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(71) **Applicant** *(for all designated States except US)*: **INTELLIGENTNANO INC.** [CA/CA]; c/o 11216 - 23A Avenue, Edmonton, Alberta T6J 4X7 (CA).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **XING, James** [CA/CA]; 11216 - 23A Avenue, Edmonton, Alberta T6J 4X7 (CA). **LU, Wiebing** [CA/CA]; c/o 11216 - 23 A Avenue, Edmonton, Alberta T6J 4X7 (CA).

(74) **Agent**: **BENNETT JONES LLP**; 1000 ATCO Centre, 10035 - 105 Street, Edmonton, Alberta T5J 3T2 (CA).

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(54) **Title**: TRANSFECTION WITH MAGNETIC NANOPARTICLES AND ULTRASOUND

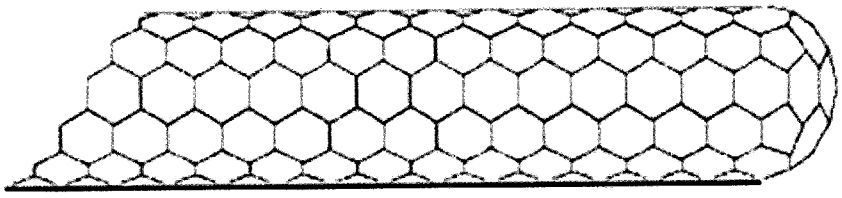


FIG 1A

(57) **Abstract**: The invention includes a magnetic nanoparticle molecular delivery vehicle to be used for transfection and delivery of therapeutic molecules across cell membranes and to specific sites in the body, using magnetic forces and ultrasound.

TRANSFECTION WITH MAGNETIC NANOPARTICLES AND ULTRASOUND

FIELD OF THE INVENTION

This invention relates to a nanostructured molecular delivery vehicle for delivering molecules to a selected site, and a method for transporting the molecular delivery vehicle across a biological membrane by applying a magnetic force and ultrasound.

BACKGROUND OF THE INVENTION

Transfection is the introduction of foreign genetic material into eukaryotic cells using a vector as a means of transfer. The term transfection is most often used in reference to mammalian cells, while the term transformation is preferred to describe DNA transfer in bacteria and non-animal eukaryotic cells such as fungi, algae and plants.

Existing methods of transfection must overcome problems with the permeability of the cell membrane and the solubility of the transfected particle.

Drug delivery often involves transportation of the drug across cell membranes. The most basic method *in vivo* method is to introduce the drug into the blood stream by oral or intravenous methods and then hope it is absorbed by the correct cells. This non-discriminatory technique requires relatively large doses of the drug and simply does not work for some molecules such as DNA, which is used in gene therapy.

Existing methods to transfect material into a cell can be grouped into two categories: viral and non-viral. The utilization of viruses to transfect material into a cell has been shown to be extremely efficient; however, the possibility of a immune response to viruses and the insertion of mutagens into the body have proven to be serious concerns, especially in clinical trials. Non-viral drug delivery methods include naked DNA injection and electroporation. Unfortunately, naked plasmid DNA injection has shown

to have a relatively low efficiency of gene delivery, while following electroporation tissue damage caused by the electric pulses has been observed.

Microinjection is a mechanical technique that utilizes a precision tool to place the molecule directly into the cell. This works excellently for DNA, however it is impractical in many situations as it can only be applied to one cell at a time.

A gene gun is a mechanical device that fires a particle bonded to the bio-molecule into the cell. These particles are relatively large and often damage cells. They also require large doses to be effective.

Electroporation is a physical method, which creates pores in the cell membrane by applying an electric shock to the cell. These pores allow the increased diffusion of materials into the cell. This increased permeability allows for easier transfection.

Sonoporation is similar to electroporation except it uses ultrasound to stimulate the cell membrane. The ultrasound also creates turbulence in the fluid surrounding the cell, which increases the rate of diffusion across the membrane.

Calcium phosphate transfection is a chemical method, which is very cheap. It uses calcium phosphate bonded to DNA. This molecule in some cases is able to transfect cells; however, this method is often ineffective and limited.

Viral delivery is a very effective method because viruses naturally are a carrier of genetic information and are very adept at entering cells. This makes them an obvious choice to help deliver DNA molecules into cells. However, the use of viral vectors is sometimes undesirable because of their immunogenicity and their potential mutagenicity. Furthermore, viral delivery is non-specific and can trigger side effects in the host.

Yet another method uses magnetic force and a molecular delivery vehicle to cross the cell membrane. A version of this method is described in United States Patent

Application 2007/0231908 A1, and requires that the molecular delivery vehicle be oriented before it penetrates the biological membrane.

For most of the above methods, the effectiveness is extremely variable depending on the cell type being transfected. Some cells are known to be harder to transfect then others and these are usually the cells that hold the greatest reward.

Therefore, there is a need in the art for methods of transporting biomolecules and other molecules of interest into cells which mitigate the difficulties of the prior art.

SUMMARY OF THE INVENTION

The present invention provides for transfection of cells using nanoparticles and magnetic forces to direct the nanoparticles through a cell wall or membrane. In one embodiment, the nanoparticle is directed through a cell membrane, a nuclear membrane, or a cell membrane *in vivo* such as the blood-brain barrier. In one embodiment, the invention further comprises the use of ultrasound to increase the permeability of the biological membranes. This results in greater efficiency or molecular delivery or transfection.

This invention comprises the following aspects (a) a method of creating nanoparticles, which are nontoxic, magnetic, and bondable to biological molecules or other molecules of interest; (b) a method of bonding such molecules to this nanoparticle; and (c) a system to force these nanoparticles through a membrane using a magnetic field. In one embodiment, ultrasound in the form of low-intensity pulsed ultrasound (LIPUS) is used increase the permeability of the membrane.

In one aspect, the invention comprises a method of delivering a molecule across a cell membrane using a delivery vehicle comprising a magnetic nanoparticle, the method comprising the steps of:

(a) fixing the molecule to the nanoparticle;

(b) positioning the nanoparticle in the immediate vicinity of the cell membrane;

(c) subjecting the nanoparticle and cell membrane magnetic field; and

(d) simultaneously subjecting the nanoparticle and cell membrane to ultrasound.

The nanoparticle comprises bonding sites so that the molecule can be attached to this nanoparticle. The number of bonding sites is variable as is the spacing between bonding sites. In addition, the type of bond may be covalent, ionic or another bond which is capable of fixing the molecule to the nanoparticle. In one embodiment, the molecule may comprise a genetic material such as DNA or RNA, proteins, or any other biological molecule.

The nanoparticle may comprise nanotubes, such as carbon nanotubes, or single-walled carbon nanotubes. In one embodiment, the nanoparticles may be biodegradable or biocompatible, and may comprise silica. The nanoparticles may display low or no toxicity to cells *in vivo* or *in vitro*.

On a macroscopic scale, this magnetic force can be used to control the molecular delivery vehicles to move to certain parts of a body. On a microscopic to nanoscale level, this force can be used to force the molecular delivery vehicles through a biological membrane. If necessary or desired, the molecular delivery vehicle can be further transported into the nucleus of the cell by moving it with a magnetic force.

This membrane may be the cell wall or the wall of the nucleus inside the cell, or another biological membrane such as the mitochondrion's double membrane. This membrane could also be the blood-brain barrier. Thus, this invention may allow for the transportation of molecules into the central nervous system.

Thus using this method, a bio-molecule can be delivered to a specific target.

In one embodiment, the invention comprises a molecular delivery vehicle which comprises a nanostructure which is magnetic and has bonding sites so that a bio-molecule can be attached to this molecular delivery vehicle. The number of bonding sites is variable as is the spacing between bonding sites. In addition, the type of bond may be covalent, ionic or another bond which is capable of holding the biomolecule.

Using this magnetic force the magnetic nanoparticle can be controlled in numerous ways. In one embodiment, the delivery vehicles can be collected in one location using a magnetic force that attracts to that location, such as an organ or specific tissue in vivo.

In one aspect, the invention comprises a method for using the molecular delivery system to deliver molecules into cells or transfect such cells *in vitro* or *in vivo*. *In vitro* cells may be supported on solid or liquid media.

BRIEF DESCRIPTIONS OF THE DRAWINGS

In order that the above-recited and other features and advantages of the present invention will be readily understood, a more particular description of the invention is given. Specific examples thereof are detailed, the result of which are illustrated in the appended figures. Any example is only a single embodiment of the invention, and is not to be considered in any way the limit of its scope. In the accompanying figures:

Figure 1A is a sketch of a magnetic single walled nanotube and Figure 1B is a sketch of a spherical magnetic nanoparticle after it has been functionalized.

Figure 2 shows the delivery vehicle being forced though the cell membrane. The arrows depict the magnetic field. In this depiction the carbon nanotube is being used for the delivery.

Figure 3 depicts the use of a magnet to collect the nanoparticles at a certain location in the body. In this case the particles are being collected at the top of the patients left arm.

Figures 4A, 4B, 4C, and 4D show schematic processes for functionalizing a single-walled nanotube.

Figure 5A and 5B show XPS and IR spectra for carboxylated SWNTs.

Figures 6A and 6B show IR and UV-vis spectra for FITC labelled SWNT. The vertical axis A shows absorption.

Figure 7A shows a confocal microscopy image showing control cells. Figure 7B shows cells a confocal microscopy image showing cells with FITC labelled nanoparticles in the cytoplasm. Figures 7C and 7D show confocal microscopy of MCF-7 control cells and cells transfected with nanoparticles bound with GFP plasmid.

Figure 8A show distribution of FITC labelled nanoparticles in control MCF-7 cells and Figure 8B shows distribution in MCF-7 cells exposed FITC labelled magnetic nanoparticles and a magnetic field.

Figure 9 shows a graph of percentage uptake by MCF-7 cells.

Figures 10A, 10B, and 10C show FITC labelled nanoparticles delivered into hematopoietic stem cells in a control, after 3 hours and after 6 hours, respectively.

Figure 11 shows a graph demonstrating viability of MCF-7 cells after FITC labelled nanoparticle uptake compared to control cells.

Figure 12A shows FACS results for Negative control sample contained no GFP plasmid, no Definity, and was not sonicated. FACs results: Marker: M1, % Gated: 0.16. Extremely high cell viability is observed. Figure 12B shows FACS results for Positive control sample contained 2 μg of GFP plasmid, no Definity, the lipofection agent PEI, and was not sonicated. FACs results: Marker: M1, % Gated: 33.12%. Very low cell viability is observed.

Figure 13 shows FACS results FACs results for sample sonicated at 0.5 W/cm2, with a 20% duty cycle for 60 seconds. DNA plasmid concentration was varied. Figure 13A - DNA plasmid concentration: 2 μg/mL, marker: M1, % Gated: 16.20. Figure 13B - DNA plasmid concentration: 15 μg/mL, marker: M1, % Gated: 26.93. Figure 13C - DNA plasmid concentration: 30 μg/mL, marker: M1, % Gated: 32.51. A high amount of cell viability is seen in all cases.

Figure 14 shows FACs result for sample sonicated at 0.3 W/cm2, with a 100% duty cycle for 60 seconds. DNA plasmid concentration was 30μg/mL. FACs results: marker: M1, % Gated: 14.67. Cell viability is observed to have decreased.

Figure 15 FACs result for sample sonicated at 0.5 W/cm2, with a 100% duty cycle for 60 seconds. DNA plasmid concentration was 30 μg/mL. FACs results: marker: M1, % Gated: 32.12. Cell viability is observed to be low.

Figure 16 shows a picture of a biocompatible silica nanotube.

Figure 17 shows a graph of IR spectra of Si-NT which has been carboxylated.

Figure 18 shows HeLa cells which have been transfected with Si-NT-GFP plasmid, compared with a control.

Figure 19 shows a graph demonstrating low toxicity of the Si-NT after 48 and 72 hours of incubation.

DETAILED DESCRIPTION OF PREFERED EMBODIMENTS

This invention comprises a method to deliver biomolecules or other molecules of interest into cells using a a molecular delivery vehicle, which is magnetically drivable and capable of bonding to at least one bio-molecule. This molecular delivery vehicle can pass through the cell wall with the aid of an external magnetic force.

"Biomolecule" – a biological molecule that performs some function which influences the behavior or nature of a biological system.

"Magnetic nanoparticle" – any particle on the nanoscale (having one dimension less than about 100 nm) the motion of which is influenced by a magnetic field.

"Nanoscale" – the range of lengths used to measure objects from 0.1nm up to 1000nm where 1 nm is 10^{-9} meters.

"Transfect" – a process to introduce foreign genetic material into a cell.

The present invention relates to the use of magnetic nanoparticles to transport biomolecules and other molecules of interest across a cell membrane.

In one embodiment of the present invention, the magnetic nanoparticles take the form of a metal core coated in a material such as carbon as shown in FIG 1B. These nanoparticles are then functionalized so that a bio-molecule can be bonded to them.

In one embodiment of the present invention, the magnetic nanoparticles are carbon nanotubes, such as single-walled carbon nanotubes (SWNT) embedded with magnetic metal atoms (FIG 1A). In one embodiment, the magnetic metal atoms comprise nickel, iron or cobalt.

