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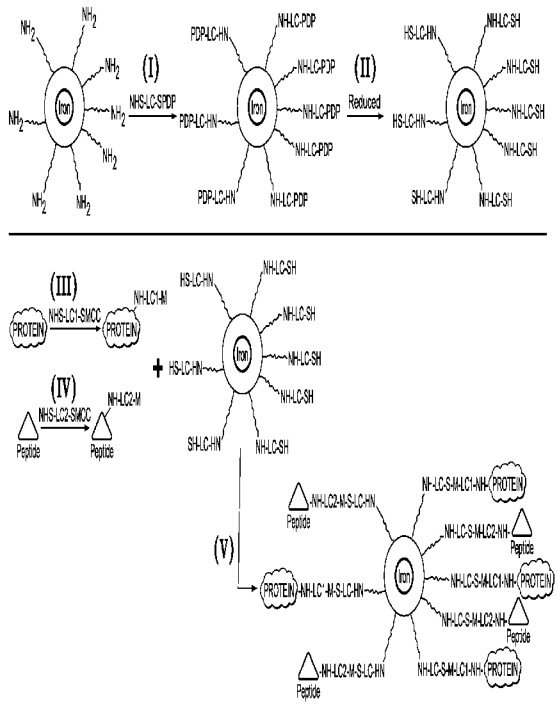

(57) Abstract: Functionalized biocompatible nanoparticles capable of penetrating through a mammalian cell membrane and delivering intracellularly a plurality of bioactive molecules for modulating a cellular function are disclosed herein The functionalized biocompatible nanoparticles comprise: a central nanoparticle ranging in size from about 5 to about 50 nm and having a polymer coating thereon, a plurality of functional groups covalently attached to the polymer coating, wherein the plurality of bioactive molecules are attached to the plurality of the functional groups, and wherein the plurality of bioactive molecules include at least a peptide and a protein, and wherein the peptide is capable of penetrating through the mammalian cell membrane and entering into the cell, and wherein the protein is capable of providing a new functionality within the cell. The protein may be a transcription factor selected from the group consisting of Oct4, Sox2, Nanog, Lin28, cMyc, and Klf4.

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FUNCTIONALIZED NANOPARTICLES FOR INTRACELLULAR DELIVERY OF BIOLOGICALLY ACTIVE MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 61/550,213, filed on October 21, 2011, which application is incorporated herein by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] The present invention relates generally to organic synthesis and nanobiotechnology, and, more particularly, to functionalized nanoparticles for the delivery of bioactive molecules into cells for modulation of cellular function, as well as to methods related thereto.

BACKGROUND OF THE INVENTION

The ability of cells to normally proliferate, migrate and differentiate to various cell types is critical in embryogenesis and in the function of mature cells, including but not limited to the cells of hematopoietic and/or cardiovascular systems in a variety of inherited or acquired diseases. This functional ability of stem cells and/or more differentiated specialized cell types is altered in various pathological conditions, but can be normalized upon intracellular introduction of biologically active components. For example, abnormal cellular functions such as impaired survival and/or differentiation of bone marrow stem/progenitor cells into neutrophils are observed in patients with cyclic or severe congenital neutropenia who may suffer from severe life-threatening infections and may evolve to develop acute myelogenous leukemia or other malignancies [Aprikyan et al., Impaired survival of bone marrow hematopoietic progenitor cells in cyclic neutropenia. Blood, 97, 147 (2001); Goran Carlsson et al., Kostmann syndrome: severe congenital neutropenia associated with defective expression of Bcl-2, constitutive mitochondrial release of cytochrome C, and excessive apoptosis of myeloid progenitor cells. Blood, 103, 3355 (2004)]. Inherited or acquired disorders such as severe congenital neutropenia or Barth syndrome are triggered by various gene mutations and are due to deficient production and function of patients' blood and/or cardiac cells leading to subsequent neutropenia, cardiomyopathy and/or heart failure

[Makaryan et al., The cellular and molecular mechanisms for neutropenia in Barth syndrome. Eur J Haematol. 88:195-209 (2012)]. Severe congenital neutropenia disease phenotype can be caused by different substitution, deletion, insertion or truncation mutations in the neutrophil elastase gene, HAX1 gene, or Wiskott-Aldrich Syndrome Protein gene [Dale et al., Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood.* 96:2317-2322 (2000); Devriendt et al., Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet.* 27:313-7 (2001); Klein et al., HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease) *Nat Genet.* 39:86-92 (2007)].

[0003] Other inherited diseases like Barth syndrome, a multi-system stem cell disorder induced by presumably loss-of-function mutations in the mitochondrial TAZ gene is associated with neutropenia (reduced levels of blood neutrophils) that may cause recurring severe and sometimes life-threatening fatal infections and/or cardiomyopathy that may lead to heart failure that could be resolved by heart transplantation. In most of the cases, the mutant gene products, implicated in pathogenesis and development of inherited or acquired human diseases, affect distinct intracellular events, which lead to abnormal cellular functions and the specific disease phenotype.

[0004] Treatment of these patients with granulocyte colony-stimulating factor (G-CSF) induces conformational changes in the G-CSF receptor molecule located on the cell surface, which subsequently triggers a chain of intracellular events that eventually restores the production of neutrophils to near normal level and improves the quality of life of the patients [Welte and Dale. Pathophysiology and treatment of severe chronic neutropenia. Ann. Hematol. 72, 158 (1996)]. Nevertheless, patients treated with G-CSF may evolve to develop leukemia [Aprikyan et al., Cellular and molecular abnormalities in severe congenital neutropenia predisposing to leukemia. Exp Hematol. 31, 372 (2003); Philip Rosenberg et al., Neutrophil elastase mutations and risk of leukaemia in severe congenital neutropenia. Br J Haematol. 140, 210 (2008); Peter Newburger et al., Cyclic Neutropenia and Severe Congenital Neutropenia in Patients with a Shared *ELANE* Mutation and Paternal Haplotype: Evidence for Phenotype Determination by Modifying Genes. Pediatr. Blood Cancer, 55, 314 (2010)], which is why novel alternative approaches are being explored.

[0005] The intracellular events can be more effectively affected and regulated upon intracellular delivery of different biologically active molecules using distinctly functionalized

nanoparticles. These bioactive molecules may normalize the cellular function or may eliminate the unwanted cells when needed. However, the cellular membrane serves as an active barrier preserving the cascade of intracellular events from being affected by exogenous stimuli.

[0006] Accordingly, there is a need in the art for new types of bioactive molecules that are capable of penetrating cellular membranes and effectuating the intracellular events of interest. The present invention fulfills these needs and provides for further related advantages.

SUMMARY OF THE INVENTION

[0007] The present invention in some embodiments is directed to functionalization methods of linking proteins and/or peptides to biocompatible nanoparticles for modulating cellular functions. In some embodiments, the present invention is directed to the functionalized biocompatible nanoparticles themselves.

