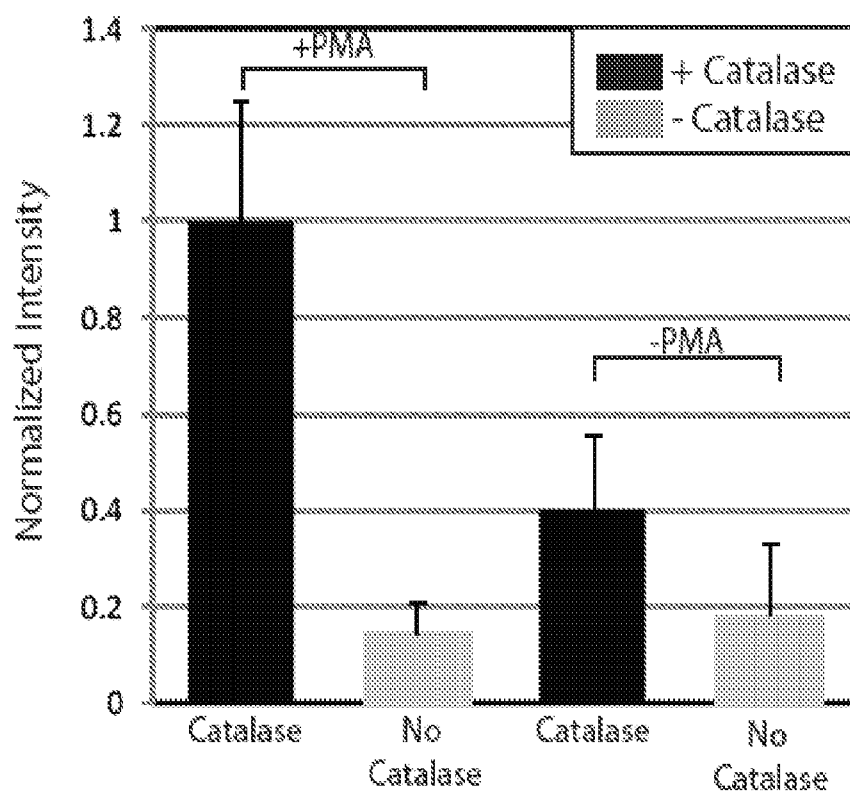


FIG. 3E

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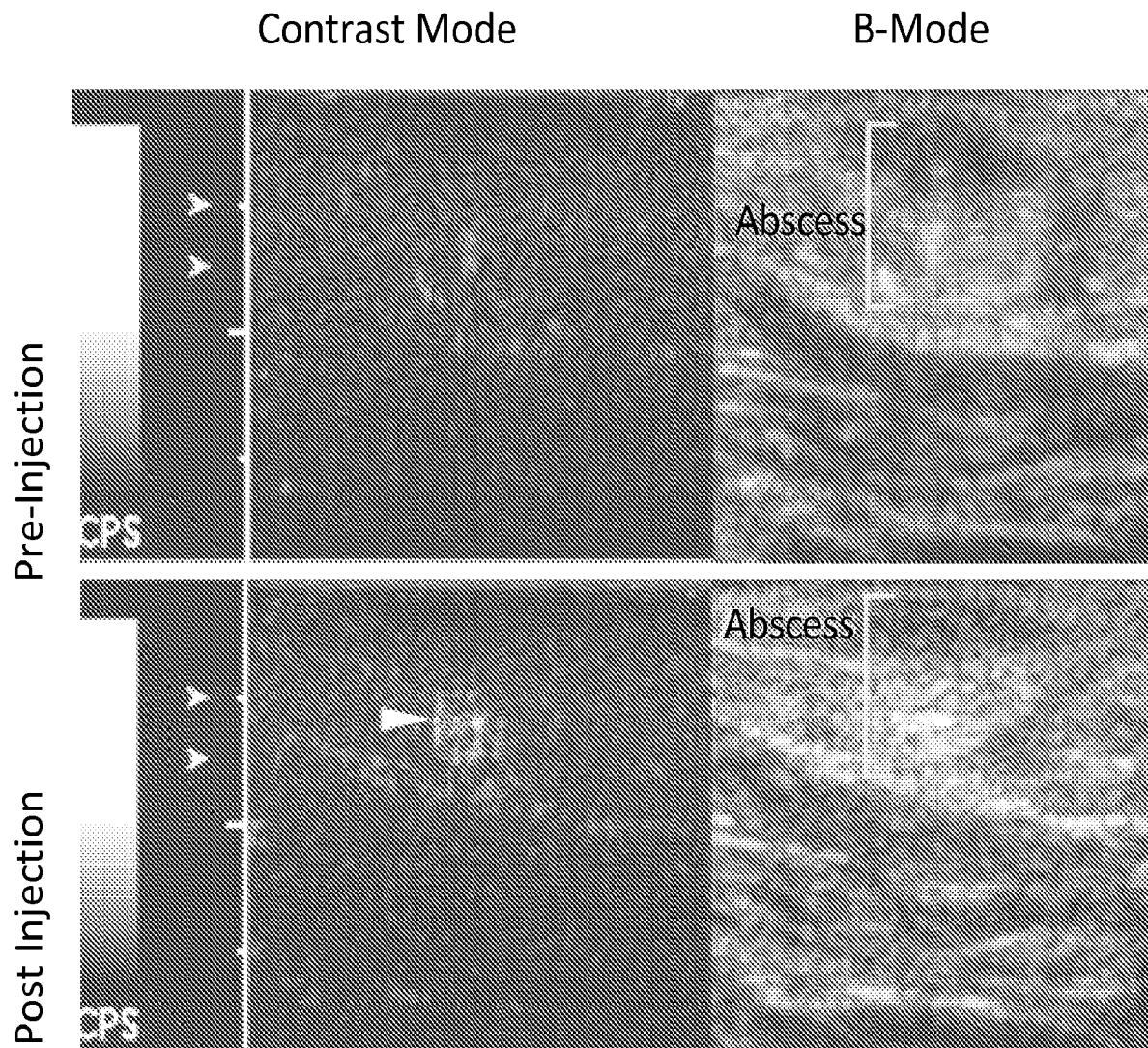