Single-walled carbon nanotubes are well known in the art and may be synthesized using any suitable technique, such as chemical vapor deposition technique (CVD). These carbon nanotubes are grown from a surface using nickel or yttrium, or both nickel and yttrium, as seed. In one embodiment, the nickel and/or yttrium is thus incorporated at least into the tip of the carbon nanotube. In one embodiment, suitable SWNTs have a diameter between about 1.2 to about 1.5 nm, and a length of about 2 to about 5 μm. The SWNTs may be either armchair or chiral nanotubes. In one embodiment, the SWNTs used are armchair (5,5) nanotubes.

The magnetic nanoparticles or carbon nanotubes are prepared for bonding to a bio-molecule by adding functional groups to them. These functional groups act as the bonding site, which will hold the bio-molecule to the nanoparticles or the carbon nanotubes. In addition, functionalization is important as many nanoparticles or carbon nanotubes, particularly SWNTs, are insoluble in water. Functionalization increases their water solubility.

In one embodiment, shown schematically in Figures 4A and 4B, functionalization is achieved by chemically altering the surface of the carbon nanotube. In one example, the surface of the magnetic carbon nanotube is carboxylated and the carboxylic acid is reacted with thionyl chloride to provide an acid chloride. The acid chloride may then be coupled with *tert*-butyl-12-aminododecylcarbamate, or *tert*-butyl (2-aminoethyl) carbamate, followed by deprotection of the Boc group to provide the amine derivative.

In an alternative embodiment, amine derivative nanotubes can be produced by reacting the acid chloride nanotube with then 2'-(ethylenedioxy)bis(ethylamine) to produce the amine derivative, as shown in Figure 4C. In a further alternative, the amine derivative may be formed using ethane – 1,2 diamine, as shown in Figure 4D.

In one example, the amine derivative is then reacted with fluoroscein isothiocynanate (FITC) giving rise to the FITC derivatized magnetic carbon nanotube.

These magnetic carbon nanotube bonded molecules may then be subjected to a magnetic field and a cell culture, as described herein.

Biomolecules such as DNA or RNA can be attached to carboxyl functional groups on the surface of the nanoparticle or carbon nanotube. In one example, plasmid vectors may be combined with carboxylated SWNTs and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 2-[N-morpholino]ethane sulfonic acid (MES) or a phosphate buffer (pH 4.5) for the aminization between the primary amine groups in the DNA molecules and carboxylic groups on the nanotubes. Alternatively, DNA or RNA can be

bound by electrostatic interaction with amine functional groups on the surface of the nanoparticle.

The nanoparticles may comprise silica or other materials which may be biodegradable or biocompatible within a cell, such as, without limitation, nanocellulose, or nanocrystalline cellulose. The term "biodegradable" as used herein means that the substance may be broken down into innocuous products by the action of living things. The term "biocompatible" means that the substance does not have toxic or injurious effects on biological function of cells either *in vitro* or *in vivo*. In one embodiment, a carbon nanotube may be coated with silica and the carbon then removed or burnt out, leaving a silica nanotube based on the carbon template. The silica nanotube may then functionalized using methods similar to those described herein for carbon nanotube, and as are known to those skilled in the art.

Once the biomolecule or other molecule of interest is bonded to the magnetic nanoparticle, the nanoparticle is placed in a solution along with the cells that are to be transfected and a magnetic force is applied so that the nanoparticles are accelerated through the solution. Inevitably, these will collide with a cell and there will be a probability that the particle will pass through the membrane into the cell, as shown schematically in FIG 2. If the particle does not enter the cell, it will be free to accelerate again to attempt to transfect another cell. A substantial majority of the cells will be transfected after a relatively short period.

The magnetic field that is used to drive the molecular delivery vehicles is configured so that it provides a magnetic force which can be static or variable in direction and magnitude. In one embodiment, the magnetic field is configured so that the magnetic force can change between being variable and static at different stages of delivery. In one embodiment, the magnetic nanoparticles can be caused to move in complex paths by constantly varying magnetic force, which is changing its magnitude and direction.

In another embodiment, the delivery vehicles can be moved in complex paths and at variable velocities and accelerations.

In one embodiment, the membrane that must be transfected can be made more permeable by applying ultrasound energy to the cell culture, such as low-intensity pulsed ultrasound. The ultrasound may be applied at higher frequencies than is known to enhance cell growth. Typically LIPUS has been used at frequencies less than about 1 MHz, however, in embodiments of the present invention, any frequency between 1 MHz to 2 MHz may be used, such as 1.5 MHz.

Ultrasound can be applied using conventional or slightly modified therapeutic ultrasound transducers. The intensity of the ultrasound energy may vary from 0.1 W/cm^2 to about 1.0 W/cm^2. In one embodiment, the intensity is between about 0.3 W/cm^2 to about 0.5 W/cm^2. Varying duty cycles and pulse repetitions may be used, such as a duty cycle between about 20% and 100% and a repetition frequency of 100 Hz. In general, higher intensities and longer duty cycles will increase movement across cell membranes, at the expense of cell viability. Total ultrasound energy, calculated as follows, should preferably not exceed a level where cell viability is substantially impaired.

Energy (J) = Intensity * Duty Cycle * Time

In one embodiment, total energy may optimally be 18000 mJ.

Suitable ultrasound contrast agents, such as Definity™ (Bristol-Myers Squibb) may be used to promote microcavitation in the vicinity of the cells.

In one embodiment, the magnetic nanoparticles may be used *in vivo* to deliver therapeutic agents such as drugs or biomolecules to a specific target. A magnet may be placed at the site where the magnetic nanoparticles are to be focused, as shown in Figure 3. As the magnetic nanoparticles circulate through the body, they will

accumulate at the site where the magnet is located. Thus, the nanoparticles deliver the biomolecules to a specific target region.

In one embodiment, this targeted delivery mechanism may be used to deliver molecules into difficult to access areas, such as across the blood-brain barrier into the central nervous system. The magnetic nanoparticles can be collected at a specific site of the blood brain barrier using a magnetic field. Then, using a magnetic force these nanoparticles can be forced across the barrier.

Once the nanoparticles have been concentrated at a specific point or region, the nanoparticles can be forced into cells at that site by using a magnetic force with rapidly alternating direction. This will excite the particles to move back and forth quickly. As they move around they will collide with the cell membrane and at least a portion of the particles will pass through the membrane into the cell. In one embodiment, the use of ultrasound and magnetic forces may be used to enhance such movement *in vivo*. Ultrasound transducers which apply ultrasound energy to the human body are well known for imaging purposes, and may be used for the molecular delivery systems described herein with little or no modification.

The present invention may be embodied in other specific forms without departing from its structures, methods, or other essential characteristics as broadly described herein and claimed hereafter. The described embodiments are to be considered in all respects only as is, therefore, indicated by the appended claims, rather than by the foregoing description. All changes that come within the meaning and equivalence of the claims are to be embraced within their scope.

EXAMPLES

The following examples are intended to be illustrative of the described invention, and not be limiting of the invention claimed herein, except where specifically recited.

[0001] Example 1 – Synthesis of FITC-labelled carbon single-walled nanotubes (SWNT) (Scheme shown in Figure 4B)

Nickel containing carbon nanotubes were refluxed with 3N HNO_3 for 45 h to introduce carboxylic acid groups. After refluxing, the solution was diluted with deionized water, filtrated and washed several times with deionized water. The acid treated SWNTs were collected and dried under vacuum.

100 mg of SWNTs were stirred in 20 mL of $SOCl_2$ (containing 1 mL of dimethylformamide) at 70 ^{0}C for 24 h. After centrifugation, the brown-colored supernatant was decanted and the remaining solid was washed with anhydrous tetrahydrofuran. After centrifugation, the pale-colored supernatant was decanted. The remaining solid was dried under vacuum.

A mixture of the resulting SWNTs and 1 g of *tert*-butyl-2-aminoethylcarbamate was heated at 100° C under an argon atmosphere for 100 h. After cooling to room temperature, the excess *tert*-butyl-2-aminoethylcarbamate was removed by washing with methanol. The resulting black solid was dried under vacuum.

The coupling product of SWNTs with *tert*-butyl-2-aminoethylcarbamate was suspended in MeOH and a solution of HCl in dioxane (4 N) added, the resulting mixture was stirred at room temperature for 4 h. Then anhydrous ethyl ether was added, the resulting precipitate was collected and dried under vacuum.

The amine groups-containing SWNTs were suspended in a mixture of DMF and diisopropylethylamine and a solution of fluoroisothiocyanate (FITC) in DMF was added. The resulting mixture was stirred for 4 h at room temperature in darkness. Then anhydrous ethyl ether was added, the resulted precipitate was collected by centrifugation and washed thoroughly with ethyl ether and methanol, dried under vacuum to give FITC-labeled SWNTs.

In an alternative method, shown schematically in Figure 4C, SWNTs from Aldrich were oxidized to form carboxylic acid groups on the surface. These nanotubes were reacted with thionyl chloride and then 2'-(ethylenedioxy)bis(ethylamine) to produce amine-terminated nanotubes. The amine was then reacted with FITC to attach FITC to SWNTs.

Example 2 – IR, XPS andUV-vis Characterization

To validate the all synthesis take place, all of the intermediates shown in Fig. 4C and final product (SWNT-FITC) were characterized by Infrared (IR), X-ray photoelectron spectroscopy (XPS) and UV-vis and the results are shown in Figures 5 and 6. IR data clearly show that SWNTs were successfully functionalized to give carboxylic groups and XPS data show that about 6.1 % of the carbon atoms are present as carboxyl groups. The UV-vis spectrum of the FITC-labeled SWNT in water is shown in Figure 8, for comparison, the UV-vis spectrum of the FITC in water is shown in the same figure.

Example 3 - Fluorescent Staining and Imaging

FITC-labeled SWNTs (CNT-FITC) as prepared using the method described in Example 1 (Fig. 4B) were used to stain and image a human breast adenocarcinoma cell.

Materials

- Cell - MCF-7
- Medium - GIBCO 11330, DMEM/F12 (1:1)
- Formaldehyde Solution(w/v) 16%, Methanol-free, Pierce, Cat# 28906
- Hoechst - Invitrogen Cat# 33342
- Rhodamine Phalloidin - Invitrogen Cat# R-415

  (Rhodamine Phalloidin 300U was dissolved in 1.5ml Methanol to form concentration of 200 units/ml, distributed them into 10ul each vial, store at -20 ^{o}C)

- PBS buffer
- Block buffer - PBS/0.5%BSA
- Magnets- Applied Magnets Cat#ND075 (www magnet4less.com) 2 X 1 in thick disc, Grade N42, Rare earth Neodymium super strong magnet (Pull force: 176

lbs)

Round cover slips were placed into a 6-well or 24-well plate, one cover slip into one well and MCF-7 cells into each well, cell number: $1X10^5$/ml, and incubated at 37°C over night. Add Hoechst into each well (1ul Hoechst in 1ml medium) and icubate at 37°C for 1h. 1ml of *CNT-FITC* was added into each well of the plate (except the control) and incubate at 37°C for 1h. Each well was washed 3 times with PBS.

A cover slip picked out of one well with tweezers, and vertically inserted into a beaker containing 10ml serum-free medium supplemented with *CNT-FITC* (10:1, medium: *CNT-FITC*) was placed on hotplate (magnetic stirrer) with the cells facing the incoming nanotubes for 3 min. The speed of the stirrer was set at 1,200rpm. The cover slip was laid on one dish containing serum-free medium without *CNT-FITC*, and the dish was placed on a magnet for 7min. The cover slip was then washed 3 times with PBS and placed in another 24 well plate, along with cover slips which were not placed on a magnet.

The cells were fixed with 4% Formaldehyde Solution for 10min(or over night at 4 °C). The formaldehyde solution was removed and the cells washed 3 times with PBS. 250ul of PBS/0.2 TX-100 was added onto the cover slips in the wells and place at room temperature for 10min. Again the cells were washed 3 times with PBS, and blocked with 250ul of PBS/0.5%BSA for 20min. 2.5ul Rhodamine Phalloidin was added to 50ul block buffer and the mix pipetted on parafilm. The cover slip was overlaid onto the solution in place for 30min

The cover slips were then placed back to the plate and washed 3 times with PBS. The coverslips were then mounted onto slides and send for the confocal microscopy. Samples were imaged with a laser scanning confocal microscopy 510 (Carl Zeiss) equipped with Axiovert 100M microscopy (Zeiss), a F-Fluar 40X-1.3 NA oil lens and 3 different lasers (Uv, Argon/2 and HeNe1).

As shown in Figure 7A and 7B, the cell nuclei fluoresce strongly as a result of the Invitrogen stain which combines with double-stranded DNA. In Figure 8B, fluorescence of the FITC moities may be plainly seen within the cells cytoplasm, indicating that the CNT-FITCs have passed through the cell membranes and into the cytoplasm.

In another example, SWNT were conjugated to GFP plasmid (pDRIVE5-GFP) by covalent bonding using EDC and a phosphate buffer. The SWNT-GFP plasmid was then incubated with MCF-7 cells for 3 min, followed by 7 minutes with a magnetic field supplied by a magnetic stirrer. The cells were then incubated for 24 hours and confocal microscopy was used to confirm GFP expression. Figure 7D shows results of GFP fluoresence within the cells, as compared to the control cells in Figure 7C.

Example 4 – Cell Uptake Efficiency

FITC-labeled SWNT was delivered into adherent MCF-7 breast cancer cells. Following the delivery and recovery phases, the fluorescently-labelled SWNT was detected by confocal microscopy. The results are presented in Figure 8A and 8B. The data clearly shows that the SWNT crossed the cell membrane and entered the cell cytoplasm and even into the nucleus (refer to the green dots in Figure 8B; some of them are pointed by the arrows). The uptake rate is about 90% shown in Figure 9.