[0008] In an embodiment, a functionalized biocompatible nanoparticle capable of penetrating through a mammalian cell membrane and delivering intracellularly a plurality of bioactive molecules for modulating a cellular function, comprises: a central nanoparticle ranging in size from 5 to 50 nm and having a polymer coating thereon, a plurality of functional groups covalently attached to the polymer coating, wherein the plurality of bioactive molecules are attached to the plurality of the functional groups, and wherein the plurality of bioactive molecules include at least a peptide and a protein, and wherein the peptide is capable of penetrating through the mammalian cell membrane and entering into the cell, and wherein the protein is capable of providing a new functionality within the cell.

[0009] The central nanoparticle may comprise iron and be magnetic. The peptides of the present invention may be attached to the protein (as opposed to being attached to the nanoparticle). The peptides and proteins may each be attached to the nanoparticle by way of one or more interposing linker molecules. The peptide may include five to nine basic amino acids in some embodiments, whereas in other embodiments the peptide includes nine or more basic amino acids. The protein may be a transcription factor such as, for example, a transcription factor selected from the group consisting of Oct4, Sox2, Nanog, Lin28, cMyc, and Klf4.

[0010] In another aspect, the present invention is directed to a method of changing a cellular functionality within a mammalian cell. The novel method comprises administering an

effective amount of functionalized biocompatible nanoparticles to the cell and changing the cellular functionality within the cell. The changing of the cellular functionality may involve a change in a physico-chemical property of the cell, a change in proliferative property of the cell, a change in surviving ability of the cell, or a change in morphological phenotypical property of the cell. The changing of the cellular functionality may involve an acquired ability of the cell to make a new cell type including a stem cell or a more specialized cell type.

[0011] These and other aspects of the present invention will become more evident upon reference to the following detailed description and attached drawings. It is to be understood, however, that various changes, alterations, and substitutions may be made to the specific embodiments disclosed herein without departing from their essential spirit and scope. Finally, all of the various references cited herein are expressly incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 depicts a multi-step functionalization of nanoparticles scheme based on the simultaneous attachment of peptide and protein molecules to a nanoparticle in accordance with an embodiment of the present invention.

[0013] Figure 2A depicts a reaction of a nanoparticle containing amine groups with equimolar ratios of long chain LC1-SPDP and Iodoacetic acid nanoparticle in accordance with an embodiment of the present invention.

[0014] Figure 2B depicts a reduction of the disulfide bond of PDP to provide a free SH group nanoparticle in accordance with an embodiment of the present invention.

[0015] Figure 2C depicts a reaction of long chain LC1-SMCC with the lysine groups of a protein nanoparticle in accordance with an embodiment of the present invention.

[0016] Figure 2D depicts a reaction of a multifunctional nanoparticle with the protein that had been reacted with SMCC and contains a terminal reactive maleimide group nanoparticle in accordance with an embodiment of the present invention.

[0017] Figure 2E depicts a reaction of an amino group of a peptide with LC2-SMCC. The reaction is then subsequently followed by a reaction with mercaptoethanol to convert the terminal maleimide to an alcohol nanoparticle in accordance with an embodiment of the present invention.

[0018] Figure 2F depicts a reaction of a functional bead (and protein attached) with a modified peptide to the free carboxyl group on the nanoparticle nanoparticle in accordance with an embodiment of the present invention.

[0019] Figure 3A depicts a reaction of a nanoparticle containing amine groups with LC1-SPDP nanoparticle in accordance with an embodiment of the present invention.

[0020] Figure 3B depicts a reduction of the disulfide bond of PDP to provide a free SH group nanoparticle in accordance with an embodiment of the present invention.

[0021] Figure 3C depicts a reaction of long chain LC2-SMCC with the lysine groups of a protein nanoparticle in accordance with an embodiment of the present invention.

[0022] Figure 3D depicts a reaction of a multifunctional nanoparticle with the protein that had been reacted with SMCC and contains a terminal reactive maleimide group nanoparticle in accordance with an embodiment of the present invention.

[0023] These and other aspects of the present invention will become more readily apparent to those possessing ordinary skill in the art when reference is made to the following detailed description in conjunction with the accompanying drawings.

DETAILED DESCRIPTION OF THE INVENTION

[0024] In order to deliver biologically active molecules intracellularly, the inventors of the present invention present a universal device based on cell membrane-penetrating nanoparticles with covalently linked biologically active molecules. To this end, the inventors present herein a novel functionalization method that ensures a covalent linkage of proteins and peptides to nanoparticles. The modified cell-permeable nanoparticles of the present invention provide a universal mechanism for intracellular delivery of biologically active molecules for regulation and/or normalization of cellular function.

[0025] The ability of cells to normally proliferate, migrate and differentiate to various cell types is critical in embryogenesis and in the function of mature cells, including but not limited to the stem/progenitor and more differentiated cells of hematopoietic and cardiovascular systems in a variety of inherited or acquired diseases. This functional ability of stem cells and/or more differentiated specialized cell types is altered in various pathological conditions due to aberrant alterations in intracellular events, but can be normalized upon intracellular introduction of biologically active components. For example, the impaired survival and differentiation of human bone marrow progenitor cells into

neutrophils that is observed in patients with cyclic or severe congenital neutropenia who suffer from severe life-threatening infections and may evolve to develop leukemia, may be normalized by cell membrane-penetrant small molecule inhibitor of neutrophil elastase, which interferes with aberrant intracellular events and apparently restores the normal phenotype. Nevertheless, such small molecules specific to target mutant products causing the disease are rarely available which is why alternative efficient cell membrane-penetrant devices are needed for intracellular delivery of biologically active molecules capable of modulating cellular function.

[0026] The methods disclosed herein utilize biocompatible nanoparticles, including for example, superparamagnetic iron oxide particles similar to those previously described in scientific literature. This type of nanoparticles can be used in clinical settings for magnetic resonance imaging of bone marrow cells, lymph nodes, spleen and liver [see, e.g., Shen et al., Monocrystalline iron oxide nanocompounds (MION); physicochemical properties. Magn. Reson. Med., 29, 599 (1993); Harisinghani et. al., MR lymphangiography using ultrasmall superparamagnetic iron oxide in patients with primary abdominal and pelvic malignancies. Am. J. Roentgenol. 172, 1347 (1999)]. These magnetic iron oxide nanoparticles contain ~5 nm nucleus coated with cross-linked dextran and having ~45 nm overall particle size. Importantly, it has been demonstrated that these nanoparticles containing cross-linked cell membrane-permeable Tat-derived peptide efficiently internalize into hematopoietic and neural progenitor cells in quantities of up to 30 pg of superparamagnetic iron nanoparticles per cell [Lewin et al., Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat. Biotechnol. 18, 410 (2000)]. Furthermore, the nanoparticle incorporation does not affect proliferative and differentiation characteristics of bone marrow-derived CD34+ primitive progenitor cells or the cell viability [Maite Lewin et al., Nat. Biotechnol. 18, 410 (2000)]. These nanoparticles can be used for in vivo tracking the labeled cells.

[0027] The labeled cells retain their differentiation capabilities and can also be detected in tissue samples using magnetic resonance imaging. Here we present novel nanoparticle-based devices which are now functionalized to carry peptides and proteins that can serve as excellent vehicles for intracellular delivery of biologically active molecules for cell reprogramming solutions to target intracellular events and modulate cellular function and properties.

