VERSATILE NANOPARTICULATE BIOMATERIAL FOR CONTROLLED DELIVERY AND/OR CONTAINMENT OF THERAPEUTIC AND DIAGNOSTIC MATERIAL

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ABSTRACT

The invention provides compositions for controlled delivery and/or containment of therapeutic and/or diagnostic agents comprising the agent or agents encapsulated by a matrix containing chitosan, polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and tetra-methoxy-ortho-silicate (TMOS) or tetra-ethylxq-ortho-silicate (TEOS), as well as methods for preparing the compositions, and uses of the compositions for therapy and imaging.
FIGURE 1
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
FIGURE 7A-7B

A

B

Tadalafil Nanoparticles
(54 minutes)

Tadalafil Nanoparticles
(54 minutes)
FIGURE 8

- Before tadalafil-nanoparticles
- 30 min after tadalafil-nanoparticles
- 60 min after tadalafil-nanoparticles
Corpora Spongiosum With Urethra
Corpora Cavernosum
Cavernous vein

FIGURE 9
VERSATILE NANOPARTICULATE BIOMATERIAL FOR CONTROLLED DELIVERY AND/OR CONTAINMENT OF THERAPEUTIC AND DIAGNOSTIC MATERIAL

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority of U.S. Provisional Patent Application No. 61/214,238, filed on Apr. 21, 2009, the content of which is incorporated by reference.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under grant numbers R01DK-077665, R01HL-73732 and TW007129-01 awarded by the National Institutes of Health, U.S. Department of Health and Human Services. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Current imaging contrast reagents and therapeutics can have limitations with respect to deployment and efficacy. For example, some imaging reagents are limited by rapid host clearance, and therapeutics that can only be delivered intramuscularly and intravenously have well-documented adverse effects associated with their use as a consequence of being delivered systemically. Furthermore, liposomal delivery systems can be unstable with limited applicability, and most existing nanoparticle based delivery systems are very limited in terms of applications and can be comprised of materials that may be toxic.

Thus, there is a need for a new platform that addresses limitations associated with clinical applicability arising from limited delivery options, rapid degradation/biologic clearance, patient compliance with dosing schedules, systemic toxicities, and lack of targeting. The present invention addresses this need by providing a versatile nanoparticulate biomaterial for controlled and versatile delivery and/or containment of therapeutic and diagnostic agents.

SUMMARY OF THE INVENTION

The present invention provides compositions for controlled delivery and/or containment of one or a combination of therapeutic and/or diagnostic agents comprising an agent or agents encapsulated in a matrix comprising a) chitosan, b) polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and c) tetra-methoxy-ortho-silicate (TMOS) or tetra-ethoxy-ortho-silicate (TEOS).

The invention further provides methods for preparing compositions for controlled delivery and/or containment of one or more therapeutic and/or diagnostic agent, the methods comprising: (a) admixing the agent or agents with i) chitosan, ii) polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and iii) tetra-methoxy-ortho-silicate (TMOS) or tetra-ethoxy-ortho-silicate (TEOS) in a buffered solution; (b) drying the mixture of step (a) to produce a gel; and (c) heating the gel to produce a glassy material, or lyophilizing the gel to produce a particulate material. Alternatively, the mixture may be spray-dried to produce a particulate matter. The invention further provides compositions prepared by the disclosed methods.

Without in any way limiting the present invention, the emergent properties of the compositions of the invention are understood to be the result of introducing into a porous hydrogel, molecules such as chitosan that can form a strong glass-like hydrogen bonding network with the molecular components of the hydrogel polymer (e.g., Si—OH groups). The hydrogen bonding network creates the blockage of the open hydrogel channels that typically allow facile diffusion of substrates in and out of the hydrogel. The formation of the hydrogen bonding network within the robust framework of the hydrogel is directly responsible for the slow water initiated release of contents due to the slow water induced disruption of the glassy network of hydrogen bonds. Different silanes and different polysaccharides/polymers capable of forming hydrogen bonding networks can be used to modulate the stability and release profiles of the nanoparticles. For example, trimethoxy silanes could be used that have a fourth site containing a side chain that has enhanced hydrogen bonding capacity or the capacity to hydrogen bond with a different class of introduced polymers. Additionally, other silanes or polymers could be used to control the pH sensitivity of the release profiles.

In addition, the invention provides methods for treating and imaging subjects comprising administering compositions disclosed herein to the subject. Additional objects of the invention will be apparent from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Release of Badan-label GSH. Fluorescence emission scans were performed with excitation at 395 nm. The individual curves represent the emission scans at different times (0 minutes, 30 minutes, 75 minutes, 24 hours) after mixing 20 mg of particles with 0.6 mL of water.

FIG. 2A-2B. MRI of a mouse injected with Magnevist® (gadopentetate dimeglumine) (A) and a mouse injected with nanoparticles loaded with gadopentetate dimeglumine (B). The left images are pre-injection, center images are 4 hours post injection, and right images are 24 hours post injection. Significant contrast was maintained at 24 hours post-injection with the gadopentetate dimeglumine-loaded particles while contrast with Magnevist® returned to pre-injection levels at 24 hours post-injection.

FIG. 3. Demonstration of dose dependent percent attenuation of radiation by melanin-containing nanoparticles in vitro. The efficacy of X-ray radiation penetration was inversely proportional to the concentration of melanin encapsulated and the density of nanoparticles, as compared to controls, including empty nanoparticles and aluminum.

FIG. 4. Protective effects of melanin-containing nanoparticles from therapeutic dose of gamma radiation. Arbitrary desquamation and erythema score after localized limb irradiation (30 Gy) in (BALB/c) mice following hindlimb injection of melanin-containing nanoparticles (lower trace) or empty control nanoparticles (upper trace). Vertical axis-Arbitrary score. Scoring criteria appear in Table 1, below.

FIG. 5. Nanoparticles loaded with Adriamycin® (doxorubicin) killed HeLa cells (controls are empty nanoparticles). Error bars represent SEM.

FIG. 6. Sialorphin nanoparticles induce erectile activity in an in vivo erectile dysfunction model. A continuous trace of intracorporeal pressure (ICP) (upper panel) and blood pressure (BP) (lower panel) over the course of an experiment following administration of the sialorphin-nanoparticles perfomed topically on the rat penis. The nanopar-
articles were applied at time=0 as a 200 ml suspension. Increased ratio of ICP/BP is indicative of erectile activity.