In addition to delivery of FITC to adherent cells, like MCF-7 cells, we also successfully delivered FITC into difficult-to-transfect cells, or suspension cells, like hematopoietic stem cells (HSCs). Figure 10 shows the delivery results. The results show that SWNT can deliver FITC into HSCs. As time increases to 3 and 6 hours, more FITC enters the cell (FITC shows as green fluorescence). The control sample showed no internal fluorescence.

Example 5 – Cell Viability

Furthermore, it is worth noting that cell viability was not compromised by SWNT uptake when compared with control, as shown in Figure 11. Viability of MCF-7 cells after FITC-SWNT uptake with exposure to a magnetic field was compared to the control cells and cells exposed to SWNT alone with no magnetic field. Cells exposed to SWNT appear to substantially similar to control populations for viability after 6 hours.

Example 6 – Ultrasound Delivery (USD) - Cell preparation and DNA

USD and transfection was assessed using human breast adenocarcinoma cells (MCF-7). Cells were maintained in the IMDM medium with 10% fetal bovine serum. Cells were harvested a day before the experiment by adding 0.25% Trypsin to the culturing flask and waiting for detachment. 1 mL of cells was added to 10 mm x 35 mm dishes with an additional 1 mL of medium. Cell concentration was approximately 1.5 x 105 cells/mL.. To determine transfection, green fluorescence protein plasmid (GFP plasmid-pDRIVE5-GFP) was added to the medium 15 minutes before sonication. Various concentrations of GFP were used: 2 μg/mL, 15 μg/mL, and 30 μg/mL. The ultrasound contrast agent Definity, purchased from Lantheus Medical, was used to promote cavitation. The UCA volume used was 140 μL.

USD was performed using the Excel UltraMax therapeutic ultrasound machine, probe radius 2.5 cm. The ultrasound probe was coupled to the bottom of the cell dish using ultrasound gel. Ultrasound was applied for 60 seconds, at a 1 MHz frequency with varying output intensity: 0.3 W/cm2, and 0.5 W/cm2. The duty cycle was tested at 100% or 20% with a fixed pulse-repetition frequency of 100 Hz. As controls, we sonicated blank samples with no UCAs or GFP, and samples with GFP but no UCAs. Additionally, we ran a positive control using PEI, a lipofection agent. Finally, we prepared a sample that was not stimulated by ultrasound, but contained both Definity and GFP.

Cell counting was conducted in a fluorescence-activated cell-sorting (FACS) machine. 24hours after USD, cells were collected in FACS test tube with 0.25% trypsin and

washed once with 1xPBS. After all above, cells were resuspended in200uL 1% paraformaldehyde and tested through flow cytometry.

Cell viability was assessed by a cell count using a hemacytometer. After collecting cells in the FACS test tube, transfer 20µl of each sample into small centrifuge tubes and dilute with 0.4% trypan blue. Put 10µl in the hemacytometer and count cell number. Finally calculate the cell concentration with the following formula: Cell number counted in all squares/total number of squares counted * dilution factor* 1x104.

All the FACs test results are shown in Figs. 12 to 15. Our negative control samples did not yield any transfection, but maintained excellent cell viability, as seen in the FACs result. The PEI lipofection positive control showed GFP expression, and extreme cell death.

	GPF [µg]	Definity [µL]	W/cm^2-DC-sec	% Transfection
1.	O	0	0-0-0	0.16 %
2.	2	0	0-0-0	0.29 %
3.	2 + PEI	0	0-0-0	33.12 %

Table 1: Transfection results of positive and negative control

FACs analysis shows that as the exposure intensity increased the cell viability decreased. The maximum transfection was seen with an output intensity of 0.5 W/cm2 and a 20% duty cycle, at 32.51%. Cell viability is significantly lower at the output intensities above this level. This result suggests that the output energy achieved by a 0.5 W/cm2 and a 20% duty cycle, for 60 seconds is optimum for effective transfection.

The effect of DNA concentration on transfection efficiency was examined at every energy level. In every case, increasing the DNA concentration leads to an increase in transfection.

GFP [µg]	Definity [uL]	Output intensity, Duty cycle	Transfection %
2	140	0.5 W/cm^2, 20%	16.20%
15	140	0.5 W/cm^2, 20%	26.93%
30	140	0.5 W/cm^2, 20%	32.51%
2	140	0.3 W/cm^2, 100%	7.52%
15	140	0.3 W/cm^2, 100%	9.71%
30	140	0.3 W/cm^2, 100%	14.67%
2	140	0.5 W/cm^2, 100%	19.63%
15	140	0.5 W/cm^2, 100%	26.76%
30	140	0.5 W/cm^2, 100%	32.12%

Table 2: Transfection results for varied ultrasound output intensity, and GFP concentration.

MCF-7 cells were used to evaluate the effects of ultrasound on gene delivery. We found that the efficiency of ultrasound mediated gene delivery, depended on plasmid concentration, while the viability of the cells was directly related to the ultrasound's output intensity. The latter could be due to the fact that the other physical effects of ultrasound, such as transient increase of local temperatures and pressure, are detrimental to cells, or that the pores the cavitation effect opened were unable to re-seal.

The results from the negative control samples show that the DNA plasmid GFP is unable to diffuse across the cell membrane on its own. The USD results show that the application of ultrasound with UCAs allow the DNA plasmid to transfect and be expressed by the cell. Furthermore, our results demonstrate that there is an optimum ultrasound exposure level for transfection and cell viability; the existence of optimum exposure parameters is consisted with other literary results. The FACs results exhibit that any output energy greater than 18000 mJ is detrimental to cell viability, where:

*Energy (J) = Intensity * Duty Cycle * Time*

Due to the nature of the FACs analysis, the transfection results obtained from the 0.5 W/cm2, 100% duty cycle sample may be skewed. Since a high percentage of cells in this sample were dead, transfection percentage we obtained is misrepresented and cannot be compared to our results obtained with higher cell viability.

Plasmid concentration was an important factor in determining transfection efficiency. In every case, transfection rate increased with DNA concentration. This result leads us to consider the importance of DNA proximity to the cells during USD. However, it is expected that the effect of increasing plasmid concentration to increase transfection efficiency will eventually plateau.

The findings from the lipofection agent, PEI, revealed two results. First, it confirms that the plasmid GFP can be expressed by the MCF-7 cells, but more importantly it highlights the importance of USD. The FACs results show an extremely high amount of cell death due to PEI. In contrast, USD was able to obtain similar transfection efficiency while maintaining a much lower cell death rate.

Example 7 – Formation of Silica nanotubes

An amount of magnetic single-walled carbon nanotube powder was mixed with ground $Na_2SiO_3 \cdot 9H_2O$ ($Na_2SiO_3 \cdot 9H_2O$/carbon nanotube ratio was 0.2 by volume). The mixture was ground carefully for 10 min to mix the reactants uniformly. Excessively ground NH_4Cl (NH_4Cl /$Na_2SiO_3 \cdot 9H_2O$ = 3 by volume) was then added to the mixture. After being ground carefully for 50 min, the product was aged for 5 h and then washed three times with distilled water. Silicon dioxide coated nanotubes (Si-NT) were obtained after being dried at 60°C for 5 h.

Particles core level spectra were measured using X-ray photoelectron spectrometer (VG ESCALAB MK II). The excitation source was a Mg X-ray anode and HV equalled to 20 eV.

To determine crystallite sizes and phase purity of the powders, the X-ray diffraction spectrum was obtained with a Rigaku D/max-rA X-ray diffractometer using Cu Kα (λ =1.54056 Å) radiation.

Si-NT' morphology was observed with JEOL JEM 2010 transmission electron microscope (TEM) operating at 200 kV, as shown in Figure 16. TEM samples were prepared by dispersing a small amount of powder in ethanol. A drop of the dispersion was then transferred onto coated grid and died for observation.

Example 8 – Si-NT Functionalization

Oxidation of the Si-NTs: The Si-NTs (200 mg) were refluxed to introduce carboxylic groups. After refluxing, the solution was diluted with deionized water, filtered over a 0.2 μm polycarbonate filter (Millipore) and washed several times with deionized water. The sample was collected and dried overnight in a vacuum oven at 80°C to give Si-NT-2(170 mg).

The carboxylated Si-NT underwent IR spectrum analysis, with the results shown in Figure 17.

Reaction with thionyl chloride to give Si-NT-COCl: A suspension of Si-NT-2 (100 mg) in 20 mL of $SOCl_2$ together with 5 drops of dimethylformamide (DMF), was stirred at 70°C for 24 h. The mixture was cooled and centrifuged at 2000 rpm for 30 min. The excess $SOCl_2$ was decanted and the resulting black solid was washed with anhydrous THF (3 x 20 mL), dried overnight in a vacuum oven at 80°C to give Si-NT-3 (78 mg).

Coupling with ethylenediamine: The mixture of Si-NT-3 (50 mg) and anhydrous ethylenediamine (120 mL) was heated at 100°C for 100 h. During this time, the liquid phase became dark. After cooling, the mixture was poured into methanol (100 mL), centrifuged to give a black solid, which was washed several times with methanol. The resulting solid was dried overnight in a vacuum oven at 80°C to give Si-NT-4 (42 mg).

Functionalization with GFP plasmid: A suspension of the Si-NT-4 (25 mg) and GFP plasmid (5 mg) in anhydrous DMF (10 mL) was stirred in dark for 5 h, then the reaction mixture was poured into anhydrous ethyl ether (40 mL), centrifuged to give a black solid, which was washed with methanol until TLC (10 % MeOH in dichloromethane) showed no free GFP left. The product was dried overnight in a vacuum oven at 80 0C to get the final product (23 mg), Si-NT-GFP.

Example 9 – Transfection of HeLa

HeLa cells were grown in RPMI 1640 supplemented with 10% FB in 35mm Petri dish with a cover slip.

Si-NT-GFP solution was prepared by weighing 3mg Si-NT-GFP powder into 50ml centrifuge tube. 3ml of sterilized DI water was added and sonicated until the silica tube powder dissolve and incubated for 1hr at room temperature. The final volume was brought to 50ml using RPMI 1640 medium w/o FBS. A similar solution with Si-NT was prepared as a control. The test and control silica tube solutions were added to 100ml beakers.

200,000 cells were seeded per dish and cultured overnight allowing cells to attach. A volume of test or controls solutions were added to the dishes and the cells were then magnetically treated for 3min vertically by putting dishes on top of magnetic stir hot plate and followed by 7mins with Petri dishes on top of a stirring magnet.

The cells were washed twice with PBS, and replaced with 2 ml of culture medium. The dishes were returned to incubator and incubated for 24hr and 48hr, respectively.

Each of the samples were prepared for and viewed with confocal microscope observation of the GFP signal. The results are shown in Figure 18.

Toxicity studies showed that increasing concentrations of Si-NT had little effect on cell survival rate, as shown in Figure 19.

References - The following references are representative of the level of skill in the art and are incorporated herein as if reproduced in their entirety (where permitted).

R. King, "Gene Delivery to Mammalian Cells by Microinjection", from book "Gene Delivery to Mammalian Cells: Volume 1: Nonviral Gene Transfer Techniques", ISBN: 978-1-58829-086-1

E. Heleniusi, M. Boije, V. Niklander-Teeri, E. Tapio Palva and T. H. Teeri, "Gene Delivery into Intact Plants Using the Helios™ Gene Gun", Plant Molecular Biology Reporter, 18: 287a–287l, 2000

VF Tendeloo, P Ponsaerts, F Lardon, G Nijs, M Lenjou, C Broeckhoven, DR Bockstaele, ZN Berneman, "Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells," Blood. 2001 Jul 1; 98(1):49-56

H Pan, Y Zhou, F Sieling, J Shi, J Cui, C Deng, "Sonoporation of Cells for Drug and Gene delivery", Engineering in Medicine and Biology Society, 2004. IEEE Conference on EMBS, Vol 2, Sept. 2004 Page(s): 3531 – 3534

F Scherer1, M Anton, U Schillinger, J Henke, C Bergemann, A Krüger, B Gänsbacher and C Plank, "Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo", Gene Therapy, January 2002, Volume 9, Number 2, Pages 102-109

A Watson and D Latchman, "Gene Delivery into Neuronal Cells by Calcium Phosphate-Mediated Transfection", Methods, Volume 10, Issue 3, December 1996, Pages 289-291

G Beattie, E Goetzman, Q Tang, T Conlon, M Campbell-Thompson, D Matern, J Vockley, TR Flotte, "Recombinant adeno-associated virus-mediated gene delivery of long chain acyl coenzyme A dehydrogenase (LCAD) into LCAD-deficient mice", The Journal of Gene Medicine, Volume 10 Issue 10, Pages 1113 – 1123, Aug 2008

T Bettinger, R Carlisle, M Read, M Ogris, and L Seymour, "Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells", Nucleic Acids Res. 2001 September 15; 29(18): 3882–3891.

Z Liu, A Fan, K Rakhra, S Sherlock, A Goodwin, X Chen, Q Yang, D, Felsher, H Dai, "Supramolecular Stacking of Doxorubicin on Carbon Nanotubes for in vivo cancer therapy", Angew. Chem. Int. Ed., Volume 48, Issue 41, Pages:7668-7672, September 28, 2009.