[0028] General Description of Nanoparticle-Peptide/Protein Conjugates:

[0029] Nanoparticles based on iron or other material with biocompatible coating (e.g. dextran polisaccharide) with X/Y functional groups, to which linkers of various lengths are attached, which, in turn are covalently attached to proteins and/or peptides (or other small molecules) through their X/Y functional groups.

[0030] Functional groups that may be used for crosslinking include:

[0031] -NH_2 (e.g. , lysine, a—NH_2),

[0032] -SH,

[0033] -COOH,

[0034] -NH-C(NH)(NH_2),

[0035] carbohydrate,

[0036] -hydroxyl (OH),

[0037] -attachment via photochemistry of an azido group on the linker.

[0038] Crosslinking reagents may include:

[0039] SMCC [succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate] Also available is Sulfo-SMCC, the Sulfosuccinimidylderivative for crosslinking amino and thiol groups.

[0040] LC-SMCC (Long chain SMCC). Also Sulfo-LC-SMCC.

[0041] SPDP [N-Succinimidyl-3-(pypridyldithio)-proprionate] Also Sulfo-SPDP. Reacts with amines and provides thiol groups.

[0042] LC-SPDP (Long chain SPDP). Also Sulfo-LC-SPDP.

[0043] EDC [1-Ethyl Hydrocholride-3-(3-Dimethylaminopropyl)carbodiimide] Reagent used to link –COOH group with—NH_2 group.

[0044] SM(PEG)n where n=1,2,3,4…….24 glycol units. Also the Sulfo-SM(PEG)n derivative.

[0045] SPDP(PEG)n where n=1,2,3,4…….12 glycol units. Also the Sulfo-SPDP(PEG)n derivative.

[0046] PEG molecule containing both carboxyl and amine groups.

[0047] PEG molecule containing both carboxyl and sulfhydryl groups.

[0048] Capping and blocking reagents include:

[0049] Citraconic Anhydride---specific for NH

[0050] Ethyl Maleimide---specific for SH

[0051] Mercaptoethanol—specific for maliemide

[0052] In view of the foregoing, we have treated biocompatible nanoparticles to produce functional amines on the surface, which in turn were used to chemically bind proteins and short peptides.

[0053] In the case of attaching proteins, for example Green Fluorescent Protein or a transcription factor, to superparamagnetic or alternative nanoparticles, the following protocol can be used: Superparamagnetic beads containing amino functional groups on the exterior can be commercially purchased from various manufacturers. They can range from 20-50 nm in size and 10^{15}-10^{20} nanoparticles per ml with 10 or more amine groups per nanoparticle. The nanoparticles are placed into the correct reaction buffer (0.1 M phosphate buffer, pH 7.2) by use of an Amicon centrifugal filter unit (microcolumn) with a molecular cutoff of 10,000 dalton. Approximately 4 washes are generally required to ensure proper buffering system. Nanoparticles are removed from the filter unit as recommended by manufacturer (inverting column/filter device by spinning at low speed).

[0054] SMCC (from Thermo Fisher) was dissolved in dimethylformamide (DMF) obtained from ACROS (sealed vial and anhydrous) at the 1 mg/ml concentration. Sample is sealed and used almost immediately.

[0055] Ten (10) microliters of the solution are added to nanoparticles in 200 microliter volume. This provides a large excess of SMCC to the available amine groups present, and the reaction is allowed to proceed for one hour. Excess SM and DMF can be removed using an Amicon centrifugal filter column with a cutoff of 3,000 daltons. Five exchanges of volume are generally required to ensure proper buffer exchange. It is important that excess of SMCC be removed at this stage.

[0056] Any peptide based molecule, as an example commercially available Green Fluorescent Protein (GFP) or purified recombinant GFP or other proteins are added to the solution containing a certain amount of ethylene glycol for freezing at -30 ^{O}C. To 3 micrograms of the protein in 14 microliters, 10 microliters of a freshly prepared DTT (dithiothreitol, Cleland's reagent) solution in PBS are added with vigorous vortexing. Because the proteins usually contain more than one cysteine, there is a tendency to crosslink different GFP molecules. Therefore, the excess DTT reduces the dithiol linkage and frees GFP. Reaction is allowed to proceed for two hours at 4^{O}C and then excess reagent is removed by an Amicon centrifugal filter unit with a 3,000 MW cutoff.

[0057] The activated nanoparticles and the protein solutions are combined and allowed to react for two hours, after which the unreacted protein is removed by an Amicon centrifugal filter unit having an appropriate MW cutoff (in the example with GFP it is 50,000 dalton cut-off). Sample is stored at -80^{O}C. Instead of using Amicon spin filter columns, small spin columns containing solid size filtering components, such as Bio Rad P columns can also be used. These are size exclusion columns. It should also be noted that SMCC also can be purchased as a sulfo derivative (Sulfo-SMCC), making it more water soluble. DMSO may also be substituted for DMF as the solvent carrier for the labeling reagent; again, it should be anhydrous.

[0058] All the other crosslinking reagents can be applied in a similar fashion. SPDP is also applied to the protein/applicable peptide in the same manner as SMCC. It is readily soluble in DMF. The dithiol is severed by a reaction with DTT for an hour or more. After removal of byproducts and unreacted material, it is purified by use of an Amicon centrifugal filter column with 3,000 MW cutoff.

[0059] Another more direct and controlled means of labeling a nanoparticle with a peptide and protein would be to use two different bifunctional coupling reagents. The reaction sequence is somewhat similar to that of Figure 1. Iodoacetic acid is used to introduce a select number of "carboxyl" groups on the nanoparticle surface.

[0060] The peptide containing the LC-SMCC is treated with aminomercaptoethanol. This creates a linkage through the sulfhydryl group and provides a free amino group. This amino group is then coupled to the carboxyl group on the nanoparticle using EDC. EDC is known as 1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride. This coupling step is performed last in the reaction scheme.

[0061] Figure 1 shows the general description of the magnetic nanoparticles---protein/peptide adducts. The magnetic nanoparticle is coated with a polysaccharide and then functionalized. It can be purchased with amines on the surface. They can also be altered/morphed into any other functional formats. The extender/connector physically binds the two units together.

[0062] Various functional groups may be used to chemically attach the nanoparticle to the protein via crosslinking reactions. The variety of functional groups available does allow for numerous proteins/peptides to be attached to the nanoparticle, one step at a time.

[0063] Similarly, various crosslinking reagents or reactive catalysts may be used to crosslink nanoparticles with proteins/peptides via hetero-bifunctional reagents. It should also be noted

that these crosslinking reagents come in various lengths. For instance many contain the LC notation, referring to extenders or "long chains". The pegylated compound is also available in various lengths. In this way linkers of various lengths may be added to the nanoparticles and provide differing attachment lengths for larger molecules, such as proteins and small molecules, such as peptides.

[0064] Often time different proteins may contain the same functional groups, making it difficult to label the nanoparticle with the various proteins. There are reagents which allow a change in functional groups; hence, we can change the functional groups on proteins, thus giving us the selectivity in a step wise fashion without interference from the other proteins. This requires changing the functional groups on proteins.

[0065] Various reagents can be used to alter proteins so that different chemistries may be used to attach proteins with like functional groups. For instance, a compound, such as SPDP, can be used to convert and amine to a sulfhydryl, which is then receptive towards reaction with a maleimide moiety.