Preferably, the composition is in the form of particles having a diameter of 10 nm to 100 μm in their dry form, and more preferably 10 nm to 1 μm or 10 nm to 100 nm.

The invention also provides a method for preparing a composition for controlled delivery and/or containment of an agent or agents, such as one or more therapeutic and/or diagnostic agent, the method comprising: (a) admixing the agent or agents with i) chitosan, ii) polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and iii) tetra-methoxy-ortho-silicate (TMOS) or tetra-ethoxy-ortho-silicate (TEOS) in a buffered solution; (b) drying the mixture of step (a) to produce a gel; and (c) heating the gel to produce a glassy material, or lyophilizing the gel to produce a particulate material. Alternatively, the mixture may be spray dried to produce a particulate material.

Preferably, the gel is heated in step (c) to a temperature of less than or equal to 70°C, preferably to 55-70°C, and more preferably to about 60°C.

The method can optionally further comprise grinding or milling the material of step (c) to produce particles of a desired size, preferably a diameter of 10 nm to 100 μm, and more preferably a diameter of 10 nm to 1 μm or 10 nm to 100 nm.

In preferred embodiments of the method, TMOS or TEOS is diluted with an additional silane. The additional silane can be chosen, for example, to either alter the internal environment of the resulting particles with respect to properties such as hydrophobicity and polarity or to introduce reactive groups (e.g., amino, carboxyl, sulfhydryl) that allow the covalent attachment of additional molecules to the particles. Molecules that can be attached include, for example, those that are suitable for fluorescence labeling (e.g. BADAN), radioactive labeling (labeled peptides), tissue targeting (peptides), linkers for attachment of the particles to cell delivery vehicles such as bacteria, and additional PEG molecules to coat the surface of the particles. Preferably, the additional groups contain an alkyl side chain, a PEG chain having a molecular weight of 200 to 400 Daltons, a sugar, an amino terminus, or a reactive sulfhydryl or carbonyl group. The additional silane can be, for example, a hydrophobic silane, such as, for example, trimethoxymethyl isopropyl silane, trimethoxymethyl butyl silane or trimethoxymethyl fluoropropyl silane.

Preferably, the chitosan is at least 50% deacetylated. More preferably, the chitosan is at least 80% deacetylated. Most preferably, the chitosan is at least 85% deacetylated.

Preferably, the concentration of chitosan in the composition is 0.05 g-1 g chitosan/100 ml of composition (dry weight).

Preferably, the concentration of TMOS or TEOS in the composition is 0.5 ml-5 ml of TMOS or TEOS/24 ml of composition (dry weight).

PEGs of various molecular weights, conjugated to various groups, can be obtained commercially (see, for example, Nektar Therapeutics, Huntsville, Ala.). Preferably, the concentration of polyethylene glycol (PEG) in the composition is 1-5 ml of PEG/24 ml of composition (dry weight).

The agent can be, for example, a hydrophobic agent or a hydrophilic agent. Therapeutic and/or imaging agents may be used in combination. Therapeutic agents that can be used include, for example, but are not limited to, one or more of therapeutic peptides; small molecules such as nitric oxide; hormones such as growth hormone; growth factors; cytokines; chemo therapeutic agents such as e.g. doxorubicin; melanin; agents for treating erectile dysfunction such as e.g.
tadalafil, sialorphin or nitric oxide; agents for treating urological disorders; plasmids; antioxidants; anti-inflammatory agents; radioprotectants such as melanin and amifostine; and antimicrobial agents; and combinations thereof. Imaging agents that can be used include, for example, but are not limited to, one or more of MRI (e.g., gadolinium based MRI contrast reagents), PET, SPECT, optical, ultrasound and radioactive imaging agents. The composition can comprise a label such as, for example, a dye such as a fluorescent label or a radioactive label. Particles comprising compositions of the present invention can also include an antibody, aptamer or other targeting agent.

[0030] When the agent is melanin, the melanin can be formed, for example, by polymerization of L-dopa in the solution of step (a) of the method.

[0031] The invention provides compositions prepared by any of the methods disclosed herein.

[0032] The invention also provides methods of treating a disease or disorder in a subject comprising administering a therapeutic amount of a composition herein disclosed to the subject. The subject can have, for example, a cancer, a microorganism infection, or erectile dysfunction or the subject can be at risk for exposure to ionizing radiation. The cancer can be, for example, bladder, breast, head and neck, leukemia, liver, lung, lymphoma, mesothelioma, multiple myeloma, neuroblastoma, ovary, pancreas, prostate, sarcoma, stomach, testis, cervix, thyroid or uterus cancer. The infection can be, for example, a bacterial, viral, fungal or parasitic infection.

[0033] The invention also provides methods of imaging a subject comprising administering a composition herein disclosed comprising an imaging agent to the subject. Imaging methods are known in the art as appropriate for the different imaging agent that can be used, such as, for example, MRI (e.g., gadolinium based MRI contrast reagents), PET, SPECT, optical, ultrasound and radioactive imaging agents.

[0034] The compositions described herein can be delivered to a subject by a variety of routes of delivery, including but not limited to topical, subcutaneous, transdermal, intraperitoneal, intravenous, oral and aerosol administration. The compositions can be incorporated, for example, in a cream, lotion, solution, foam, oil, ointment, transdermal patch, or implantable biomedical device.

[0035] When the subject has erectile dysfunction, the composition can be administered by topical application to the penis. Agents that can be used for treating erectile dysfunction include, but are not limited to, sialorphin, tadalafil and nitric oxide, or combinations thereof. Where the agent is sialorphin, erection can occur about 4-15 minutes after administration of the composition. Where the agent is tadalafil, erection can occur within about 1 hour after administration of the composition. The agents are effective in subjects that have damage to nerves that facilitate penile erection (cavernous nerves), which can be damaged, for example, during prostate surgery or as a result of diabetic neuropathy.