M Prato; K Kostarelos; A Bianco; C D. Partidos, "Biomedical applications of functionalized carbon nanotubes", Chemical Communications, Volume 5, Pages 571 - 577, 2005.

Y. Sakakima, S. Hayashi, Y. Yagi, A. Hayakawa, K. Tachibana, and A. Nakao, "Gene therapy for hepatocellular carcinoma using sonoporation enhanced by contrast agents", Cancer Gene Therapy (2005) , 884–889

B. Patrick, P.C. Valerie, G. Adolfo, et al. "Naked DNA Injection for liver metastases treatment in rats". Hepatology. 2002; 35:1144–1152.

Y. Yamashita, M. Shimada, K. Tachibana, et al. "In vivo gene transfer into muscle via electrro-sonoporation", Hum Gene Ther. 2002;13:2079–2084.

M. W. Miller, D. L. Miller, and A. A. Brayman. "A review of in vitro bioeffects of inertial ultrasonic from a mechanistic perspective". Ultrasound Med. Biol. 22:1131–1154, 1996

T. Leighton, "The Acoustic Bubble". Academic Press, San Diego, 1997

"Gene therapy progress and prospects: Ultrasound for gene transfer Revised and Expanded", Marcel Dekker, Inc., pp4

D. Dalecki, S. Z. Child, C. H. Raeman, C. Cox, E. L. Carstensen, "Ultrasonically induced lung hemorrhage in young swine", Ultrasound Med Biol 1997a;23:777–781.

P. E. Huber, P. Pfisterer, "In vitro and in vivo transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound". Gene Ther 2000;7:1516 –1525.

H.D. Liang, Q. L. Lu, S. A. Xue, and M. Halliwell "Optimization of Ultrasound-mediated Gene Transfer (Sonoporation) in Skeletal Muscle Cells",

T. Kodama, D. O. Cosgrove, H. J. Stauss, T. A. Partridge and M. J. K. Blomley, Ultrasound in Med. & Biol., Vol. 30, No. 11, pp. 1523–1529, 2004

WE CLAIM:

1. A method of delivering a molecule across a cell membrane using a delivery vehicle comprising a magnetic nanoparticle, the method comprising the steps of:

(a) fixing the molecule to the nanoparticle;

(b) positioning the nanoparticle in the immediate vicinity of the cell membrane;

(c) subjecting the nanoparticle and cell membrane magnetic field; and

(d) simultaneously subjecting the nanoparticle and cell membrane to ultrasound.

2. The method of claim 1 wherein the ultrasound comprises low-intensity pulsed ultrasound.

3. The method of claim 1 wherein the nanoparticle comprises a single-walled carbon nanotube.

4. The method of claim 1 wherein the nanoparticle comprises a biodegradable or biocompatible material.

5. The method of claim 4 wherein the nanoparticle comprises silica.

6. The method of one of claims 1 to 5 wherein the molecule comprises a DNA or a RNA molecule.

7. The method of one of claims 1 to 5 which is practiced *in vivo*, and the delivery vehicle are concentrated in a specific region by a magnetic force placed adjacent the specific region, and forced across a cell membrane by a magnetic field.

8. The method of claim 7 wherein the magnetic field alternates direction across the specific region after concentration of the delivery vehicle.

9. The method of claim 7 or 8 wherein the specific region is behind or associated with the blood-brain barrier.

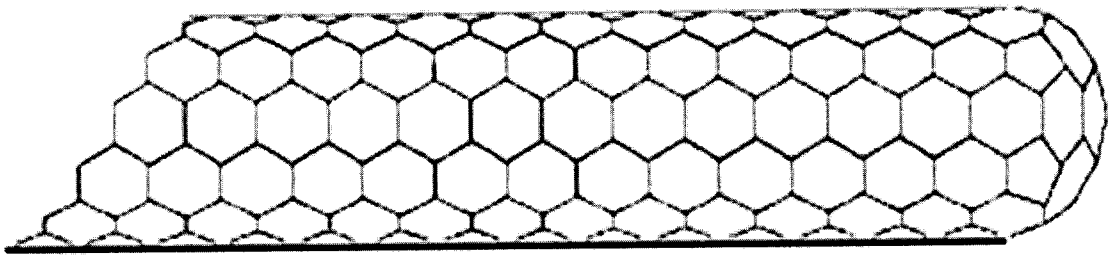


FIG 1A

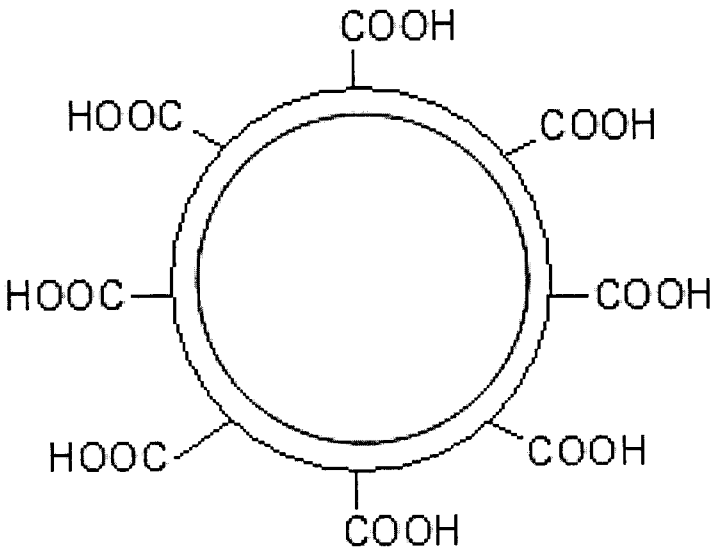


FIG 1B

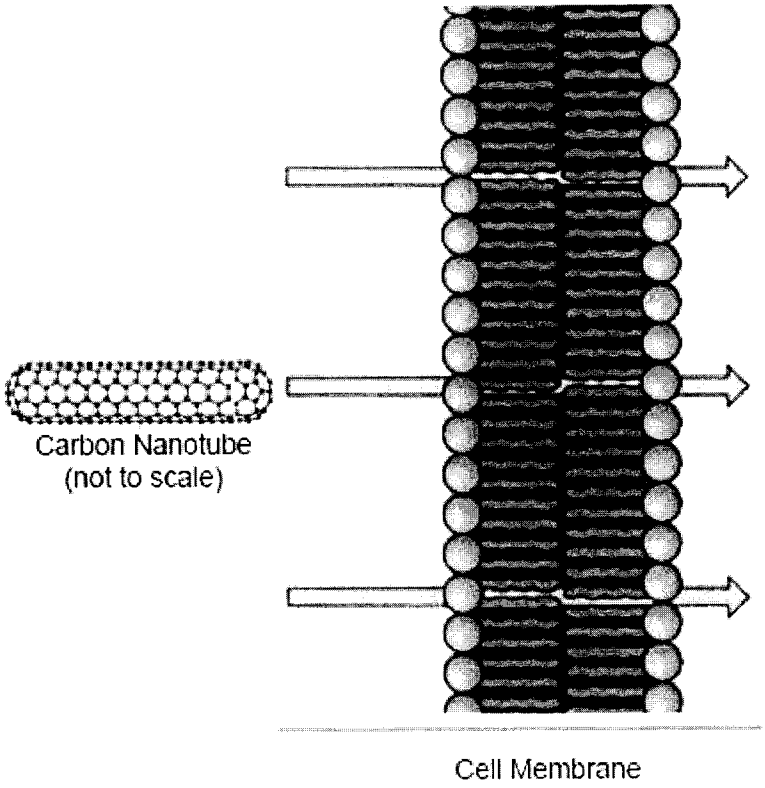


FIG 2

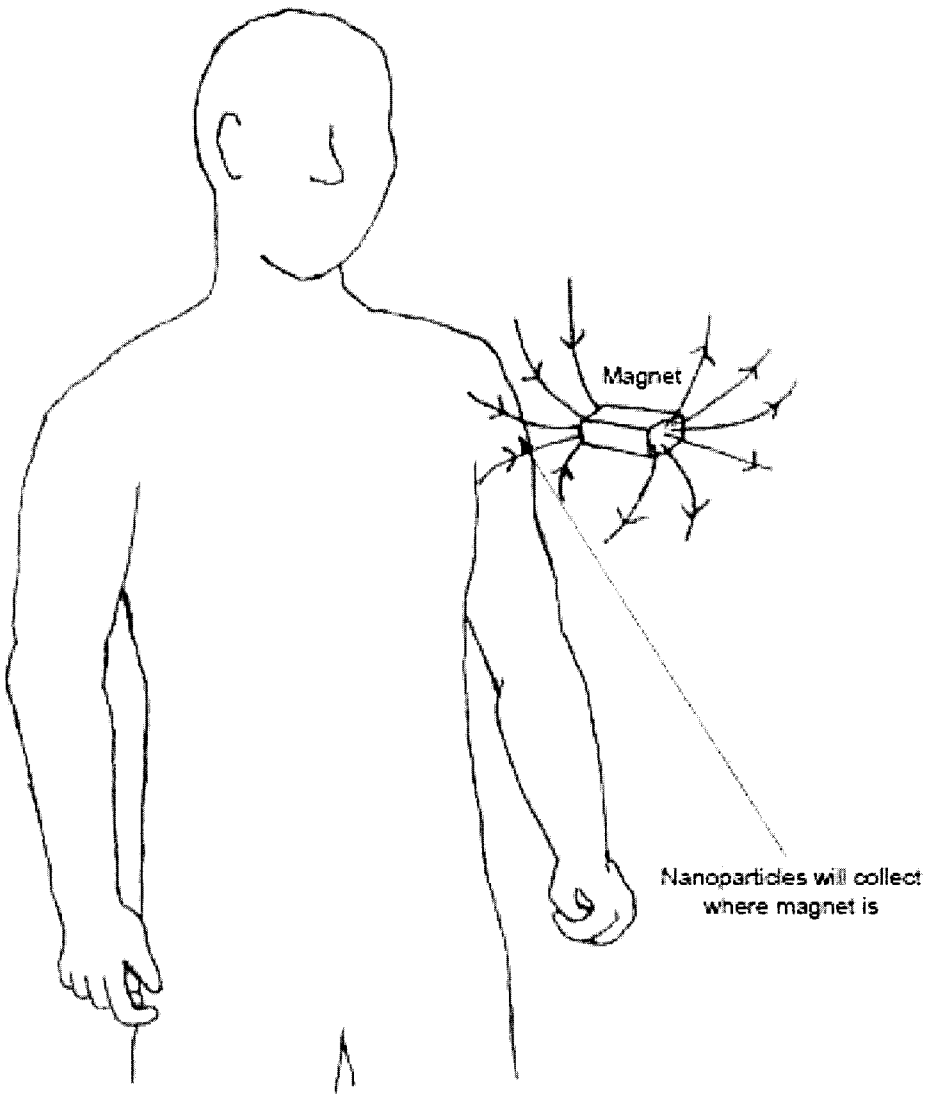


FIG 3

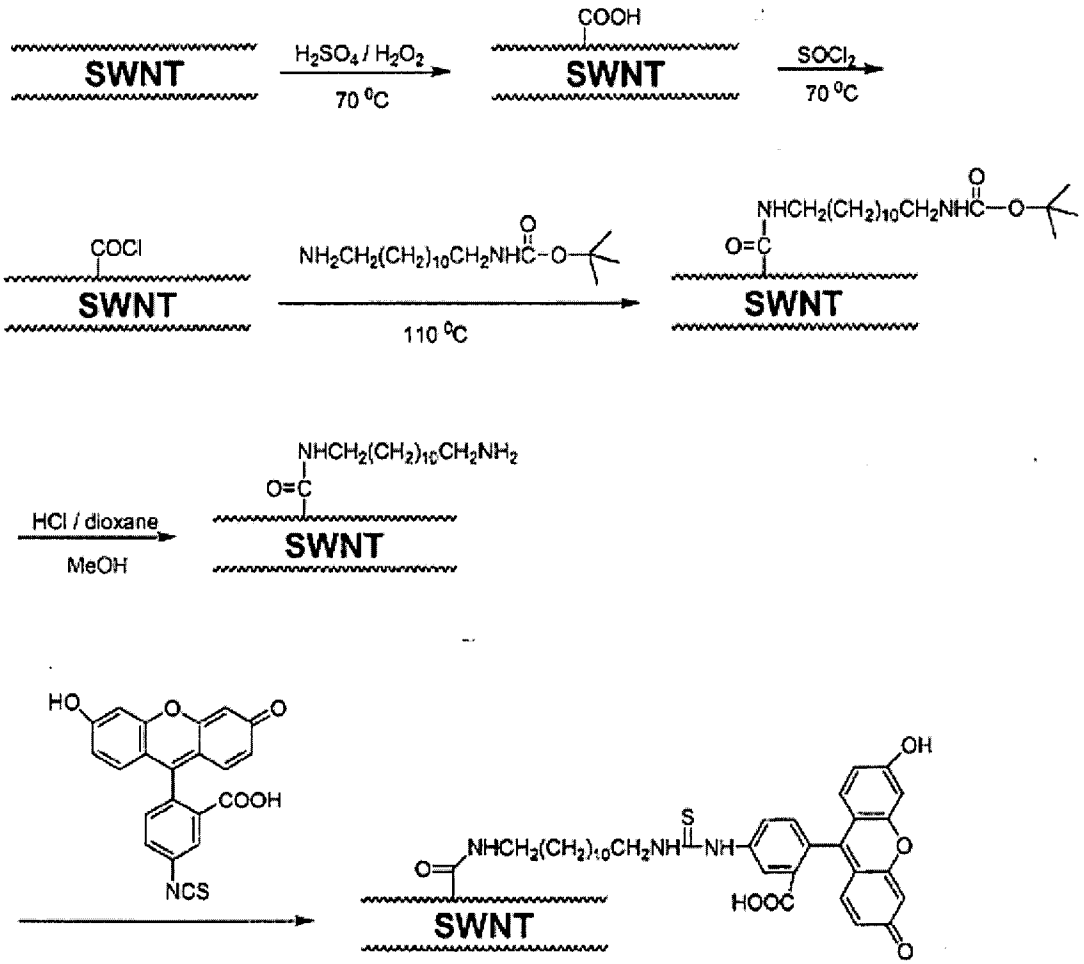

SWNT
H_2SO_4 / H_2O_2
70 °C
COOH
SWNT
$SOCl_2$
70 °C
COCl
SWNT
$NH_2CH_2(CH_2)_{10}CH_2NHC(=O)-O-$
110 °C
$NHCH_2(CH_2)_{10}CH_2NHC(=O)-O-$
O=C
SWNT
HCl / dioxane
MeOH
$NHCH_2(CH_2)_{10}CH_2NH_2$
O=C
SWNT
HO
O
O
COOH
NCS
OH
S
NH
$NHCH_2(CH_2)_{10}CH_2NH$
O
SWNT
HOOC
O