[0066] When attaching proteins to the bead (nanoparticle) in a stepwise fashion, often residual and active groups of proteins that were attached previously may interfere with the coupling chemistries. Thus permanent or reversible capping reagents may be used to block these active moieties from interference with reagents that are about to be used to attach a second or third protein to the nanoparticle.

[0067] Numerous different capping compounds may be used to block the unreacted moiety. They need to be used judiciously as the capping compounds may also interfere with protein activity. Used most often when a second chemical attachment step is required and this functional group may interfere.

[0068] To show that proteins can be attached to beads (nanoparticles) using the chemistries noted above, we provided the synthesis of magnetic nanoparticles, which contained Green Fluorescent protein derived from Jelly fish. LCC-SMCC was used in this synthesis scheme.

[0069] The N-hyhroxysuccinimide is chemically reacted with the free amine groups on the nanoparticle in order to form a chemical bond. This provides a maleimide end group that can react with GFP. It is known that GFP has two cysteines and the cysteines from various GFP molecules may react to form disulfide bonds. To remove such interference, the molecule is first reduced with Cleland's reagent.

[0070] The protein is purified and then allowed to react with beads containing the LC-maleimide group. The reaction is allowed to proceed for 1 hour and reaction purified on Amicon spin filter (50K cutoff). Pictures were taken on the fluorescence electron microscope.

[0071] Multiple types of functional groups can be created on a nanoparticle. This allows the addition of three or more different proteins to be attached.

[0072] One first starts out with an amine on the surface.

[0073] Traut's reagent may be used to convert some of those amines to sulfhydryl. In addition iodoacetic acid may be used to convert some amines to carboxylic acid

[0074] For both proteins and peptides, the amines are converted to the functional groups with different linker length as described in more detail below. This will serve as a generalized group to attach proteins and peptides.

[0075] Figure 1 depicts schematic representation of nanoparticle functionalization and binding of peptides and proteins to nanoparticle.

[0076] The syntheses and coating are performed as follows: NHS-LC-SPDP commercially available through Thermo Fisher is a long chain extender with bifunctional coupling reagents on either side, which are specific for amines and a disulfide that can be converted to a sulfide.

[0077] One end has an N-Hydroxysuccinimide ester, while the other end of the extender contains a pyridyldithiol group. This dithiol group can be reduced to produce a sulfhydryl. NHS-LC-SPDP is allowed to react with the nanoparticles and the reaction can be cleared from unincorporated NHS-LC-SPD. The coupled nanoparticles are then reduced as shown in Figure 1.

[0078] Production of Coupled Proteins: The biologically active proteins purified using affinity columns contain a free epsilon-amine group from carboxy-terminal lysine residue added to facilitate binding to the nanoparticles. NHS-LC-SMCC is used as the bifunctional coupling reagent. The molecule has an LC1 chain extender. One end has the N-Hydroxysuccinimide reagent specific for amines. The other end contains the maleimide group, very specific for sulfhydryl groups. Once the material is coupled to a protein and separated from the reaction mixture, the maleimide coupled protein will be added to the sulfhydryl containing nanoparticle. The resultant material s separated by gel filtration.

[0079] Peptide Coupling to Nanoparticle: In this case the peptide also contains a carboxy-terminal lysine that will serve as the base for the NHS ester-LC-maleimide coupling. The

molecule has an LC2 chain extender. All procedures are similar to those describe above for the protein.

[0080] During the optimization, the membrane-permeable peptide and the proteins will be mixed at different ratios to achieve the maximum number of molecules coupled to nanoparticle. Based on previously published studies, 3-4 molecules of surface-bound cell-penetrating peptide per nanoparticle are sufficient for efficient intracellular delivery of superparamagnetic nanoparticles.

[0081] The use of LC2-extender arm provides an important means to increase the number of bound peptide-based molecules. The use different concentration of NHS-LC-SPDP allows increased number of anchored peptide and protein molecule to the surface of nanoparticles, and therefore, more efficient penetration and consequently, more robust cell reprogramming activity.

[0082] Attachment of Peptides and Proteins on One Nanoparticle: This may be accomplished using the procedure shown in Figure 1. In this case, ratios of SMCC labeled proteins and peptides are added to the beads and allowed to react.

[0083] Another more direct and controlled means of labeling a nanoparticle with a peptide and protein would be to use two different bifunctional coupling reagents (Figures 2A-F). The reaction sequence is somewhat similar to that of Figure 1 with some modifications described below.

[0084] Iodoacetic acid is used to introduce a select number of "carboxyl" groups on the nanoparticle surface. This is performed at step I; see Figures 2A-F, steps (I-VII).

[0085] The peptide containing the NH-LC-SMCC is treated with aminoethanol. This creates a linkage through the sulfhydryl group and provides a free amino group. This amino group is then coupled to the carboxyl group on the nanoparticle using EDAC (EDC). EDAC is known as 1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride. This coupling step is performed last in the reaction scheme.

[0086] In another aspect, the present invention is also directed to a method of delivering bioactive molecules attached to functionalized nanoparticles for modulation of intracellular activity. For example, human cells, fibroblasts or other cell types that are either commercially available or obtained using standard or modified experimental procedures are first plated under sterile conditions on a solid surface with or without a substrate to which the cells adhere (feeder cells, gelatin, martigel, fibronectin, etc.). The plated cells are cultured for

a time with a specific factor combination that allows cell division/proliferation or maintenance of acceptable cell viability. Examples are serum and/or various growth factors, which can later be withdrawn or refreshed and the cultures continued. The plated cells are cultured in the presence of functionalized biocompatible cell-permeable nanoparticles with bioactive molecules attached using various methods described herein in the presence or absence of magnetic field. The use of a magnet in case of superparamagnetic nanoparticles renders an important increase in the contact surface area between the cells and nanoparticles and thereby reinforces further improved penetration of functionalized nanoparticles through the cell membrane. When necessary, the cell population is treated repeatedly with the functionalized nanoparticles to deliver the bioactive molecules intracellularly.

[0087] The cells are suspended in culture medium, and non-incorporated nanoparticles are removed by centrifugation or cell separation, leaving cells that are present as clusters. The clustered cells are then resuspended and recultured in fresh medium for a suitable period. The cells can be taken through multiple cycles of separating, resuspending, and reculturing, until a consequent biological effect triggered by the specific bioactive molecules delivered intracellularly is observed.

[0088] One use of the invention is the screening of a compound (or compounds) for an effect on cell reprogramming. This involves combining the compound attached to the nanoparticle using one or more of the methods in disclosed herein with a cell population of interest, culturing for suitable period and then determining any modulatory effect resulting from the compound(s). This may include initiation of the cell reprogramming and generation of pruripotent stem cells, differentiation or transdifferentiation of cells to more specialized or different specialized cell types, examination of the cells for toxicity, metabolic change, or an effect on contractile activity and other functions.

[0089] Another use of the invention is the formulation of specialized cells as a medicament or in a delivery device intended for treatment of a human or animal body. This enables the clinician to administer the cells in or around the damaged tissue (whether heart, muscle, liver, etc.) either from the vasculature or directly into the muscle or organ wall, thereby allowing the specialized cells to engraft, limit the damage, and participate in regrowth of the tissue's musculature and restoration of specialized function.