[0036] The invention further provides a method of treating a subject with tissue damage, comprising: a) incorporating any of the compositions disclosed herein into stem cells, wherein the composition is in the form of nanoparticles and the nanoparticles comprise a therapeutic agent; and b) administering the stem cells of step a), or derivatives or descendants thereof, to the subject in an amount and manner effective to treat tissue damage in the subject. The tissue damage can include, for example damage to the subject’s heart, such as can occur for example during ischemic heart disease or Cushing’s disease. Preferred therapeutic agents for use in treating tissue damage include one or more of nitric oxide, growth hormone, or a growth factor or cytokine.

[0037] The invention also provides a method of evaluating distribution and retention of stem cells in a subject, comprising: a) incorporating any of the compositions disclosed herein into stem cells, wherein the composition is in the form of nanoparticles and the nanoparticles comprise an imaging agent; b) administering the stem cells of step a), or derivatives or descendants thereof, to the subject; and c) imaging the subject over time to evaluate the distribution and retention of the stem cells in the subject. The imaging agent can be one or more of a MRI (such as e.g. gadolinium based MRI contrast reagent), PET, SPECT, optical (such as e.g. a fluorescent label), ultrasound or radioactive imaging agent.

[0038] Preferred stem cells include mononuclear or mesenchymal stem cells, which can be isolated from bone marrow. The nanoparticles can have a diameter, e.g., of 10 nm to 100 μm, 10 nm to 1 μm, or 10 nm to 100 nm. The stem cells containing the nanoparticles can be administered systemically to the subject, for example by intraperitoneal or intravenous administration. Alternatively, the stem cells can be administered to a localized area of the subject, for example to a site of tissue damage, or adjacent to such site.

[0039] The present invention also provides for the use of the disclosed compositions for therapy or imaging as disclosed herein, and for the use of these compositions for the preparation of therapeutic or imaging compositions.

[0040] The invention is illustrated in the following Experimental Details section, which is set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims that follow thereafter.

EXPERIMENTAL DETAILS

1. Overview

[0041] The following material describes: i) preparative protocols for micro/nano particles; ii) protocols for characterization of the particles; iii) protocols for measuring release profiles from the protocols; and iv) examples of pre-clinical data associated with this platform. The presented technology can serve as a flexible platform for generating delivery/transport vehicles suitable for both diagnostic and therapeutic materials. The expansive range of clinically relevant materials for which this platform can be used is very broad and as a result the applications are also correspondingly expansive.

II. Platform Protocols

[0042] Basic protocol for preparation of hydrogel/glass composites: Tetramethoxysilane TMOS (5 mL) is mixed with an HCl solution (560 μL of 0.2 mM HCl added to 600 μL of deionized water) and then immediately sonicated for 45 minutes in a cool water bath after which the sonicated mixture is placed on ice. The material to be incorporated as a therapeutic (e.g. peptides, chemo therapy agent, plasmids, antioxidants, anti-inflammatory agents, radioprotectants, antimicrobials) or imaging agent (e.g. Magnevist®) is dissolved in 50 mM phosphate buffer (pH 7.5). Polyethylene glycol (e.g., PEG 400) is then added at a ratio of 1 mL PEG/20 mL of buffered solution. Chitosan (5 mg of chitosan/mL acidified distilled water (with 1 M HCl) pH 4.5) is then added at a ratio of 1 mL chitosan solution/20 mL of buffered solution. After the buffered solution is well stirred, the previously sonicated TMOS is slowly
introduced at a ratio of 2 mL TMOS/20 mL buffer. The combined mixture is then stirred immediately and set aside. The resulting mixture gels within 1-2 hours. These monolith (block) sol-gels samples are then taken out of their containers and dried by blotting with paper towels prior to either heating or lyophilization. Control samples are made with the same overall protocol, but with some lacking a specific individual component such as the therapeutic/imaging agent, chitosan or PEG. Samples are also made with different molecular weight PEG chains. The samples are prepared as above, replacing PEG 400 with the same volume of PEGs with molecular weights ranging from e.g., 200-3000. The PEG 400 and PEG 200 are pure liquid samples (approximately 1.1-1.2 g/mL), while the solid PEG 3000 is dissolved in ultrapure water to saturation at 50 mg/mL. Samples are also prepared using polyvinyl alcohol (PVA) instead of PEG. PVA, a well-known biocompatible plasticizer, is anticipated to enhance the strength and flexibility of the particles. Samples are also prepared with the same basic protocol but with the TMOS diluted with other silanes (typically 90:10 vol:vol) chosen to either alter the internal environment of the resulting particles with respect to properties such as hydrophobicity and polarity or to introduce reactive groups (e.g. amino, carboxyl, sulfhydryl) that allow to the covalent attachment of additional molecules to the particles. Molecules to be attached include those that are suitable for fluorescence labeling (e.g. BADAN), radioactive labeling (labeled peptides), tissue targeting (peptides), linkers for attachment of the particles to cell delivery vehicles such as bacteria, and additional PEG molecules to coat the surface of the particles.

Preparation of heat treated hydrogel/glass composites: The dried solid samples are heated in a closed convection oven at 70°C until the gel becomes a hard, white, glassy material (24-48 hours). Excessive heating resulted in a brown discoloration indicative of caramelization of the sugar. Caramelization was never observed when the sample was heated at temperature at or below 70°C. Discolored materials are discarded. The material is then placed in a planetary ball mill (Fritsch, “Pulverisette 6”) for several minutes at a speed of 140 rpm.

Preparation of lyophilized hydrogel/glass composites: The dried hydrogel monoliths generated using the above described protocols are placed into lyophilization flasks and lyophilized for 24 hours. The resulting material is a mix of compacted fine white particulate matter. This mixture is then ground with a mortar and pestle resulting in a fine white powder.

Purpose of the components: The silane is the building block for the sol-gel matrix that provides the robust frame work for the particles. Doping with modified silanes is used to alter the polarity/hydrophobicity/lipophilicity/viscosity within the particles. Doping with silanes containing reactive groups will allow for attachment of additional molecular groups on the surface of the particles. The chitosan has two functions: i) it creates the glassy interior within the sol-gel that plugs the pores of the sol-gel matrix when the material is dry; and ii) it has desirable bio-active properties including facilitating the penetration of the particles through cell junctions and antimicrobial/antibiofilm activity. PEG contributes in any of several ways: i) small PEG chains enhances the hydrophobicity within the particles thus slowing release of therapeutics when exposed to water; ii) large PEG chains facilitate accelerated release of material when the particles are exposed to water; iii) PEG likely inhibits particle clumping and improves the hydrodynamic flow within the circulation; iv) large PEGs on the surface can inhibit clearance and should facilitate localization of particles in regions with leaky vasculature (e.g. tumors); PEG has known in vivo ability to decrease immunogenicity following the administration of PEG coated therapeutics. The PVA is a plasticizer that can slow the breakdown of the particles both in vitro and in vivo.