FIG. 4A

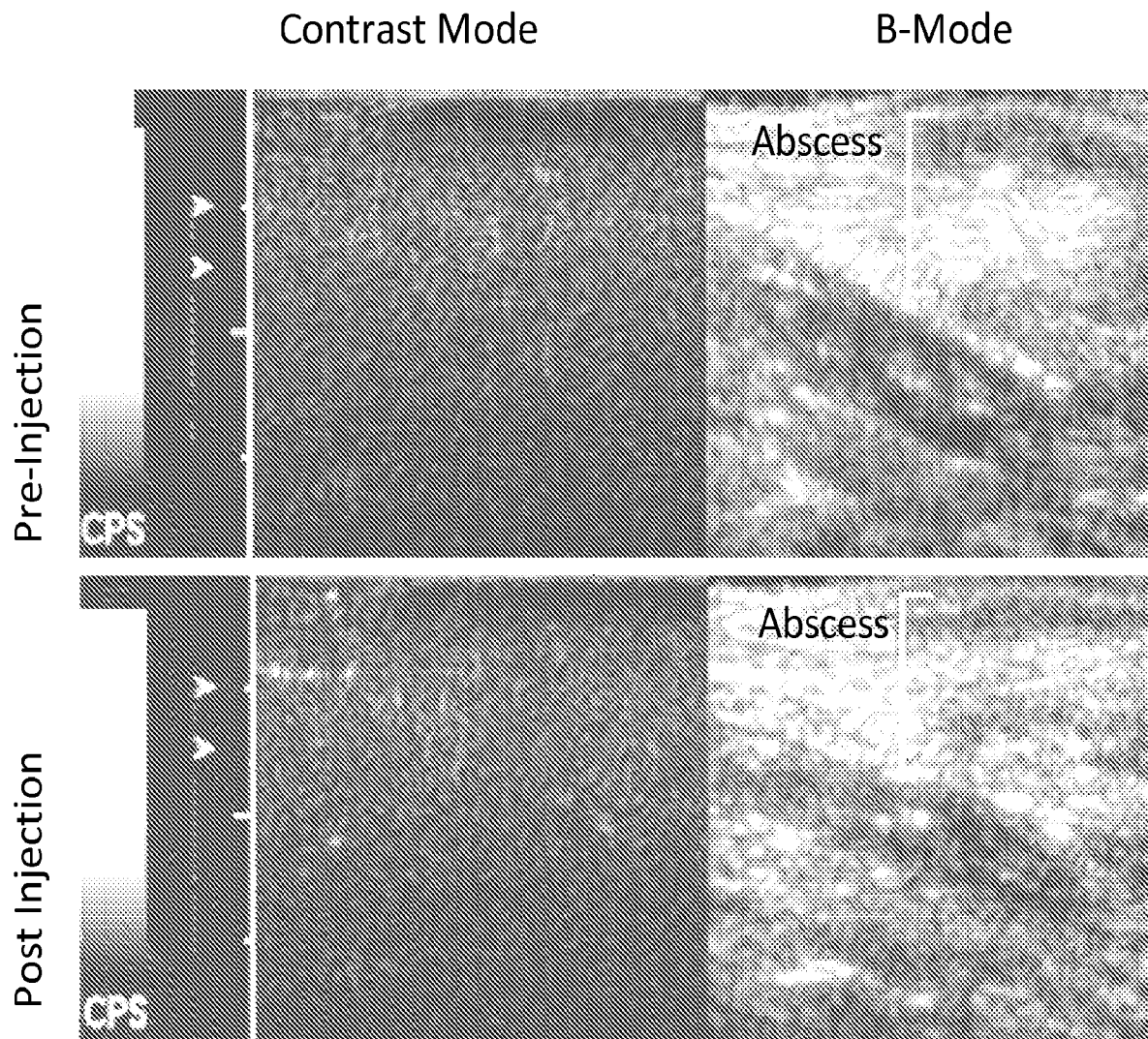


FIG. 4B