[0090] A use of the present invention involves nanoparticles functionalized with other proteins such as Oct4 and Sox2 transcription factors so as to ensure cell reprogramming and generation of stem or more differentiated cell types with preservation of intact genome.

[0091] Another use of the present invention is the screening of a compound (or compounds) for an effect on cell reprogramming. This involves combining the compound attached to the nanoparticle using the methods disclosed herein with a cell population of interest, culturing for suitable period and then determining any modulatory effect resulting from the compound(s). This may include initiation of the cell reprogramming and generation of pluripotent stem cells, differentiation or transdifferentiation of cells to more specialized or different specialized cell types, examination of the cells for toxicity, metabolic change, or an effect on contractile activity and other functions.

[0092] Still another use of the present invention is the formulation of specialized cells as a medicament or in a delivery device intended for treatment of a human or animal body. This enables the clinician to administer the cells in or around the damaged tissue (whether heart, muscle, liver, etc) either from the vasculature or directly into the muscle or organ wall, thereby allowing the specialized cells to engraft, limit the damage, and participate in regrowth of the tissue's musculature and restoration of specialized function.

[0093] As way of further illustration and not limitation, the following Examples disclose other aspects of the present invention.

[0094] EXAMPLES

[0095] Example 1

[0096] GFP was linked to the superparamagnetic particle using LC-SMM as the crosslinker (attached to the amine groups of the beads) which was then coupled directly to the sulhydryl groups on GFP. LC-SMCC (from Thermo Fisher) was dissolved in dimethylformamide (DMF) obtained from ACROS (sealed vial and anhydrous) at the 1 mg/ml concentration. Sample was sealed and used almost immediately.

[0097] Ten (10) microliters of the solution was added to nanoparticles in 200 microliter volume. This provided a large excess of SMCC to the available amine groups present, and the reaction was allowed to proceed for one hour. Excess SMCC and DMF was removed using an Amicon spin filter with a cutoff of 3,000 daltons. Five exchanges of volume were

required to ensure proper buffer exchange. It was important that excess of SMCC be removed at this stage.

[0098] Any peptide based molecule, as an example commercially available Green Fluorescent Protein (GFP) or purified recombinant GFP or other proteins) were added to the solution containing a certain amount of ethylene glycol for freezing at -30 ^{O}C. To 3 micrograms of the protein in 14 microliters, 10 microlitters of a freshly prepared DTT (dithiothreitol, Cleland's reagent) solution in PBS were added with vigorous vortexing. Because the proteins usually contain more than one cysteine, there was a tendency to crosslink different GFP molecules. Therefore, the excess DTT reduced the dithiol linkage and freed the GFP. Reaction was allowed to proceed for two hours at 4^{O}C and then excess reagent was removed by an Amicon centrifugal filter unit with a 3,000 MW cutoff.

[0099] The activated nanoparticles and the protein solutions were combined and allowed to react for two hours, after which the unreacted protein was removed by an Amicon centrifugal filter unit having an appropriate MW cutoff (in the example with GFP it is 50,000 dalton cut-off). Sample was stored at -80 ^{O}C. It should also be noted that a sulfo derivative of SMCC (Sulfo-SMCC), which is more water soluble, can be used. DMSO may also be substituted for DMF as the solvent carrier for the labeling reagent; again, it should be anhydrous.

[00100] Example 2

[00101] In this method the amino groups of lysine were used for the coupling reaction to sulhydryl groups on the bead. Beads freshly equilibrated with 0.1 M phosphate buffer at pH 7.2, were used in these studies. LC-SPDP at 1 mg/ml (in DMF) was freshly prepared. 10 microliters of SPDP solution was added to the bead suspension under vigorous vortexing and allowed to react for one hour. Subsequently, the unreacted material was removed by centrifugation and the nanoparticles washed with phosphate buffer using an Amicon Spin filter with a 10K cutoff. The disulfide bond of SPDP was broken using Clelands reagent; 1 mg was added to the solution and the reaction allowed to proceed for one hour. Byproducts and unreacted Clelands reagent were removed via an Amicon spin filter with a 10K cutoff.

[00102] While the above reaction proceeded, GFP was blocked using N-ethylmaleimide. Excess ethylmaleimide was added to the GFP solution. Reaction proceeded for one hour at room temperature and unwanted materials removed using an Amicon Spin filter with a 3K cutoff. The GFP was then allowed to react with excess SMCC for one hour. Subsequently, GFP was purified on a spin column and then reacted with PDP-nanoparticles.

Reaction proceeded for one hour and the final product purified using an Amicon spin filter with a cutoff of 50K.

[00103] Example 3

[00104] Human fibroblasts commercially available or obtained using standard experimental procedures as described [Moretti et al., Mouse and human induced pluripotent stem cells as a source for multipotent Isl1 cardiovascular progenitors. FASEB J. 24:700 (2010)] are plated at 150,000 cells density under sterile conditions on a solid surface with or without preplated feeder cells at 150,000-200,000 density in six-well plates. The feeder cells obtained either commercially or using standard laboratory procedures. The plated cells are cultured for some time with a specific factor combination that allows cell division/proliferation or maintenance of acceptable cell viability in serum-containing culture medium, which can later be withdrawn or refreshed and the cultures continued under sterile conditions in a humidified incubator with 5% CO_2 and ambient O_2.

[00105] The cells collected at the bottom of a conical tube or the plated cells are treated with 50 microliters of suspension containing functionalized biocompatible cell-permeable nanoparticles with bioactive molecules attached using various methods disclosed herein in the presence or absence of magnetic field.

[00106] The use of magnetic field in case of superparamagnetic nanoparticles renders an important increase in the contact surface area between the cells and nanoparticles and thereby ensuring improved penetration of functionalized nanoparticles through the cell membrane. Importantly, similar to poly(ethylene glycol) PEG-mediated protection of several protein-based drugs (PEG-GCSF, Amgen, CA; PEG-Interferon, Schering-Plough/Merck, NJ) to which PEG is attached, the nanoparticles used in conjunction with coupled peptides increase the size of the polypeptide and masks the protein's surface, thereby reducing protein degradation by proteolytic enzymes and resulting in a longer stability of the protein molecules used. If necessary, the cell population is treated repeatedly with the functionalized nanoparticles to deliver the bioactive molecules intracellularly.

[00107] The cells are suspended in culture medium, and non-incorporated nanoparticles are removed by centrifugation for 10 minutes at approximately 1200 x *g*, leaving cells that are present as clusters in the pellet. The clustered cells are then resuspended, washed again using similar procedure and recultured in fresh medium for a suitable period. The cells can be taken through multiple cycles of separating, resuspending,

and reculturing in a culture media until a consequent biological effect triggered by the specific bioactive molecules delivered intracellularly is observed.

[00108] In this specific example with green fluorescent protein, the cell-penetrant nanoparticles deliver the protein inside the cells, which confers acquisition of novel green fluorescence by the target cells. This newly acquired property allows subsequent sorting and separation of the cells with intracellularly delivered protein to high degree of homogeneity that can be further used for various applications. Importantly, the use of cell-permeable functionalized nanoparticles with attached protein devoid any integration into the cell genome, thereby ensuring that every cell with novel (in this case fluorescent) property maintains intact genome and preserves the integrity of cellular DNA.