III. Characterization Protocols

Characterization of the molecular environment within the particles-fluorescent-labeled glutathione (GSH): Characterization of the internal environment experienced by peptides encapsulated into the nano/microparticles can be achieved using BADAN-labeled glutathione (GS-BADAN). The BADAN fluorophore is covalently attached to solvent exposed sulfhydryl groups such as the thiol on GSH. The fluorescence peak position from the BADAN is exquisitely sensitive to the hydrophobicity and mobility of the local environment surrounding the fluorophore. In an aqueous environment, the peak position for a solvent exposed BADAN is ~530-545 nm; whereas the peak for BADAN surrounded by a dynamically frozen polar matrix such as in a dry glass derived from trehalose is ~470-500 nm. In a nonpolar environment, the peak further blue shifts to ~460 nm. Fluorescence from the encapsulated GS-BADAN is used to monitor the interior of the particles (using front face fluorescence) as a function of variations in the composition of the particles. In particular, the TMOS is doped with hydrophobic silanes (10%) such as trimethoxysilylethyl (isopropyl, butyl or fluoropropyl) silane. This approach is similarly used to determine the effect on the internal environment of: i) PEG (concentration and size); ii) addition of PVA instead of PEG; iii) and concentration effects due to other components. Variations on this approach include directly labeling the particles with BADAN by doping the silane component (95% TMOS/5% dopant) of the platform with a thiol containing silane.

Monitoring the interior of the dry particles: Front face fluorescence is used to generate the fluorescence spectrum of dry packed powder samples containing incorporated GS-BADAN. The basic protocol yields a fluorescence peak position of 465 nm indicative of a moderately hydrophobic rigid environment for the labeled glutathione. Results on sol-gels doped with silanes containing alkyl side chains, short PEG chains, sugars and thiols show that the hydrophobicity and polarity of the interior of the particles can be substantially tuned. Interestingly, the results from the PEG containing dopant indicate that PEG enhances the hydrophobicity within the particles which accounts for the improved release profiles when PEG is included in the preparative platform.

Monitoring the progressive exposure to water: With exposure to high humidity, the peak undergoes a progressive shift towards the red consistent with exposure of the interior to water. It is anticipated that a slower uptake of water in the interior of the particles would directly indicate a slower release profile for the encapsulated therapeutic reagents.

Protocol for monitoring the release of agents (e.g., peptides) from the particles: When peptide containing particles are exposed to an aqueous solvent there is the likely possibility that the peptide will be released. The release can be followed by monitoring the particle-free solvent as a function of time. Released BADAN labeled peptides such as GS-BADAN have a fluorescence peak at ~535-540 nm in contrast to the 460 nm peak for the particle entrapped peptide. The spectrum of the released peptide as a function of time subse-
quent to exposure to solvent is achieved through repetitive cycles of spinning down the particles, sampling the supranatant, and then re-suspension of particles followed by a repetition of the process after allowing the particles to remain suspended for varying delays. FIG. 1 shows the results from one such study. It can be seen that there is continued release over a 24 hour period. The spun down particles can also be assayed fluorometrically to evaluate the fractional loss of the initial “buried” peptide. Characterization protocols for the kinetics of release of other entrapped agents can be used based on the specific properties of the entrapped reagents. For example, pVAX-hSc, a plasmid for urolological applications, can be monitored through standard nucleic acid assays. Tadalafil has high absorbance at 220, 280 and 290 nm which can be used to monitor its release. Adriamycin® (doxorubicin) release from the particles was followed using variations on these protocols (see below). Additionally, biological assays represent the final tool in evaluating release profiles as reflected in time dependent efficacy. Evidence for successful release of both tadalafil and silarphin has been established for topical application to rat genitilia as part of studies of treatment of erectile dysfunction.

IV. Examples of Preclinical Studies

1) Transport of MRI Contrast Ragent

[0050] Magnetic resonance imaging (MRI) is a major diagnostic tool. MRI provides a non-invasive in vivo visualization with essentially unlimited penetration depth and high spatial resolution (micrometer rather than several millimeters). Currently, more than 40% of clinical scans are performed with the administration of a contrast agent (CA), which are formulations derived from molecules able to amplify the contrast in the image to obtain a better discrimination between tissues/organs/dominars with potential pathology and normal background tissues. Since the contrast in an MR image arises mostly from differences in the relaxation times of tissues water protons, the contrast agents that are routinely used in clinical practice are mainly paramagnetic chelates of Gd³⁺ ions. Their efficacy as a contrast agent is based on increasing the longitudinal relaxation rate of water proton (1/T1). All commercially approved gadolinium (Gd) compounds are extracellular agents with nonspecific biodistribution and rapid clearance times. There is an ongoing effort to develop CAs with increased circulation times and increased capacity for tissue specific targeting as in the case of cellular surfaces that act as early reporters of a given pathology. MRI targeting strategy is hampered by very low concentration of such reporters and by the relatively low sensitivity of Gd complexes compared to tracers used in other diagnostic imaging techniques (PET, SPECT, optical and ultrasound imaging). These limitations can be overcome using the following combination of strategies: i) increase the density of Gd based CAs within a given location; ii) increase the effective lifetime of the CA; and iii) introduce site/tissue specific targeting associated with the CAs. The nano/micro particle platform provides a means of combining all three strategies. The particles can be loaded with CAs to increase the effective density of the CA. As shown below, the particles have an extended circulation time thus enhancing the effective lifetime of the CA loaded particles. The flexible nature of the platform allows for attachment of peptides as well as large PEG chains, both of which can introduce targeting capabilities. PEGylated particles and molecules accumulate in many tumors and peptides and can be engineered to target specific tissues.