FIG 4A

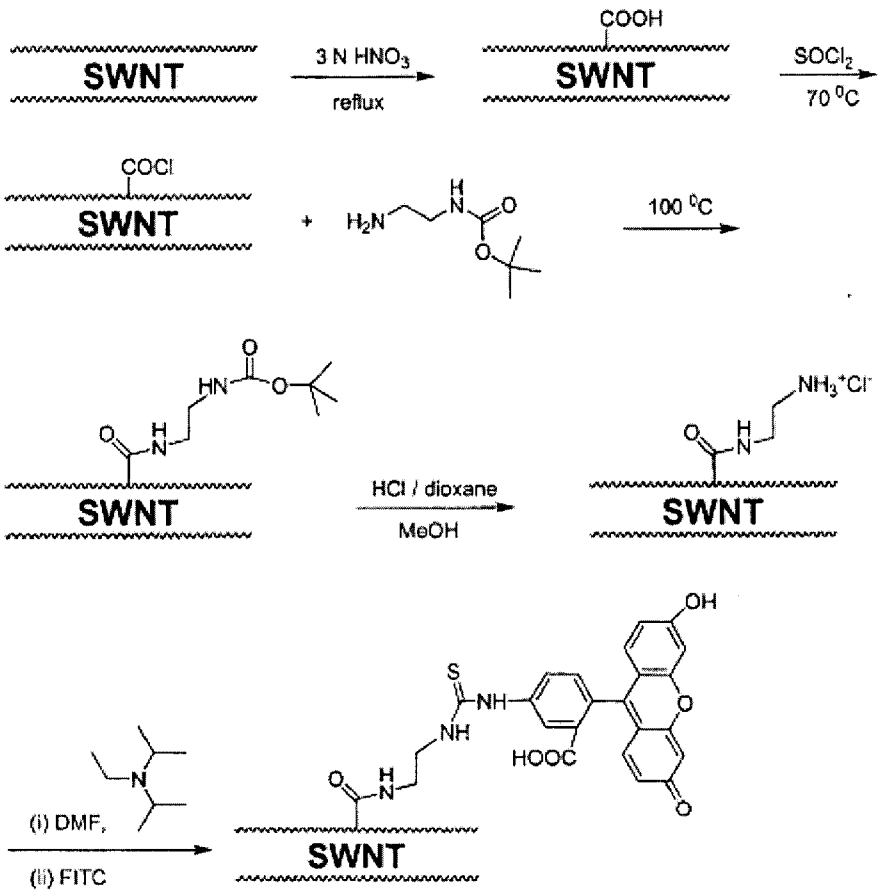

SWNT
3 N HNO3
reflux
COOH
SWNT
SOCl2
70 °C
COCl
SWNT
+
H2N
100 °C
HN
O
SWNT
HCl / dioxane
MeOH
NH3+Cl-
SWNT
OH
S
NH
NH
HOOC
O
(i) DMF,
(ii) FITC
SWNT

FIG 4B

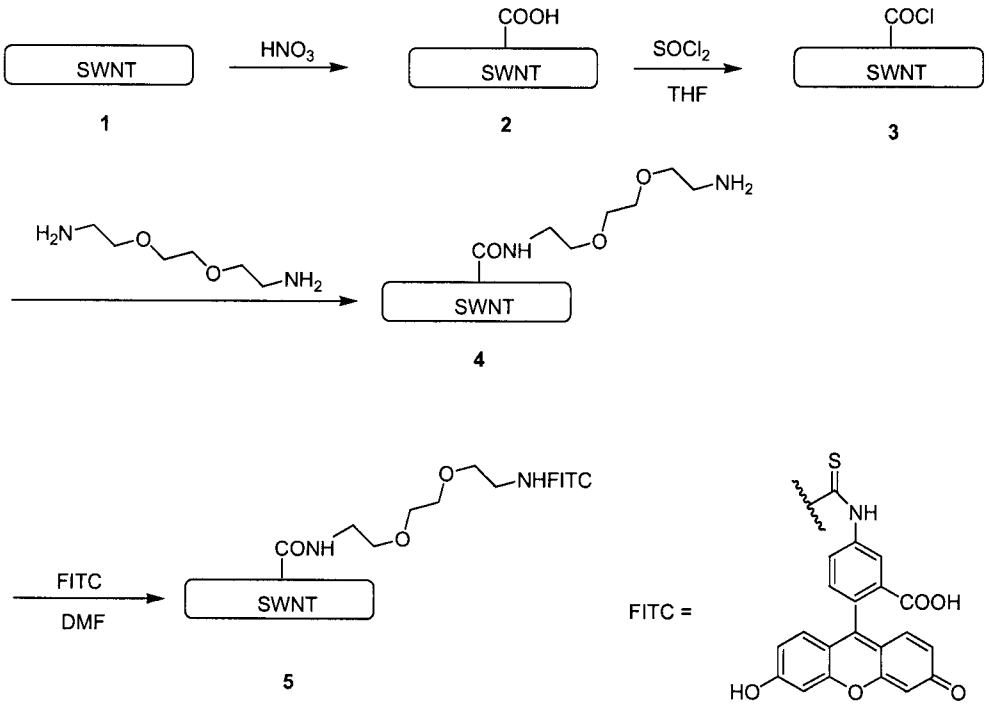

SWNT
HNO3
COOH
SWNT
SOCl2
THF
COCl
SWNT
1
2
3
H2N
O
O
NH2
CONH
O
O
NH2
SWNT
4
FITC
DMF
CONH
O
O
NHFITC
SWNT
5
FITC =
S
NH
COOH
HO
O
O

FIG 4C

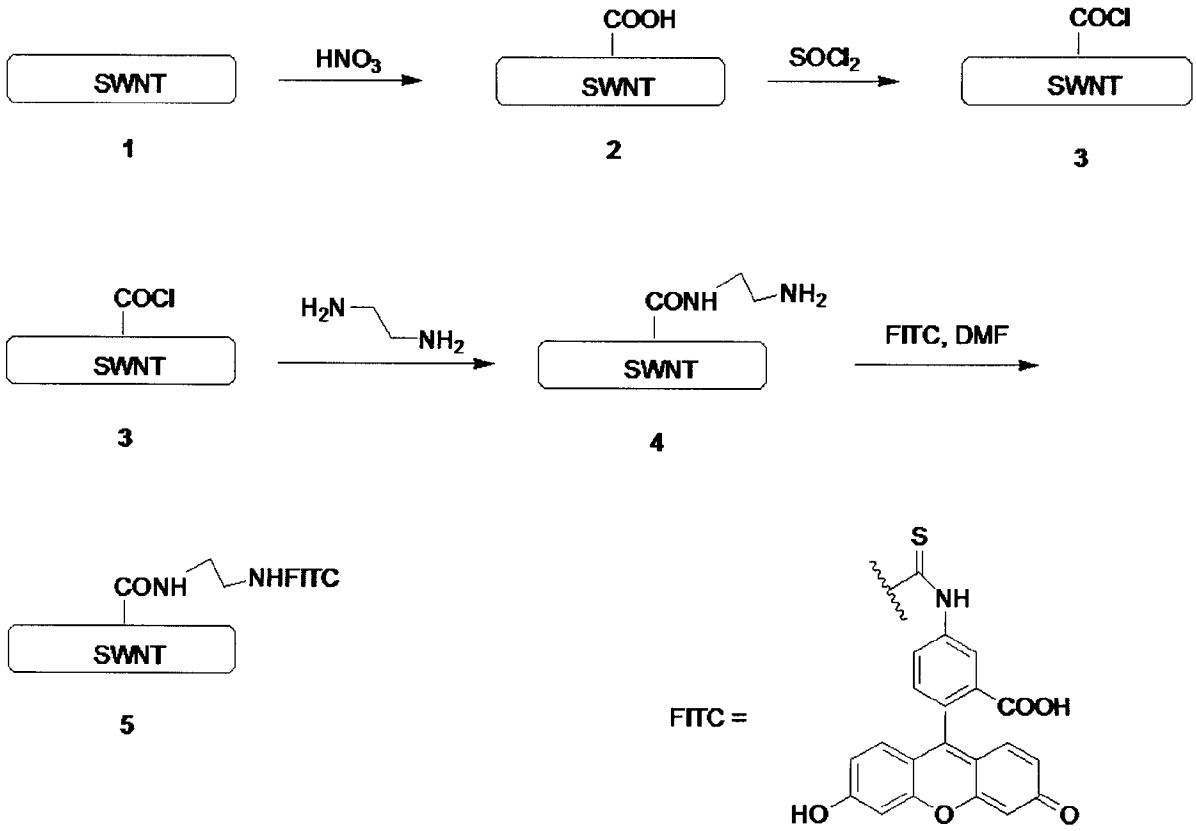

SWNT
HNO3
COOH
SWNT
SOCl2
COCl
SWNT
1
2
3
COCl
SWNT
H2N
NH2
CONH
NH2
SWNT
FITC, DMF
3
4
CONH
NHFITC
SWNT
5
FITC =
S
NH
COOH
HO
O
O

FIG 4D

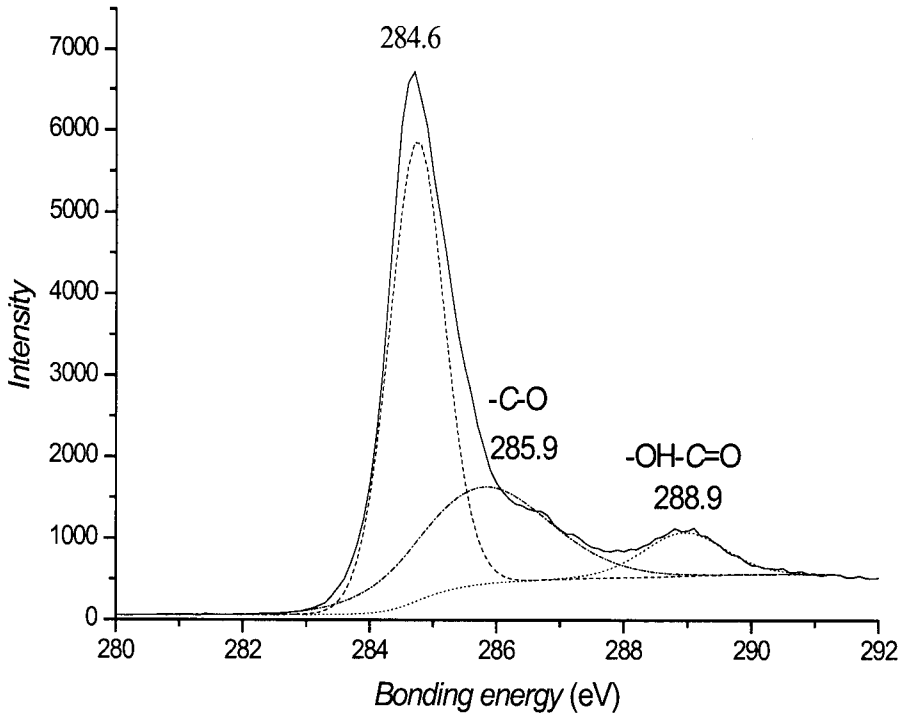

284.6
-C-O
285.9
-OH-C=O
288.9
Intensity
Bonding energy (eV)
0
1000
2000
3000
4000
5000
6000
7000
280
282
284
286
288
290
292