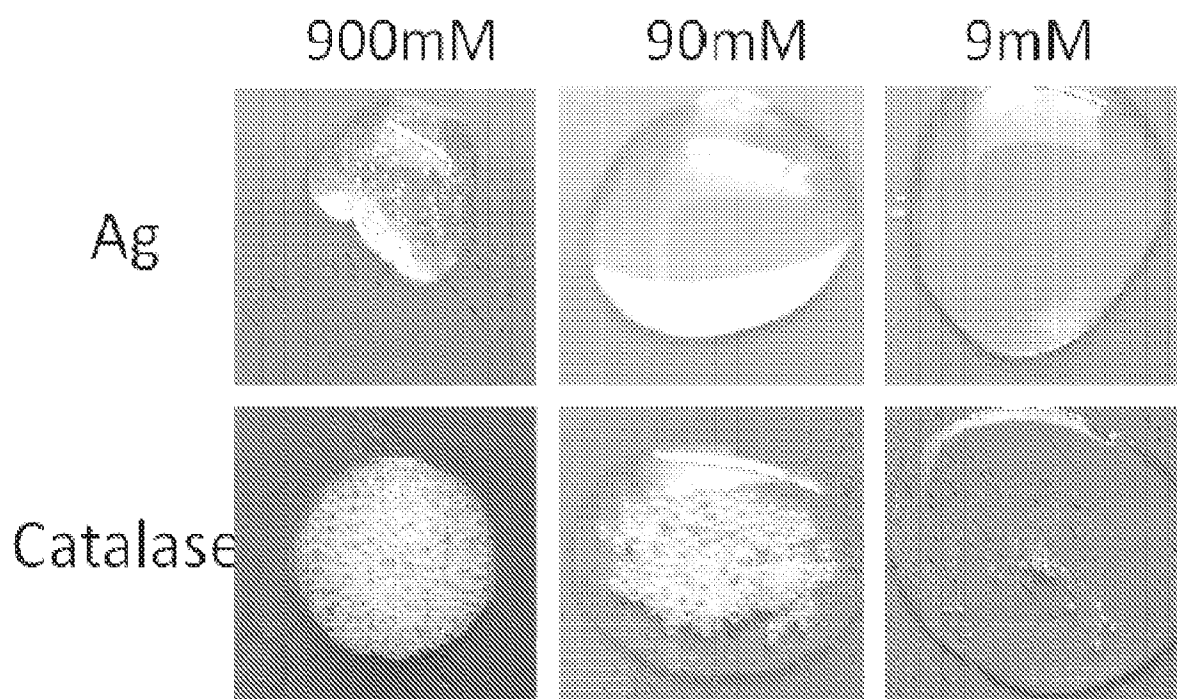
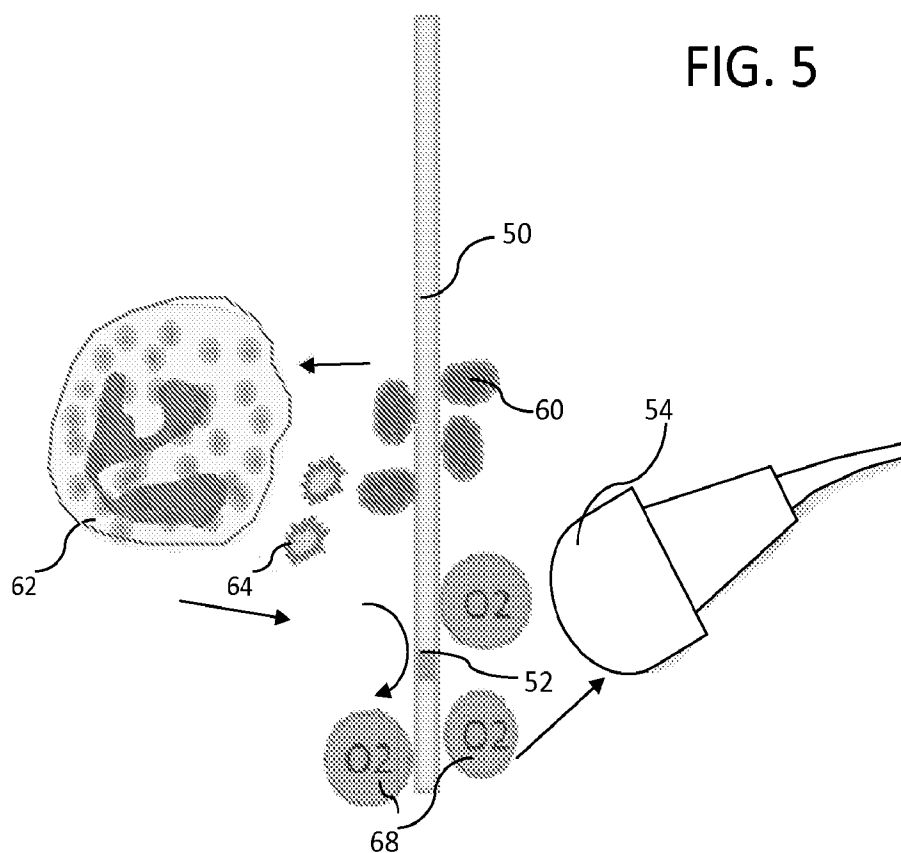