[0051] A study was conducted to address the efficacy with which CA-loaded particles can provide MRI images. MRI images of mice were acquired pre-injection. Subsequently mice were injected (via the tail vein) with one of the following: i) a suspension of empty nanoparticles; ii) a suspension of nanoparticles loaded with Magnevist® (gadopentetate dimeglumine, a commercial Gd based MRI contrast reagent); and iii) Magnevist® solution. Mice were then imaged immediately after injection of each of these materials and again at 2 hours, 4 hours and 24 hours post injection. Maximum pixel intensity of the kidney was measured (relative to the background) at each time point using imaging software. Results for two of the mice are shown here. In the mouse injected with Magnevist® solution alone, the maximum pixel intensity increased by 32% at 4 hours post-injection and returned to pre-injection levels by 24 hours post injection (FIG. 2). For the mouse injected with gadopentetate dimeglumine-loaded nanoparticles the maximum pixel intensity increased by 52% at 4 hours post-injection and increased additionally by 10% at 24 hours post-injection. Furthermore, in the mouse injected with gadopentetate dimeglumine-loaded nanoparticles there was increased signal intensity throughout the GI tract at the 24 hour time point that was not observed with Magnevist® alone. Similar results were obtained for 5 other mice injected with the nanoparticles.

2) Therapeutic Delivery—Disease State Based

A) Ultraviolet (UV)/Gamma Radiation Protection

[0052] Melanin, a ubiquitous, heterogeneous biological polymer composed of many different monomers, contains a population of stationary, semiquinone-like radicals. These radicals allow for trapping by the melanin of reactive species such as reactive oxygen species (ROS) following UV and γ-radiation exposure, therefore acting as direct molecular antagonists of these resulting reactive oxygen species. Furthermore, melanin can attenuate potentially harmful radiation wavelengths through photon absorption (U.S. No. 2007/0237829). Commercial use of melanin has been somewhat difficult due to its large molecular size and poor solubility. Encapsulation of melanin in the nano/micro particles overcomes this impediment. Melanin-containing particles were generated using either of two approaches. The first protocol generates a wet sol-gel type monolith that is filled with L-dopa. Melanin is generated in situ by allowing the L-dopa to polymerize. Once the monolith turns black, it is dried and processed as described above, using either lyophilization or oven heating to create a powder comprised of dry melanin containing particles. The second protocol is similar, but in this case the polymerization of the L-dopa is allowed to proceed in solution generating a concentrated solution of melanin, which is then added to the components that are mixed together to generate the sol-gel monolith. Once the melanin loaded monolith is formed, the same drying protocols are followed as described above. The second protocol appears to be more effective at consistently producing particles with high levels of melanin.

[0053] To demonstrate efficacy as a radiation attenuator, varying densities of brown-brown and black-brown melanin encapsulated nanoparticles (of varying concentrations of melanin) were exposed to measured levels of X-ray radiation. Penetration was documented as compared to controls (empty
particles and encapsulated carbon). A multwell radiograph depicting radiation attenuation was generated (Diagnostic X-ray-GE portable operating at 40 kVp, 10 mA at 1/60 sec exposure). The efficacy of X-ray radiation penetration was inversely proportional to the concentration of melanin encapsulated and density of nanoparticles (FIG. 3).

[0054] In vivo efficacy was demonstrated in a murine model. Set concentrations of melanin-encapsulated nanoparticles were injected into the right dorsal leg while empty nanoparticles (nanoparticles lacking melanin) were injected into the left hindleg as control. Mice were subsequently exposed to therapeutic dosing of gamma-radiation and subsequently followed for 30 days. Limbs were examined daily for radiation induced changes and graded according to the Fowler scoring system.

[0055] Arbitrary desquamation and erythema scoring (Table 1) was recorded over a 30 day period following 30 Gy irradiation over 15 minutes. Mice treated with empty nanoparticles demonstrated the expected response according to previous studies. In contrast, the treated group followed a similar timeline initially, but demonstrated both decreased erythema and desquamative changes, as well as decreased healing time as noted on day 25 (FIG. 4). Clinically, mice treated with the 200 μl subcutaneous injection of encapsulated melanin demonstrated decreased erythema and desquamation when compared to the contralateral irradiated limb. Healing times were improved, which reached a final score of 0.5, while radiation alone reached a final value of 2. Results indicated a significant difference in cutaneous skin changes between the control and treated groups (1.1-1.5 point difference on the breakdown scale; p=0.017).

<table>
<thead>
<tr>
<th>Arbitrary Score</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>mild erythema</td>
</tr>
<tr>
<td>1.0</td>
<td>erythema, mild scaling</td>
</tr>
<tr>
<td>1.25</td>
<td>severe erythema with edema</td>
</tr>
<tr>
<td>1.5</td>
<td>mild, dry breakdown of skin with scaling</td>
</tr>
<tr>
<td>1.75</td>
<td>and crusts</td>
</tr>
<tr>
<td>2.0</td>
<td>possible moist desquamation</td>
</tr>
<tr>
<td>2.5</td>
<td>breakdown of large areas of skin, not all moist</td>
</tr>
<tr>
<td>3.0</td>
<td>breakdown of most of skin with moist exudates</td>
</tr>
</tbody>
</table>

TABLE 1

B) Doxorubicin Chemotherapy

[0056] Doxorubicin (Adriamycin®) is a cytotoxic, anthracycline antibiotic used in antimitic chemotherapy. Cancers treated with doxorubicin include cancers of the bladder, breast, head and neck, leukemia (some types), liver, lung, lymphomas, mesothelioma, multiple myeloma, neuroblastoma, ovary, pancreas, prostate, surcomas, stomach, testis (gern cell), thyroid and uterus. Using absorption spectrocopry, it was determined that there is a dose-dependent and sustained release of drug from the nanoparticles over both short and long term periods of time. Results demonstrated that absorbance increased from the initial introduction of doxorubicin nanoparticles to solution over time. These samples were subsequently stored in the dark at 4°C without shaking, and then spun down at one and two weeks to be re-evaluated. The data demonstrate that with increasing concentrations of nanoparticles, there is an analogous increase in absorbance, suggesting fairly stable release of doxorubicin at the extended time points. These doxorubicin releasing nanoparticles were evaluated via killing efficacy against a cervical cancer cell line (HeLa). Cell counts obtained using the dye exclusion method demonstrated a dose-dependent reduction in cell viability when exposed to drug-containing nanoparticles as compared with control nanoparticles (FIG. 5).