FIG 5A

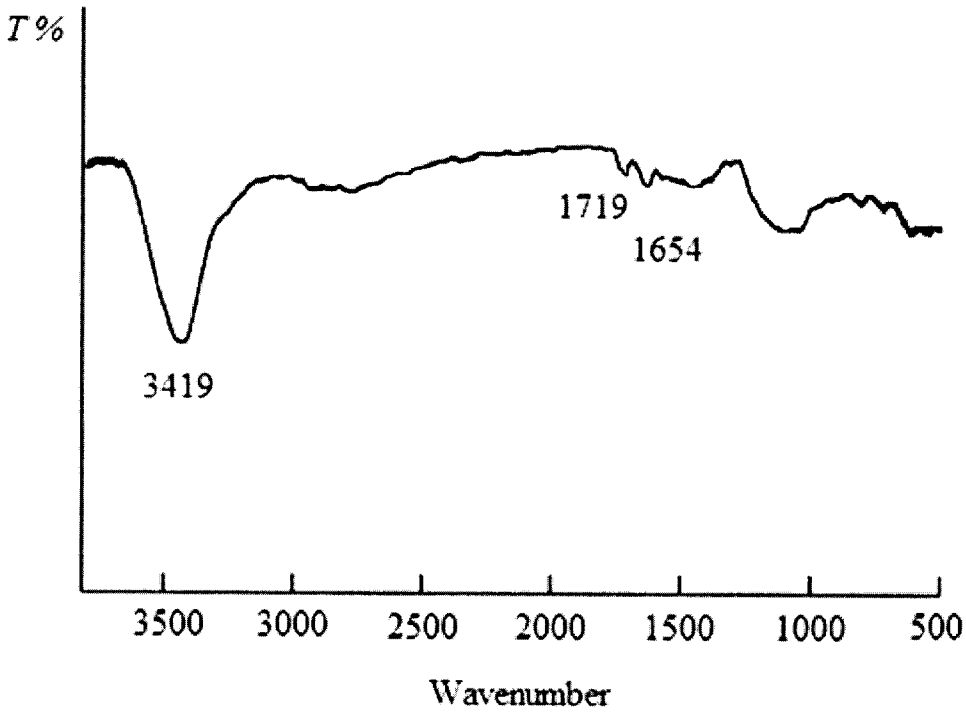

T%
1719
1654
3419
3500
3000
2500
2000
1500
1000
500
Wavenumber

FIG 5B

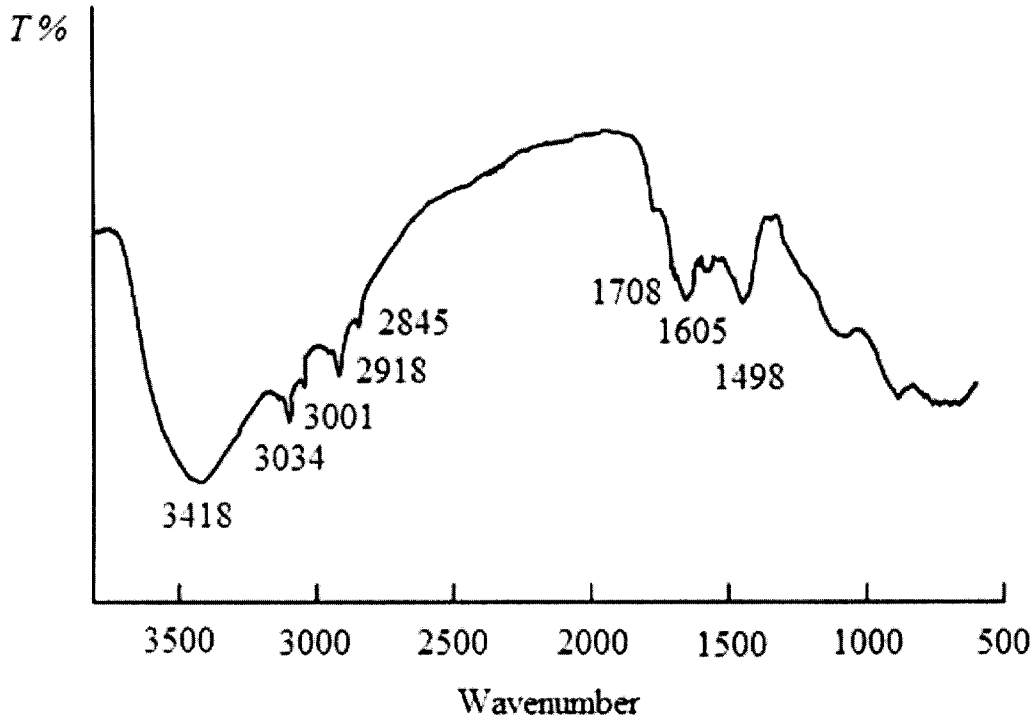

T%
3418
3034
3001
2918
2845
1708
1605
1498
3500
3000
2500
2000
1500
1000
500
Wavenumber

FIG 6A

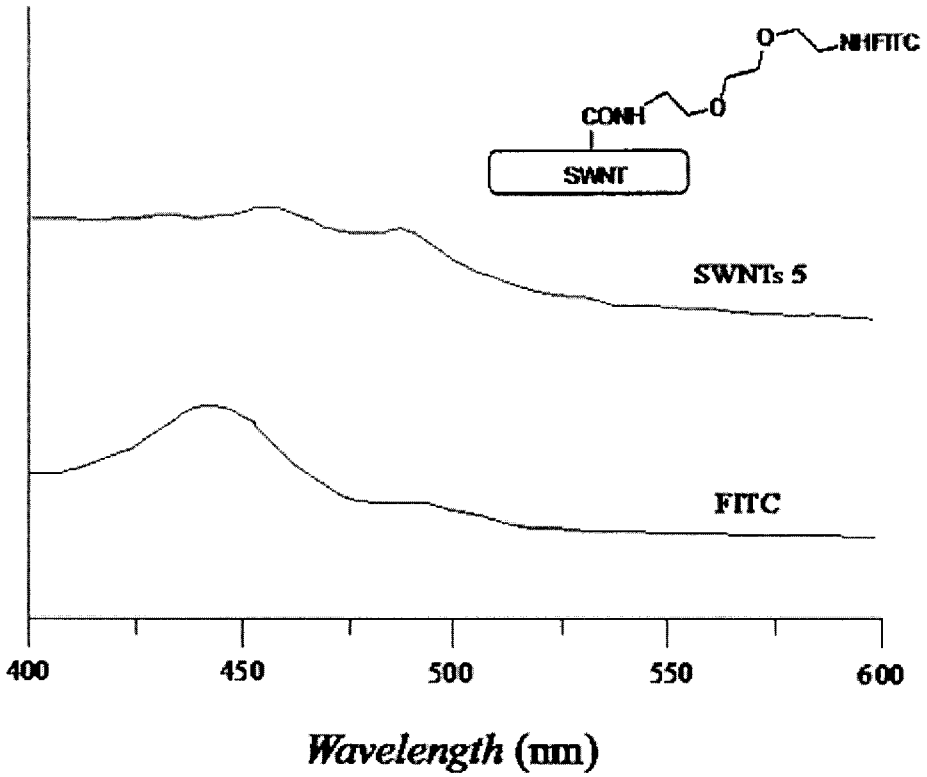

O
NHFITC
CONH
O
SWNT
SWNTs 5
FITC
400
450
500
550
600
Wavelength (nm)

FIG 6B

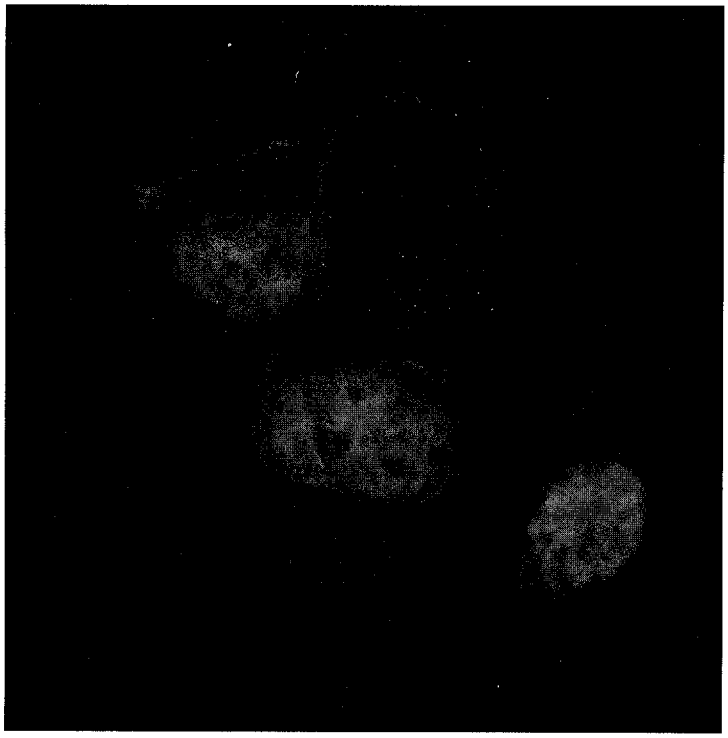


FIG 7A

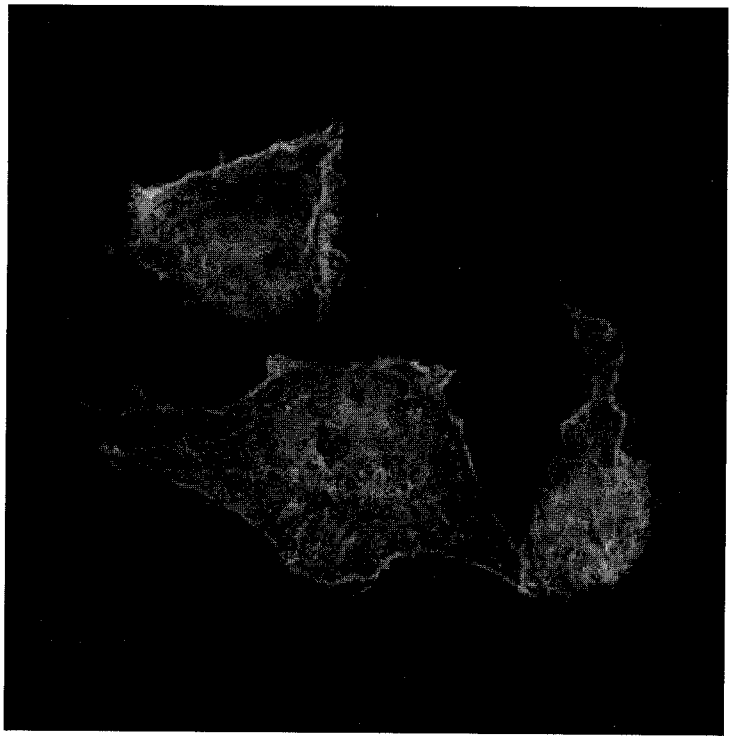
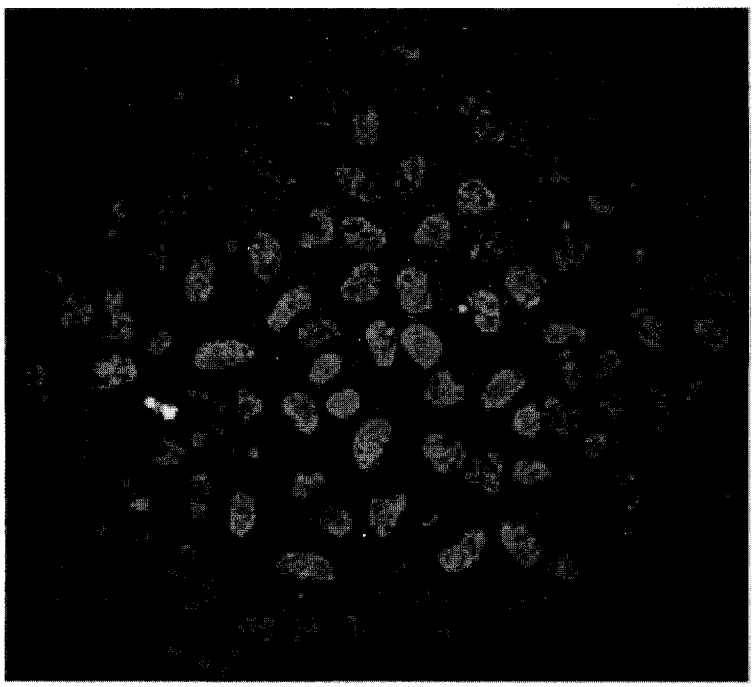
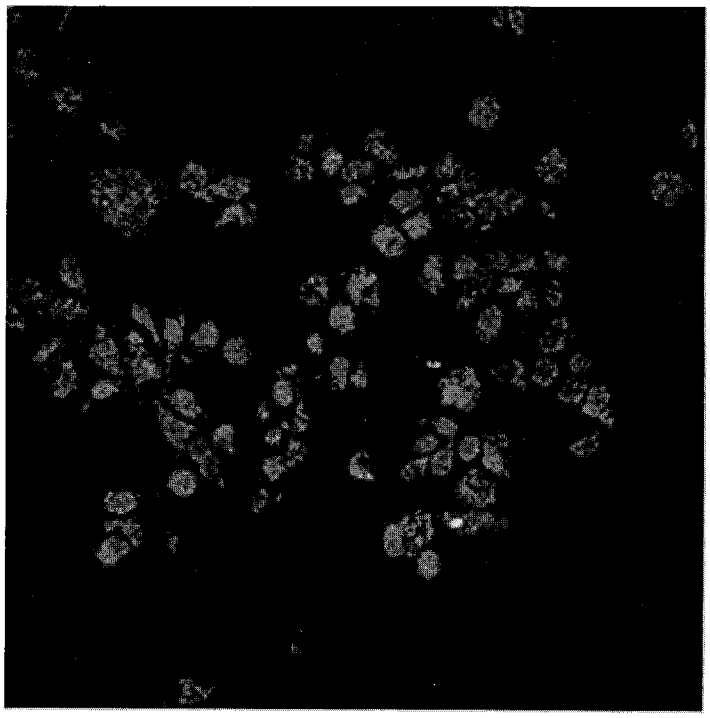