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/045274

A. CLASSIFICATION OF SUBJECT MATTER		<i>A61B 5/0215 (2006.01)</i> <i>A61B 5/0245 (2006.01)</i> <i>A61B 5/042 (2006.01)</i> <i>A61B 5/04 (2006.01)</i> <i>A61B 8/00 (2006.01)</i> <i>A61K 49/22 (2006.01)</i>																					
According to International Patent Classification (IPC) or to both national classification and IPC																							
B. FIELDS SEARCHED																							
Minimum documentation searched (classification system followed by classification symbols)																							
A61B 5/0215, 5/0245, 5/042, 5/04, 8/00, A61K 49/22																							
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																							
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)																							
Esp@cenet, PCT Online, PatSearch (RUPTO internal), PubMed, Google, USPTO, AIPN																							
C. DOCUMENTS CONSIDERED TO BE RELEVANT																							
Category*	Citation of document, with indication, where appropriate, of the relevant passages		Relevant to claim No.																				
Y	US 4340448 A (UNIVERSITY OF PITTSBURGH) 20.07.1982, abstract, claims 1, 8		1-11, 13-20, 22-32 12, 21, 33, 34																				
A																							
Y	RU 2022016 C1 (GUDKOVA LUDMILA VASILIEVNA) 30.10.1994, example 12		1-11, 13-16, 30, 31, 32																				
Y	RU 2345793 C2 (BRACCO INT. B.V.) 10.02.2009, p. 2, 3, 8, 12, 13, 15, claim 36		1-11, 13-20, 22-29, 31, 32																				
Y	RU 2016901 C1 (NAUCHNO-PROIZVODSTVENNOE OBEDINENIE "BIOPREPARAT") 30.07.1994, claims		14, 28, 30																				
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.																							
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"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																				
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																				
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																				
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																				
"P"	document published prior to the international filing date but later than the priority date claimed																						
Date of the actual completion of the international search		Date of mailing of the international search report																					
15 August 2013 (15.08.2013)		24 October 2013 (24.10.2013)																					
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1		Authorized officer T. Negulyaeva																					
Facsimile No. +7 (499) 243-33-37		Telephone No. (495)531-64-81																					

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/045274

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EA 9836 B1 (SINVESHN AG) 28.04.2008, claims 1, 4-7, 14, 15, 18, 21, 23, 24, 27	2, 3, 6-11, 16, 18, 19, 22-26, 30
A	RU 2114637 C1 (SONUS FARMASJUTIKALZ INK.) 10.07.1998, abstract, p. 1-6, claims 14, 16	1-34

专利名称(译)	用超声波检测体内过氧化氢的方法和系统		
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其他公开文献	EP2858554A1		
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摘要(译)

提供了一种方法和系统，用于通过将含过氧化氢酶的材料引入感兴趣的位置，在该位置上定位超声换能器，并产生超声图像以检测微泡，检测组织内的局部过氧化氢，其中微泡的存在指示局部过氧化氢的存在。过氧化氢酶涂覆的表面可以是微管的内表面，纳米球或微球的表面上或并入纳米球或微球中，或可植入装置。