C) Topical Application for Treatment of Erectile Dysfunction: Evidence for Effective Transcutaneous Delivery of Therapeutics

[0057] Oral phosphodiesterase inhibitors have proven very effective for the treatment of erectile dysfunction (ED); nevertheless, a considerable sub-population of patients is refractory to this therapy. Furthermore, the well-documented adverse effects associated with their use are a consequence of these medications being delivered systemically. Local therapy, such as topical application of treatments for ED, will offer several advantages over oral administration. Topical delivery of a treatment can have a localized therapeutic effect with lower dosing and without possible consequences from high systemic concentrations. The present platform for nanoparticle drug encapsulation has been tuned for the cutaneous delivery of proven erectile agents, e.g., tadalafil (pharmacological component of Cialis®), sialorphin (peptide transfer) (Davies et al., 2007) and PVAX-tSlo (gene transfer) (e.g., U.S. Pat. Nos. 6,150,338 and 7,030,906, Melman et al., 2003).

[0058] Animals and Treatment: Retired breeder male Sprague-Dawley rats weighing 650 g and over (a commonly employed animal model for the effects of aging on erectile function (Melman et al., 2003) were used in these studies and obtained from Charles River Breeding Laboratories (Wilmington, Mass., USA). All animal protocols were approved by the Animal Use Committee at the Albert Einstein College of Medicine, Bronx, N.Y.

[0059] Preparation of nanoparticles: Nanoparticles with NO were synthesized as previously described (Friedman et al., 2008, WO 2007/149520). For the tadalafil-nanoparticles, four 10 mg tadalafil capsules (Cialis®, Eli Lilly and Company, Indianapolis, Ind.) were crushed in a mortar and pestle and were subsequently dissolved in 40 mL of 0.05M phosphate buffer (pH 7). Afterwards, 2 mL of PEG, 2 mL of chitosan, and 4 mL of hydrolyzed TMOS was added and the mixture formed a solid gel. The gel was then lyophilized into a dry powder and stored away from moisture. For the sialorphin nanoparticles, 1 mg of sialorphin (Sigma-Aldrich, St. Louis, Mo.) was dissolved into 40 mL of 0.05M phosphate buffer (pH 7). PEG, chitosan, and TMOS were added as above and the resultant solid gel was lyophilized into a powder. This powder was stored away from light and moisture to protect the compound.

[0060] Measurement of ICP/BP: Rats were first anesthetized via intraperitoneal injection of sodium pentobarbital (35 mg/kg, Abbott Laboratory, Chicago, Ill., USA). A cannula was inserted into the carotid artery for systemic pressure (BP) monitoring throughout the experiment. Next an incision was made in the perineum, the ischiocavernous muscle removed to expose the corpus cavernosum crus, and a 23-gauge needle inserted to measure intracavernous pressure (ICP).

[0061] Nanoparticles were administered to the penile surface area as a 50-200 μl suspension in 1.5% Carboxymethylcellulose (CMC), a bulking agent. These suspensions represented approximately 10 nMoles NO (steady state delivery), 50 ng sialorphin, or 1 mg Cialis®. Note that the skin of the
penis was intact and at a different location to the site of measurement of intracorporal pressure. Control animals were treated with “empty” nanoparticles containing only phosphate buffer.

[0062] With tadalafil-nanoparticles, the ICP/BP response was determined both before and after administration of nanoparticles. The cavernous nerves were identified ventrolateral to the prostate gland and carefully isolated. Direct electrostimulation of the cavernous nerve was performed using a delicate stainless steel bipolar hook electrode attached to a multijointed clamp. Each probe was 0.2 mm in diameter with a 1 mm separation between the 2 poles. Monophasic rectangular pulses were delivered by a signal generator (custom-made and with built-in constant current amplifier). Stimulation was performed at 0.75 and 4 mA. Following the first ICP/BP recording, animals were treated with 200 ml suspension. Animals were then stimulated at 0.75 and 4 mA at 30 min intervals after application and the ICP/BP determined.

[0063] Effects of NO nanoparticles on erectile activity: Nitric oxide (NO) is a neurotransmitter that can initiate the development of an erection. Following administration of NO-nanoparticles, intracorporal pressure (ICP) and blood pressure (BP) were monitored for approximately one hour. None of the control animals (seven retired breeders treated with empty nanoparticles) demonstrated any visible erectile response and the ICP/BP remained less than 0.1 for the duration of the experiment. Five out of seven animals tested showed an erectile response when treated with NO-nanoparticles. Visible erections would typically begin in the animals approximately 5 minutes (average 4.5 minutes) after the administration of nanoparticles and would be followed by several other erections of diminishing intensity. The average peak ICP/BP ratio for the first erection was 0.67±/-0.14. The erections were typically of less than 2 minutes duration (average 1.42 minutes). Comparing the observed difference (i.e., an erectile response) between NO-nanoparticle treated and empty nanoparticle (control) groups using Fisher’s exact test, the p-value is p=0.02.

[0064] Effect of sialorphin-nanoparticles on erectile activity: Sialorphin is a neutral endopeptidase inhibitor which can potentially prolong the activity of signal peptides at their receptors and can increase erectile function (e.g., Davies et al. 2007). Over-expression of the gene encoding sialorphin (Vc-sal) can result in a priapic like condition in retired breeder rats (Long et al. 2006, 2008). ICP/BP was monitored for up to 2 hours following administration of sialorphin. A typical result is shown in FIG. 6. Approximately 9 minutes after administration of the sialorphin-nanoparticles a visible erection was observed, which persisted for several minutes. The average peak ICP/BP ratio was 0.72±/-0.13 (an ICP/BP ratio of 0.6 usually results in a visible erection) and a visible erection lasted an average of 8 minutes. The range of time of onset of an erection for the 5 animals investigated was between 4 and 12 minutes (average 4.5 minutes). As described above, none of the animals treated with empty nanoparticles exhibited erectile tendencies. Comparing the observed difference (i.e., an erectile response) between sialorphin-nanoparticle treated and empty nanoparticle (control) groups using Fisher’s exact test, the p-value is p=0.04.