[00109] The present invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered illustrative rather than limiting of the invention described herein. The scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within meaning and range of equivalency of the claims are intended to be embraced herein.

CLAIMS

What is claimed is:

1. A functionalized biocompatible nanoparticle capable of penetrating through a mammalian cell membrane and delivering intracellularly a plurality of bioactive molecules for modulating a cellular function, comprising:

a central nanoparticle ranging in size from 5 to 50 nm and having a polymer coating thereon,

a plurality of functional groups covalently attached to the polymer coating, wherein the plurality of bioactive molecules are attached to the plurality of functional groups, and wherein the plurality of bioactive molecules include at least a peptide and a protein, and wherein the peptide is capable of penetrating through the mammalian cell membrane and entering into the cell, and wherein the protein is capable of providing a new functionality within the cell.

2. The functionalized biocompatible nanoparticle of claim 1 wherein the nanoparticle comprises iron.

3. The functionalized biocompatible nanoparticle of claim 2 wherein the peptide is attached to the protein.

4. The functionalized biocompatible nanoparticle of claim 3 wherein the peptide and the protein are each attached to the nanoparticle by way of one or more interposing linker molecules.

5. The functionalized biocompatible nanoparticle of claim 1 wherein the peptide includes five to nine basic amino acids.

6. The functionalized biocompatible nanoparticle of claim 1 wherein the peptide includes nine or more basic amino acids.

7. The functionalized biocompatible nanoparticle of claim 5 wherein the protein is a transcription factor.

8. The functionalized biocompatible nanoparticle of claim 7 wherein the transcription factor is selected from the group consisting of Oct4, Sox2, Nanog, Lin28, cMyc, and Klf4.

9. A method of changing a cellular functionality within a mammalian cell, comprising administering an effective amount of a functionalized biocompatible nanoparticle of claim 1 to the cell and changing the cellular functionality within the cell.

10. The method of changing a cellular functionality within a mammalian cell according to claim 9 wherein the changing of the cellular functionality involves a change in a physico-chemical property of the cell.

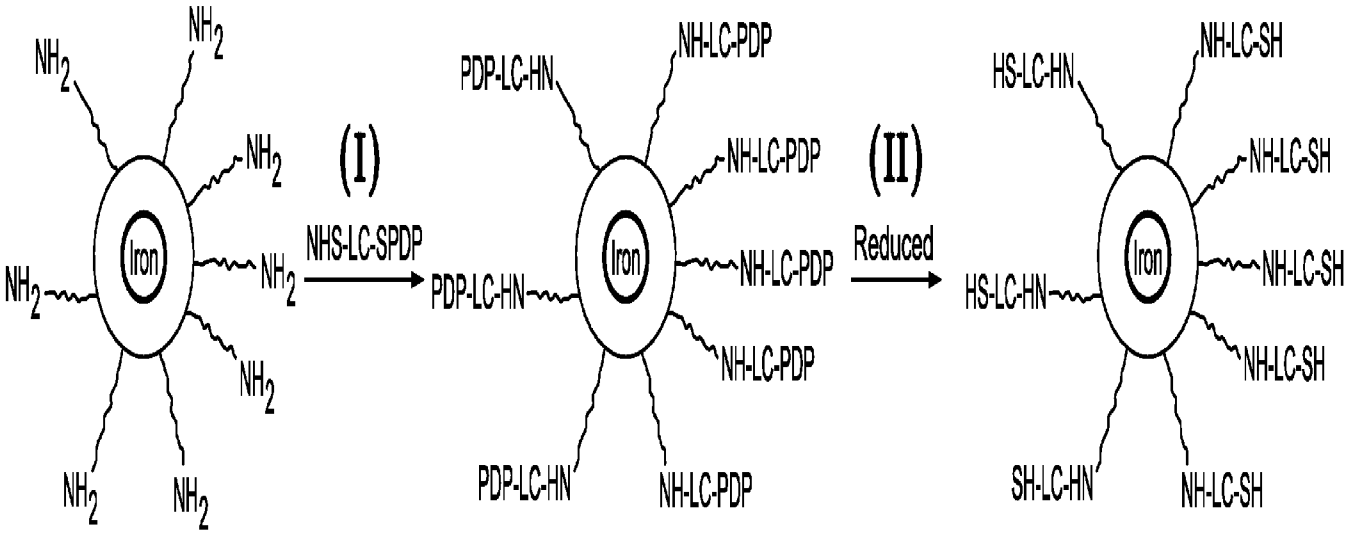
11. The method of changing a cellular functionality within a mammalian cell according to claim 9 wherein the changing of the cellular functionality involves a change in proliferative property of the cell.

12. The method of changing a cellular functionality within a mammalian cell according to claim 9 wherein the changing of the cellular functionality involves a change in

surviving ability of the cell.

13. The method of changing a cellular functionality within a mammalian cell according to claim 9 wherein the changing of the cellular functionality involves a change in morphological phenotypical property of the cell.

14. The method of changing a cellular functionality within a mammalian cell according to claim 9 wherein the changing of the cellular functionality involves an acquired ability of the cell to make a new cell type including a stem cell or a more specialized cell type.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Iron
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PDP-LC-HN
NH-LC-PDP
(II)
Reduced
HS-LC-HN
NH-LC-SH
SH-LC-HN