FIG 7B



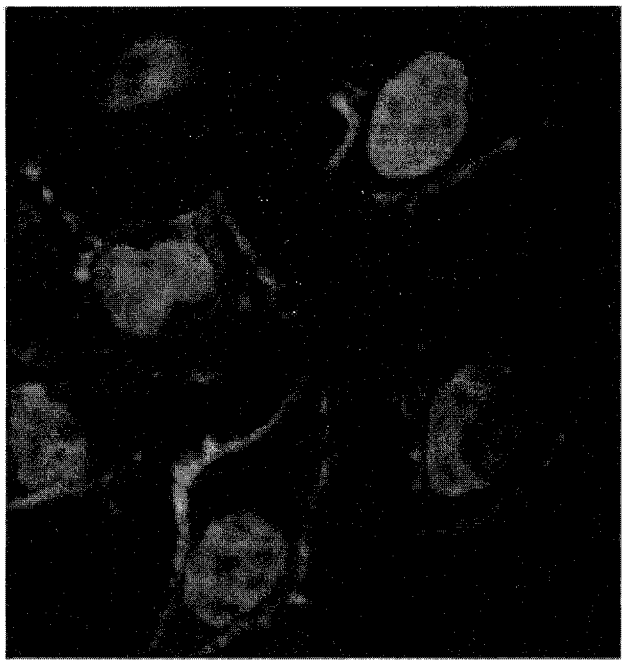
(Color) Control.
Blue: Nucleus
Green: GFP

FIG 7C



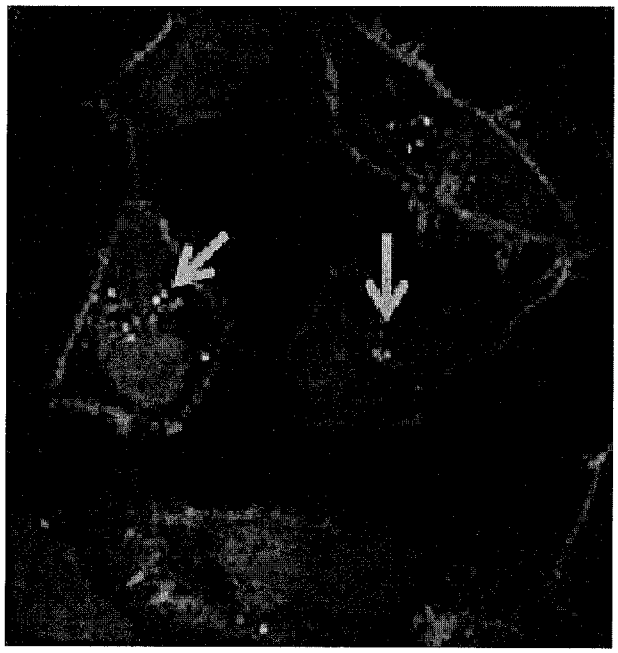
(Color) CNT-GFP (3 min incubation, 7 min applied magnetic field afterwards)
Blue: Nucleus
Green: GFP

FIG 7D



(Color) Control cells.
Blue: Nucleus.
Red: Cell membrane.

FIG 8A



(Color) SWNT 5 delivered cells. After SWNT 5 carrier exposure, FITCs are distributed in cytoplasm and even inside nucleus (green colored dots pointed by arrows).

FIG 8B

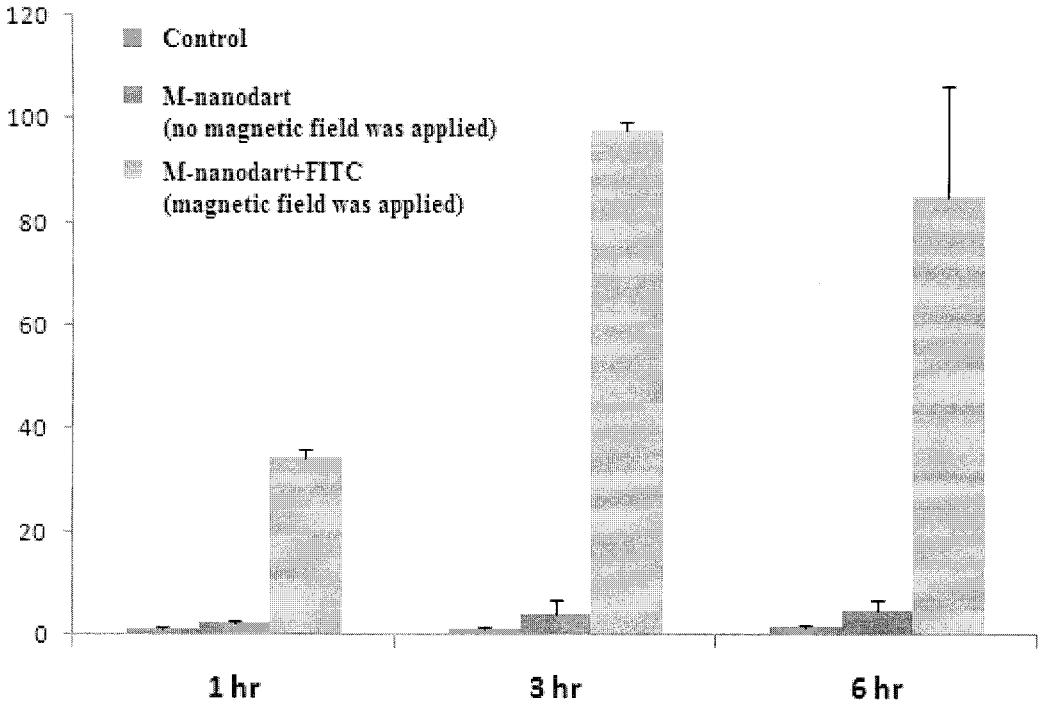

120
100
80
60
40
20
0
Control
M-nanodart
(no magnetic field was applied)
M-nanodart+FITC
(magnetic field was applied)
1 hr
3 hr
6 hr