[0065] Effect of tadalafil-nanoparticles on erectile activity: Tadalafil is known to treat erectile dysfunction (e.g., U.S. Pat. No. 5,548,490) and is the active ingredient in Cialis®. The present experimental design takes into account that whereas NO and sialorphin act at the level of neurotransmission, tadalafil’s action is at the level of signaling pathways (downstream of nervous stimulation initiating tumescence). Tadalafil is a PDE5 inhibitor which maintains intracellular levels of cGMP after neuronal stimulation to induce an erection (Turko et al., 1999). Therefore, it was reasoned that after application of the tadalafil-nanoparticles, stimulation of the cavernous nerve might be necessary to obtain an erectile response. Retired breeder rats, which have impaired erectile function, had their response to cavernous nerve stimulation at 0.75 and 4 mA measured prior to the addition of the tadalafil nanoparticles. After administration of the tadalafil particles, erectile response to cavernous nerve stimulation was monitored at approximately 30 minute intervals. A typical result is shown in FIG. 7 and the average from 5 experiments shown in FIG. 8. Prior to treatment with the tadalafil-nanoparticles, cavernous nerve stimulation at either 0.75 or 4 mA did not result in visible erections, and the ICP/BP at both stimulations were below 0.6 (a ratio which usually indicates an erection). However, after 60 min there was a significant improvement (Students t-test=0.05) in the erectile response at the 4 mA level of stimulation compared to animals treated with the empty nanoparticles (negative control). The average ICP/BP after one hour and 4 mA stimulation was 0.737+/−0.029 and visibly improved erectile responses were observed. The efficacy of transdermal delivery of tadalafil by nanoparticles was compared with orally administered tadalafil. The effect on erectile response, as determined by ICP/BP following cavernous nerve stimulation, was not significantly different between orally and topically administered tadalafil. However, there was significantly improved erectile response in both treated groups after 60 minutes at the 4 mA level of stimulation compared to untreated controls.

[0066] Nitric-Oxide delivering nanoparticles improve erectile function in an animal model of radical prostatectomy: Male Sprague-Dawley rats underwent bilateral transaction of the cavernous nerve (CN) one week prior to topical penile application of the NO-nanoparticles. Erectile function, determined by recorded intracorporal pressure/blood pressure (ICP/BP) ratio was followed in anesthetized animals for approximately 2 hours. Six out of eight animals tested showed an increased ICP/BP ratio when treated with the NO-nanoparticles. There was a statistically significant increase in ICP/BP from an average of 0.11+/−0.01 to 0.25+/−0.17 for 6 of the 8 animals studied. Two of these animals had visible spontaneous erections during the time course of the experiment. Control animals treated with empty nanoparticles did not demonstrate any visible erectile response, and the ICP/BP ratio was not significantly impacted during the course of the experiment.

[0067] Following the treatment of animals with nanoparticles, tissue was prepared for histological analysis. There was no evidence of inflammation or congestion. The tissue appeared normal.

D) Nanoparticles Traverse the Dennis

[0068] The location of nanoparticles was investigated after topical application of the nanoparticles to the rat penis. Fluorescently labeled nanoparticles were applied to the penis of rats under anesthesia. After one hour the rat was euthanized and the entire penis washed in phosphate buffered saline and fixed in 5% paraformaldehyde for 24 hours. Cross sections were taken at various points along the shaft of the penis. Cross sections were examined at 4x and 20x magnification. In order to sidestep the autofluorescence of the collagen and other
components of the tissue (fluorescent at a wide range of excitation wavelengths), a dual pass filter (green/red) was used. The green and yellow parts (green and green+red) of the tissue should represent autofluorescence while the bright red spots should represent the locations of nanoparticles. A typical result is shown in FIG. 9. Control animals (not treated with the nanoparticles) did not show any red spots. In all sections, spots could be observed at the dermis of the penis. These nanoparticles are likely to have penetrated the dermis of the skin because washing and fixing of the penis would have removed external nanoparticles. In addition, patches of red fluorescence could be seen in the corpora spongiosa and in the corpora vein. These studies demonstrate the ability of nanoparticle to traverse the dermis, and the feasibility of following biodistribution of the nanoparticles using fluorescence labeling.

E) Uptake of Nanoparticles by Stem Cells and Uses Thereof

Stem cell therapy has emerged as a potential therapeutic option for cell death-related heart diseases. Non-invasive cell tracking approaches could provide knowledge about poorly understood mechanisms responsible for the improvement described in several lesion models, and targeted drug delivery could potentially enhance functional improvement. In order to track the distribution of stem cells injected into animals, optical and magnetic resonance imaging (MRI) are being combined using nanoparticles containing near infrared fluorescent and superparamagnetic nanoparticles. Incorporation of such nanoparticles by bone marrow mesenchymal cells (MSC) and behavior of the labeled cells in vitro and in vivo after transplantation into a rat myocardial infarction model and in a mouse model of heart failure (chagasic cardiomyopathy) are being examined.

Rat or mouse MSC were isolated and cultured in DMEM-F12 with 10% fetal bovine serum and then incubated with dextran-covered iron-containing nanoparticles (Feridex®, ferumoxides injectable solution, Bayer HealthCare Pharmaceuticals) or with in-house generated nanoparticles containing Gd and near infrared fluorescent dye for 4 or 24 h, and poly-L-lysine or protamine chloride (PC) was added. Under these conditions, approximately 95% of the stem cells were labeled with nanoparticles. Incubation with nanoparticles did not affect cell proliferation and viability for up to 7 days in vitro. Nanoparticle-labeled cells were transplanted via intramyocardial injection 24 h after infarction in Wistar rats or via the tail vein two weeks after injection of mice with the protozoan parasite that causes Chagas disease. In the rat model, nanoparticle-labeled cells could be found within the heart for at least 12 days, as determined by MRI. These nanoparticles and this labeling protocol constitute an efficient tool to track injected cells in the lesioned heart.

Inclusion of nanoparticles containing therapeutic product would allow the use of stem cells as drug delivery devices where stem cells home to injured tissue as a consequence of chemotaxis toward released cues. Studies have shown efficacy of use of stem cells without nanoparticles in heart failure (dos Santos et al. 2004, Goldenberg et al. 2008, Soares et al. 2004, Tanowitz et al. 2009, Vilas-Boas et al. 2004, 2006). Nanoparticles could be used that are labeled with a therapeutic agent, such as for example nitric oxide, growth hormone, and/or a growth factor or cytokine prior to being taken up by stem cells used in therapy. Nitric oxide, for example, would be expected to increase blood perfusion to the area of tissue damage and to enhance muscle contraction.