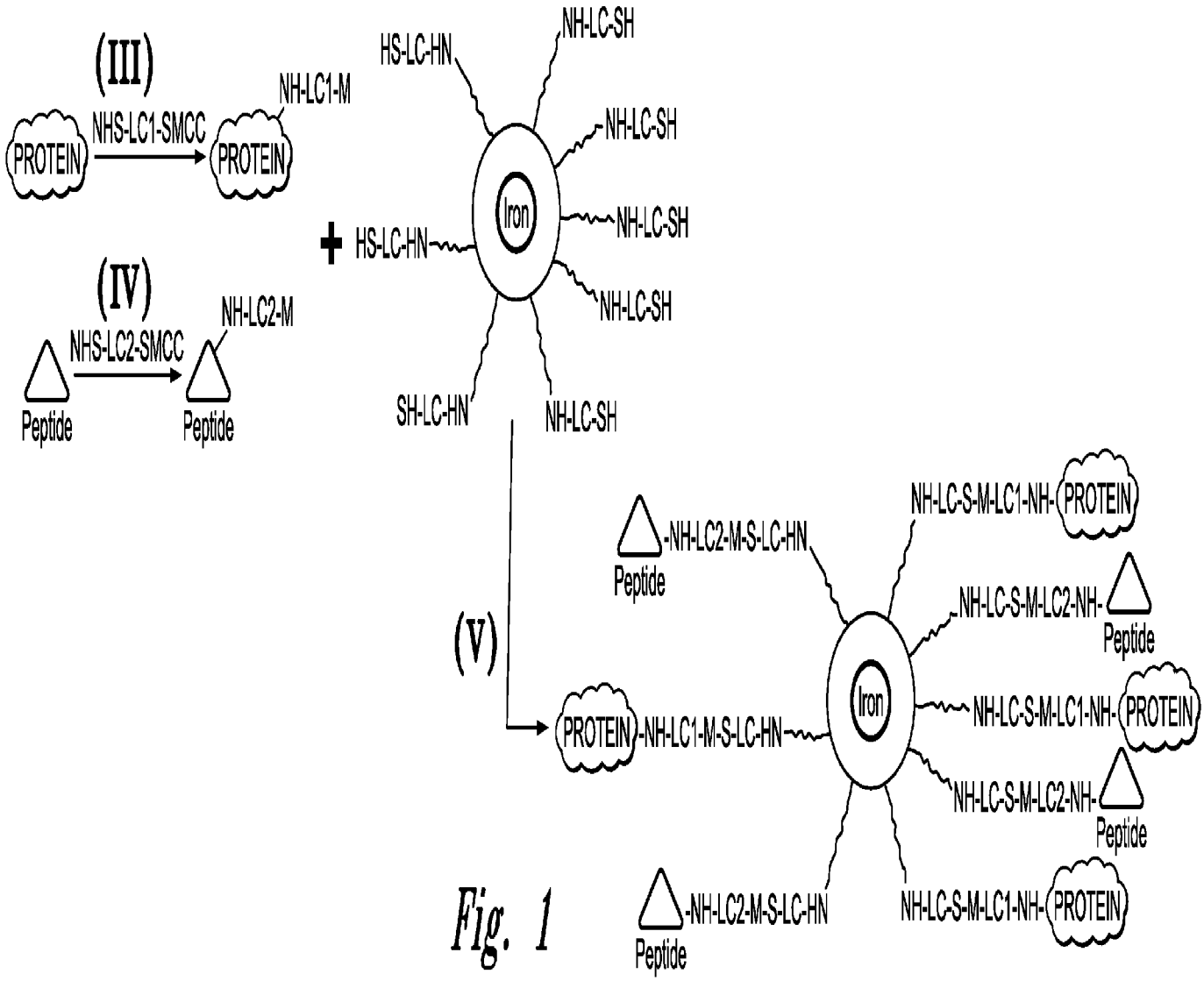

(III)
NHS-LC1-SMCC
NH-LC1-M
PROTEIN
(IV)
NHS-LC2-SMCC
NH-LC2-M
Peptide
HS-LC-HN
NH-LC-SH
Iron
(V)
NH-LC2-M-S-LC-HN
NH-LC-S-M-LC1-NH-
NH-LC-S-M-LC2-NH-
NH-LC1-M-S-LC-HN

Fig. 1

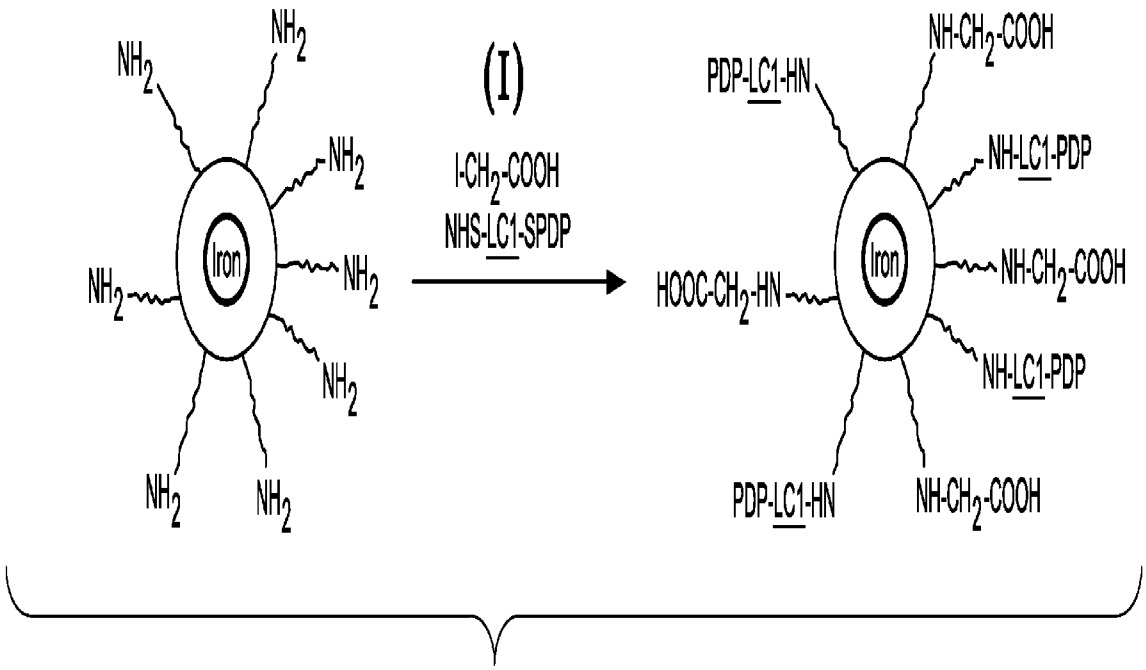
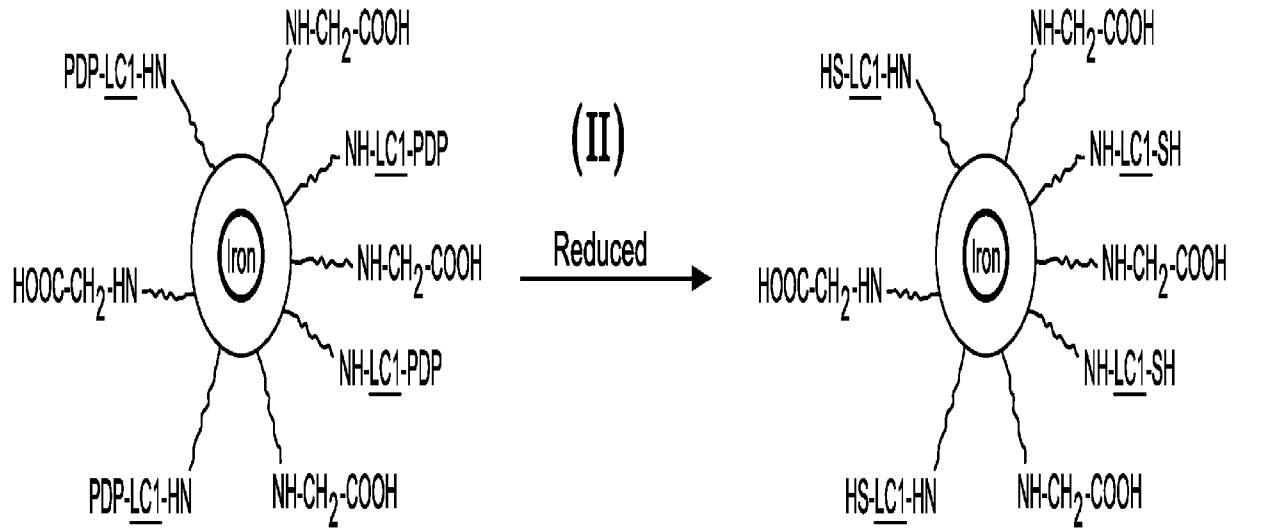