FIG 9

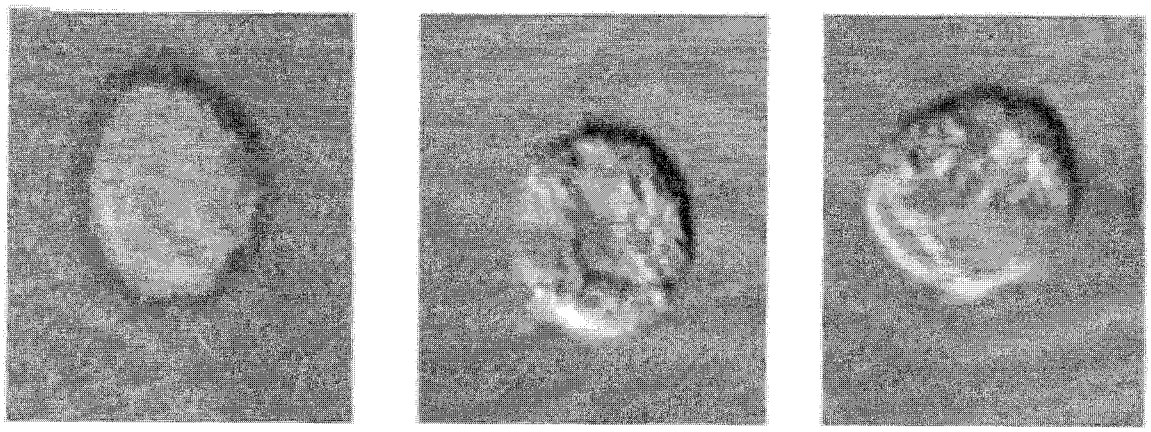

(a) Control
(b) Delivery after 3h
(c) Delivery after 6h

FIG 10

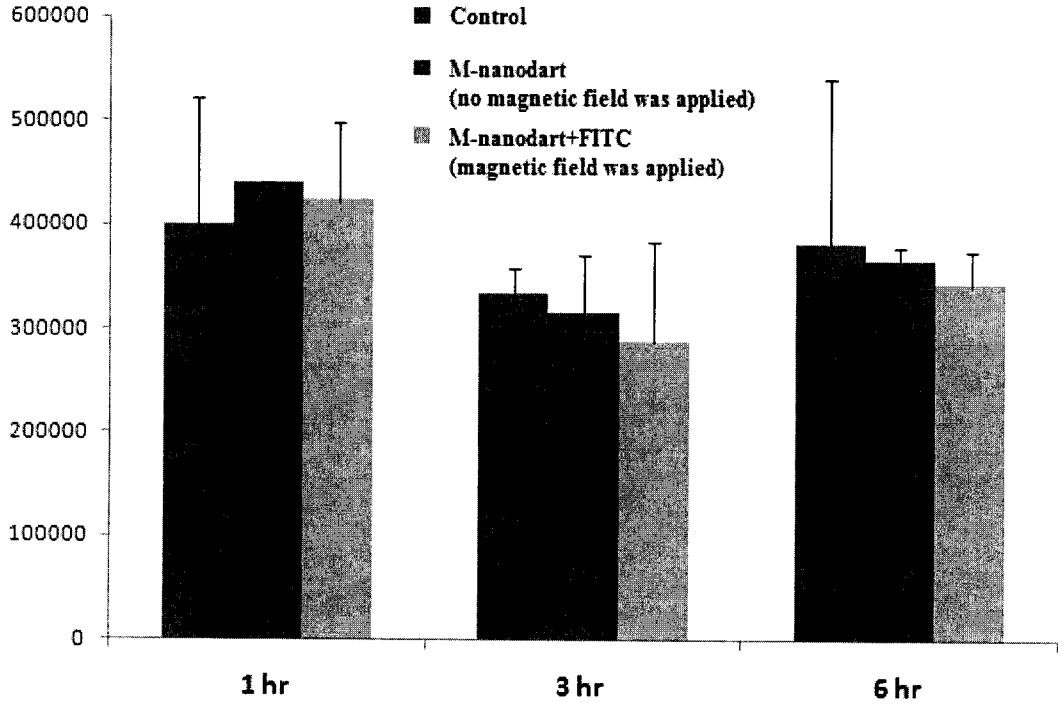


FIG 11

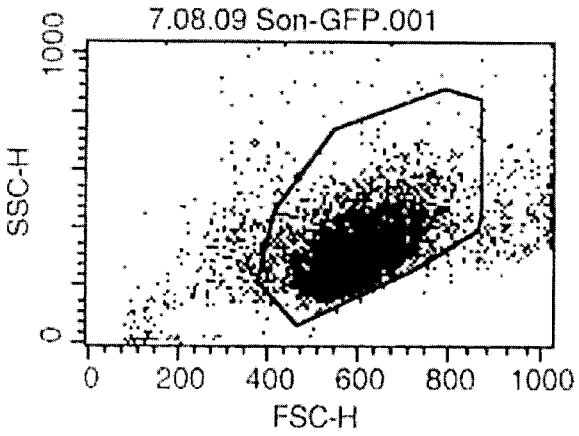


FIG 12A

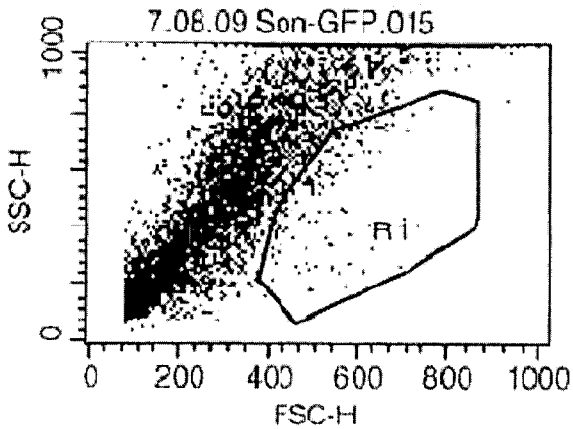


FIG 12B

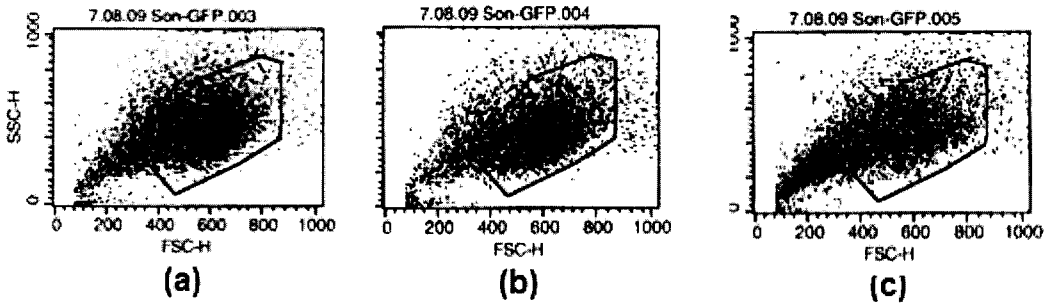

7.08.09 Son-GFP.003
7.08.09 Son-GFP.004
7.08.09 Son-GFP.005
SSC-H
FSC-H
0
200
400
600
800
1000
(a)
(b)
(c)

FIG 13

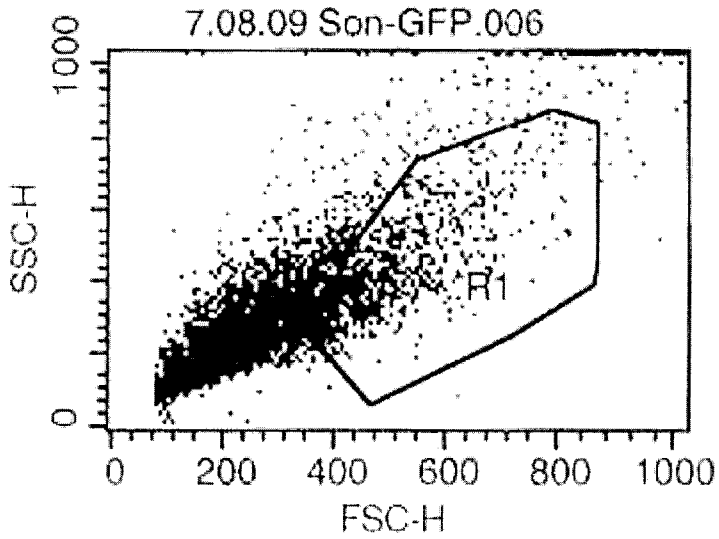

7.08.09 Son-GFP.006
SSC-H
FSC-H
R1
0
200
400
600
800
1000

FIG 14

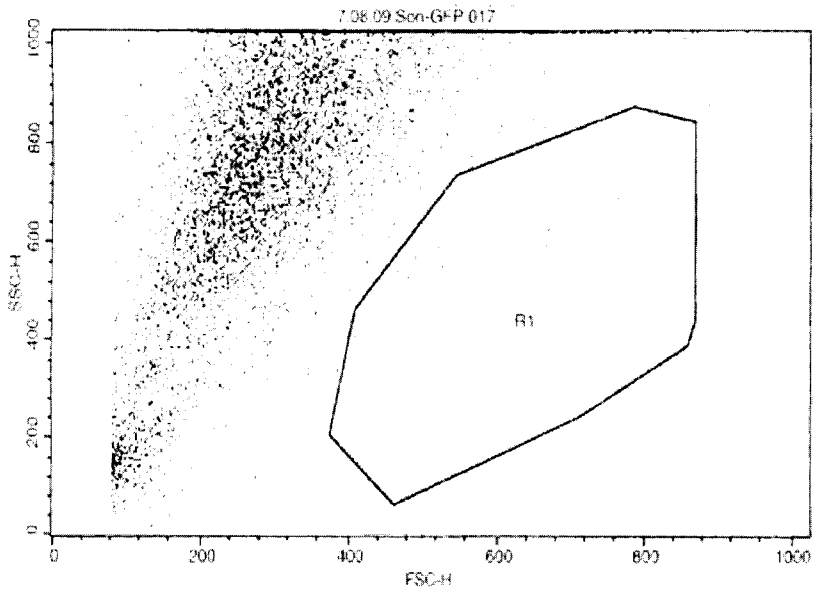


FIG 15

FIG 16

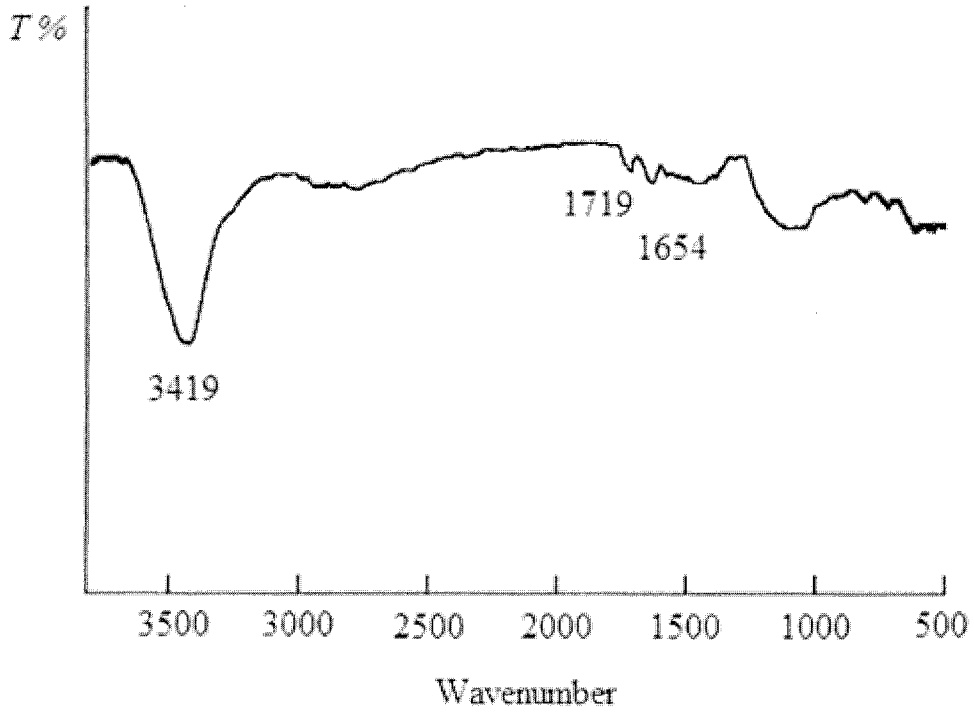
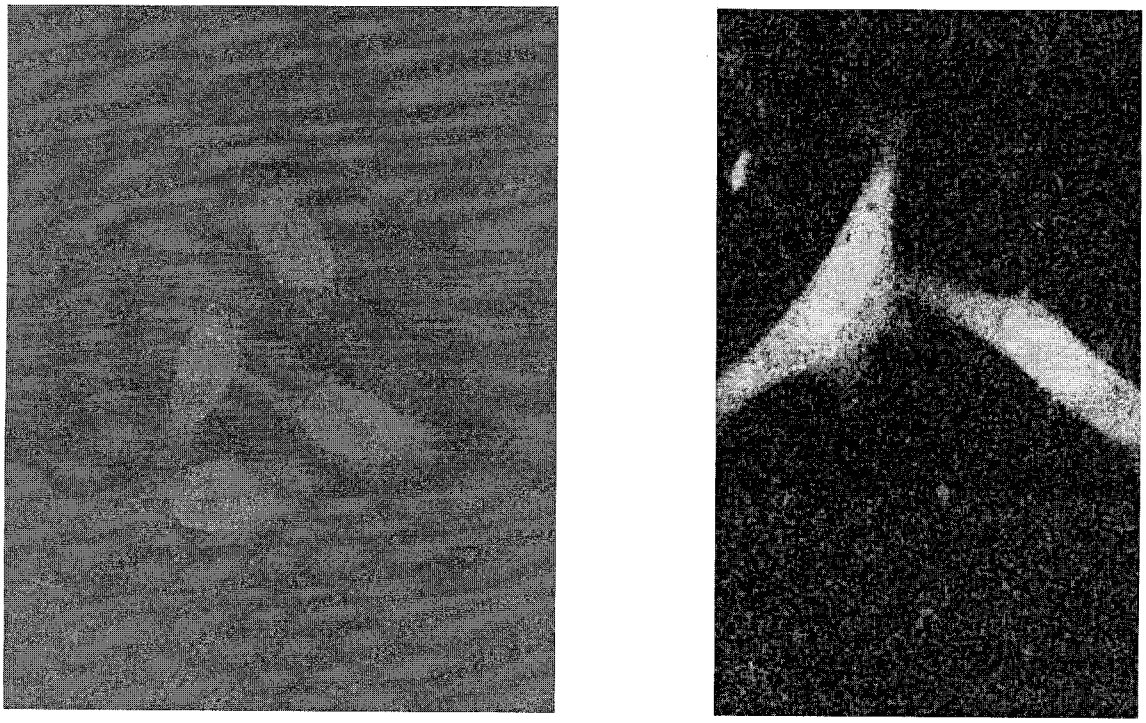


FIG 17



(a) Control

(b) Biodegradable-tube + GFP

FIG 18

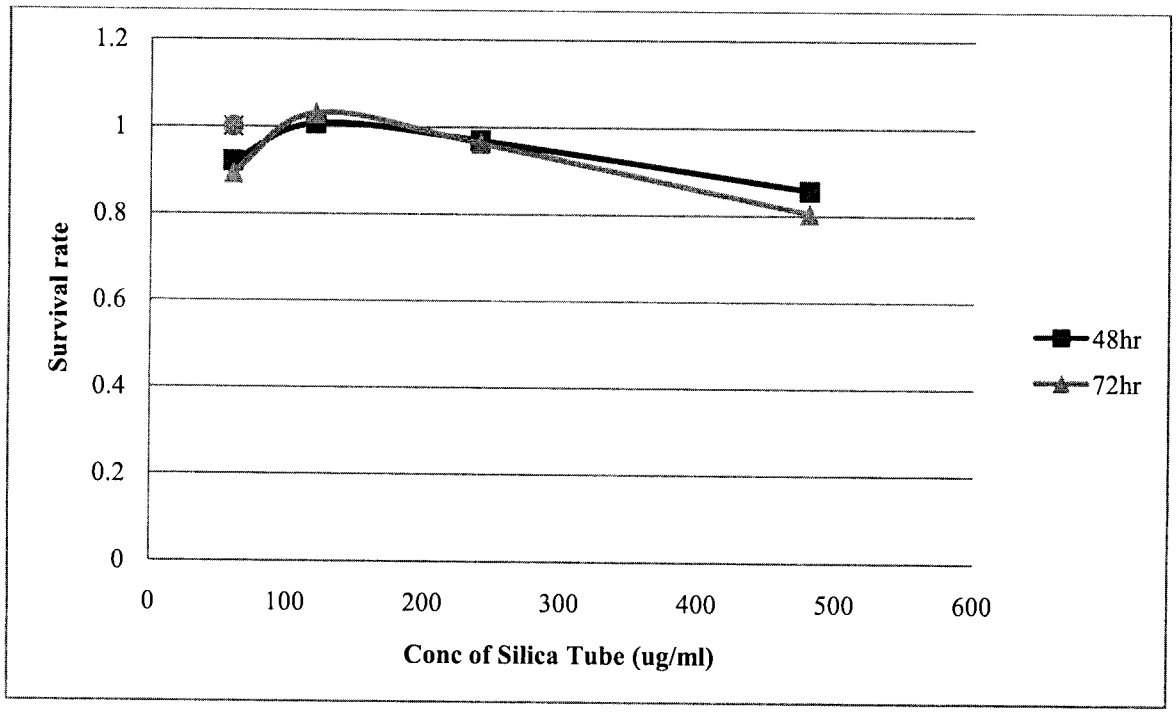

1.2
1
0.8
0.6
0.4
0.2
0
Survival rate
0
100
200
300
400
500
600
Conc of Silica Tube (ug/ml)
48hr
72hr

FIG 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001629

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12N 15/87*** (2006.01) , ***A61K 41/00*** (2006.01) , ***A61K 48/00*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2006.01): *C12N 15/87, A61K 41/00 and A61K 48/00*

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Databases: Delphion, Pubmeb, Scopus, CAplus, Medline, Biosis, Canadian Patent Database **Keywords**: transformation, transfection, magnetic, magnetofection, ultrasound, simultaneous, magnetic particle, nanoparticle, gene delivery

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STRIDE, E. et al. Enhancement of microbubble mediated gene delivery by simultaneous exposure to ultrasonic and magnetic fields. Ultrasound in Medicine and Biology, May 2009 and 17 March 2009 (electronic publication), Vol. 35(5), pages 861-868, ISSN: 0301-5629 (print) and 1879-291X (electronic). See entire document	1, 2, 4, 6, 7 and 9
Y		3, 5 and 8
Y	HUSSEINI, G.A. et al. Micelles and Nanoparticles for Ultrasonic Drug and Gene Delivery. Advanced Drug Delivery Reviews, 30 June 2008 and 4 April 2008 (electronic publication), Vol. 60(10), pages 1137–1152, ISSN: 0169-409X (Print) and 1872-8294 (Electronic). See entire document, page 5 in particular.	3 and 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 2010	9 February 2010 (09-02-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Brad Temple (819) 934-7599

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	McBAIN, S.C. et al. Magnetic nanoparticles as gene delivery agents: Enhanced transfection in the presence of oscillating magnet arrays. Nanotechnology, 1 October 2008, Vol. 19(40) ISSN: 0957-4484 (print) and 1361-6528 (electronic). See entire document	8
Y	DOBSON, J. et al. Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene therapy, February 2006, Vol. 13(4), pages 283-287, ISSN: 0969-7128 (print) and 1476-5462 (electronic). See entire document, and specifically figure 2 on page 284 and page 286, final paragraphs	1-9
Y	LARINA, I.V. et al. Enhancement of drug delivery in tumors by using interaction of nanoparticles with ultrasound radiation. Technology in Cancer Research and Treatment, April 2005, Vol. 4(2), pages 217-226, ISSN: 1533-0346 (print) and 1533-0338 (Electronic). See entire document	1-9
A	KAMINSKI, M.D. et al. Encapsulation and release of plasminogen activator from biodegradable magnetic microcarriers. European Journal of Pharmaceutical Sciences, 2 September 2008, 3 Jul 2008 (electronic publication), Vol. 35(1-2), pages 96-103, ISSN: 0928-0987 (print) and 1879-0720 (electronic).	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001629

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

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

1. [X] Claim Nos. : 1-9

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1-9 are directed to a method for treatment of the human or animal body by surgery or therapy, are not required to be searched nor is a written opinion required by this Authority. Regardless, this Authority has performed a search based on the alleged effect or purpose/use of the product defined in claims 1-9.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.