Incorporation of nanoparticles containing probes detectable, for example, by MRI (e.g., Gd, Fe, other metals) or by optical imaging (e.g., long wavelength indicators including quantum dots) will allow evaluation of distribution and retention of stem cells throughout the animal. Different agents could be included on the nanoparticles to test how such agents affect the growth and development of stem cells.

Nanoparticles tagged with antibodies or other cell recognition molecules on the surface of stem cells could enhance distribution to specific organs or pathological sites (such as tumors).

The present invention provides a flexible platform for the preparation of compositions comprised of nano- to micro-sized particles that incorporate features of both silane based hydrogels and polyol derived glasses as a general vehicle for delivery and controlled release of diagnostically and therapeutically relevant materials including but not limited to, doxorubicin, gadolinium-based MRI contrast reagents, melanin, peptides (e.g. glutathione, sirolphin), MaxiK gene therapy, nitric oxide, tadalafil, hormones such as growth hormone, growth factors and cytokines. Encapsulation of these and related materials into the nano/micro particles allows for controlled delivery in terms of route of delivery (e.g., topical, subcutaneous, transdermal, intraperitoneal, intravenous and aerosol administration), sustainability in vivo, and ultimately efficacy of delivered product through the ease with which the nanotechnology protocol can be tailored. The present platform has demonstrated long term stability as a powder and persistence of several hours and days in the circulation. It is comprised of nontoxic biocompatible materials.

The silane precursors used in the nanoparticle platform provide the chemical flexibility to allow for significant pre- or post-preparative modifications including labeling with peptides (e.g., for tissue targeting and PET imaging) and fluorophores as well as linkers for attachment of the particles to other materials. There are available numerous silanes with reactive groups (e.g., amino terminus, reactive sulfhydryl, carboxyl, etc.) that can be used as dopants which then allow for subsequent covalent modification of the particles. The hydrophobicity of the interior of the particles can be tuned thus allowing for encapsulation of both hydrophobic and hydrophilic molecules. The platform allows for the facile covalent attachments of a wide variety of organic and biochemical reagents most importantly peptides that can be used for targeting or linker molecules that can be used, for example, to attach the particles to bacteria or as means of facilitating transfer into cells.

All publications mentioned herein are hereby incorporated in their entirety into the subject application. While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art, from a reading of the disclosure, that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.

REFERENCES


[0090] U.S. Pat. No. 6,150,338, issued Nov. 21, 2000, Geliebter et al., Gene therapy for alleviating erectile dysfunction.


1. A composition for controlled delivery and/or containment of one or a combination of therapeutic and/or diagnostic agents comprising the agent or agents encapsulated in a matrix comprising a) chitosan, b) polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and c) tetra-methoxy-ortho-silicate (TMOS) or tetra-ethoxy-ortho-silicate (TEOS).

2. The composition of claim 1, wherein the polyethylene glycol has a molecular weight of 200 to 20,000 Daltons.

3. The composition of claim 1, comprising a silane in addition to TMOS or TEOS.

4. The composition of claim 3, wherein the additional silane contains an alkyl side chain, a PEG having a molecular weight of 200 to 400 Daltons, a sugar, an amino terminus, or a reactive sulfhydryl or carboxyl group.

5. The composition of claim 3, wherein the additional silane is a hydrophobic silane.

6. (canceled)

7. The composition of claim 1 comprising polyethylene glycol (PEG) and tetra-methoxy-ortho-silicate (TMOS).

8. The composition of claim 1, wherein the composition is in the form of particles having a diameter of 10 μm to 1 μm.

9. (canceled)

10. The composition of claim 1, wherein the agent is one or more of a therapeutic peptide, a chemotherapeutic agent, melanin, an agent for treating erectile dysfunction or a urological disorder, a plasmid, an antioxidant, an anti-inflammatory agent, a radioprotectant, an antimicrobial agent, nitric oxide, growth hormone, a growth factor or a cytokine.

11-12. (canceled)

13. The composition of claim 1 comprising a fluorescent label or a radioactive label or an imaging agent.

14-15. (canceled)

16. A method for preparing a composition for controlled delivery and/or containment of one or more therapeutic and/or diagnostic agent, the method comprising:

(a) admixing the agent or agents with i) chitosan, ii) polyethylene glycol (PEG) and/or polyvinyl alcohol (PVA), and iii) tetra-methoxy-ortho-silicate (TMOS) or tetra-ethoxy-ortho-silicate (TEOS) in a buffered solution;

(b) drying the mixture of step (a) to produce a gel; and

(c) heating the gel to produce a glassy material, or lyophilizing the gel to produce a particulate material.

17. The method of claim 16, wherein the polyethylene glycol has a molecular weight of 200 to 20,000 Daltons.

18. The method of claim 17, where the TMOS or TEOS is diluted with an additional silane.

19-21. (canceled)

22. The method of claim 16, wherein the gel is heated in step (c) at a temperature of 70°C or less than 70°C.

23. The method of claim 16, which further comprises grinding or milling the material of step (c) to produce particles of a desired size.

24-26. (canceled)

27. A composition produced by the method of claim 16.
28. A method of treating a subject comprising administering a therapeutic amount of the composition of claim 1 to the subject.

29. The method of claim 28, wherein the subject has cancer or erectile dysfunction or is at risk for exposure to ionizing radiation.

30-32. (canceled)

33. A method of imaging a subject comprising administering the composition of claim 1 to the subject.

34-36. (canceled)

37. A method of treating a subject with tissue damage, comprising: a) incorporating the composition of claim 10 into stem cells, wherein the composition is in the form of nanoparticles and the nanoparticles comprise a therapeutic agent; and b) administering the stem cells of step a) to the subject in an amount and manner effective to treat tissue damage in the subject.

38-39. (canceled)

40. A method of evaluating distribution and retention of stem cells in a subject, comprising:
   a) incorporating the composition of claim 13 into stem cells, wherein the composition is in the form of nanoparticles and the nanoparticles comprise an imaging agent;
   b) administering the stem cells of step a) to the subject; and
   c) imaging the subject overtime to evaluate the distribution and retention of the stem cells in the subject.

41-44. (canceled)