(I)
I-CH2-COOH
NHS-LC1-SPDP
Iron
NH2
PDP-LC1-HN
NH-CH2-COOH
NH-LC1-PDP
HOOC-CH2-HN

Fig. 2A

Fig. 2B


(II)
Reduced
Iron
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NH-LC1-PDP
HOOC-CH2-HN
HS-LC1-HN
NH-LC1-SH

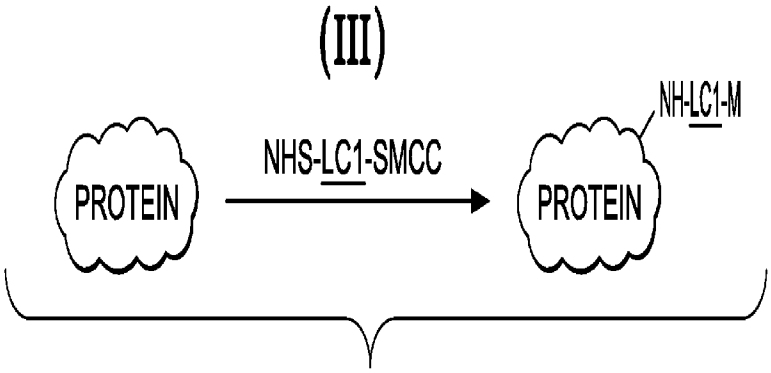
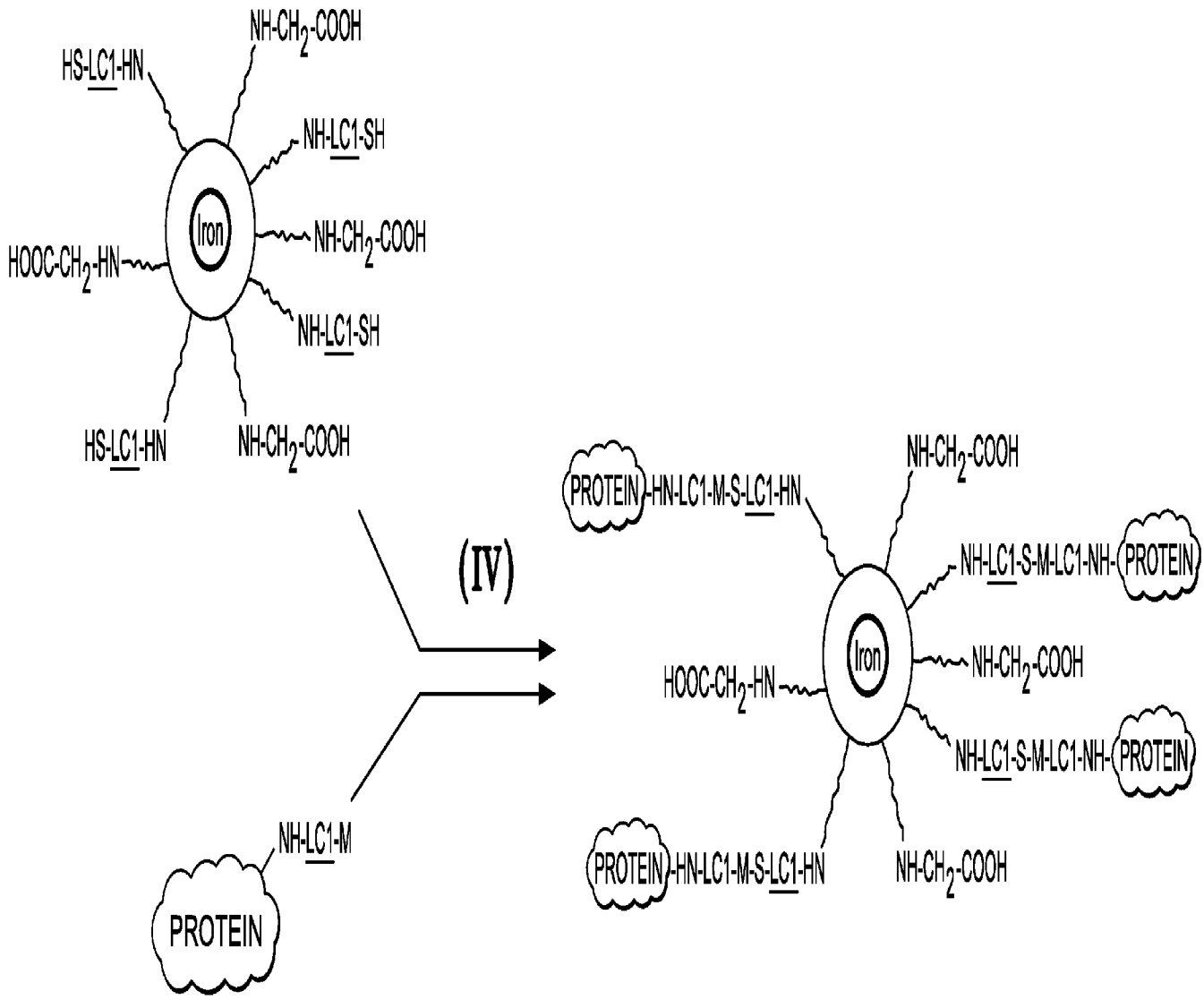

(III)
PROTEIN
NHS-LC1-SMCC
PROTEIN
NH-LC1-M

Fig. 2C

Fig. 2D


NH-CH2-COOH
HS-LC1-HN
NH-LC1-SH
Iron
HOOC-CH2-HN
NH-CH2-COOH
NH-LC1-SH
HS-LC1-HN
NH-CH2-COOH
(IV)
PROTEIN-HN-LC1-M-S-LC1-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-PROTEIN
Iron
HOOC-CH2-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-PROTEIN
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NH-CH2-COOH
NH-LC1-M
PROTEIN

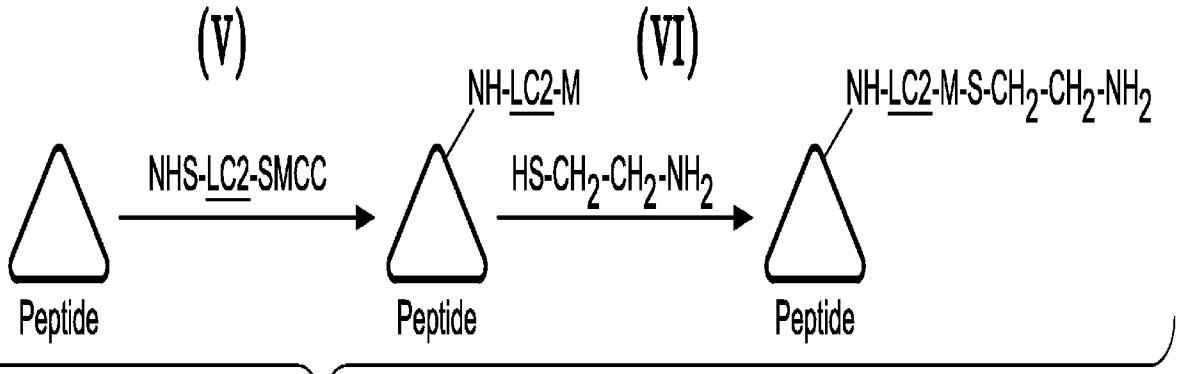
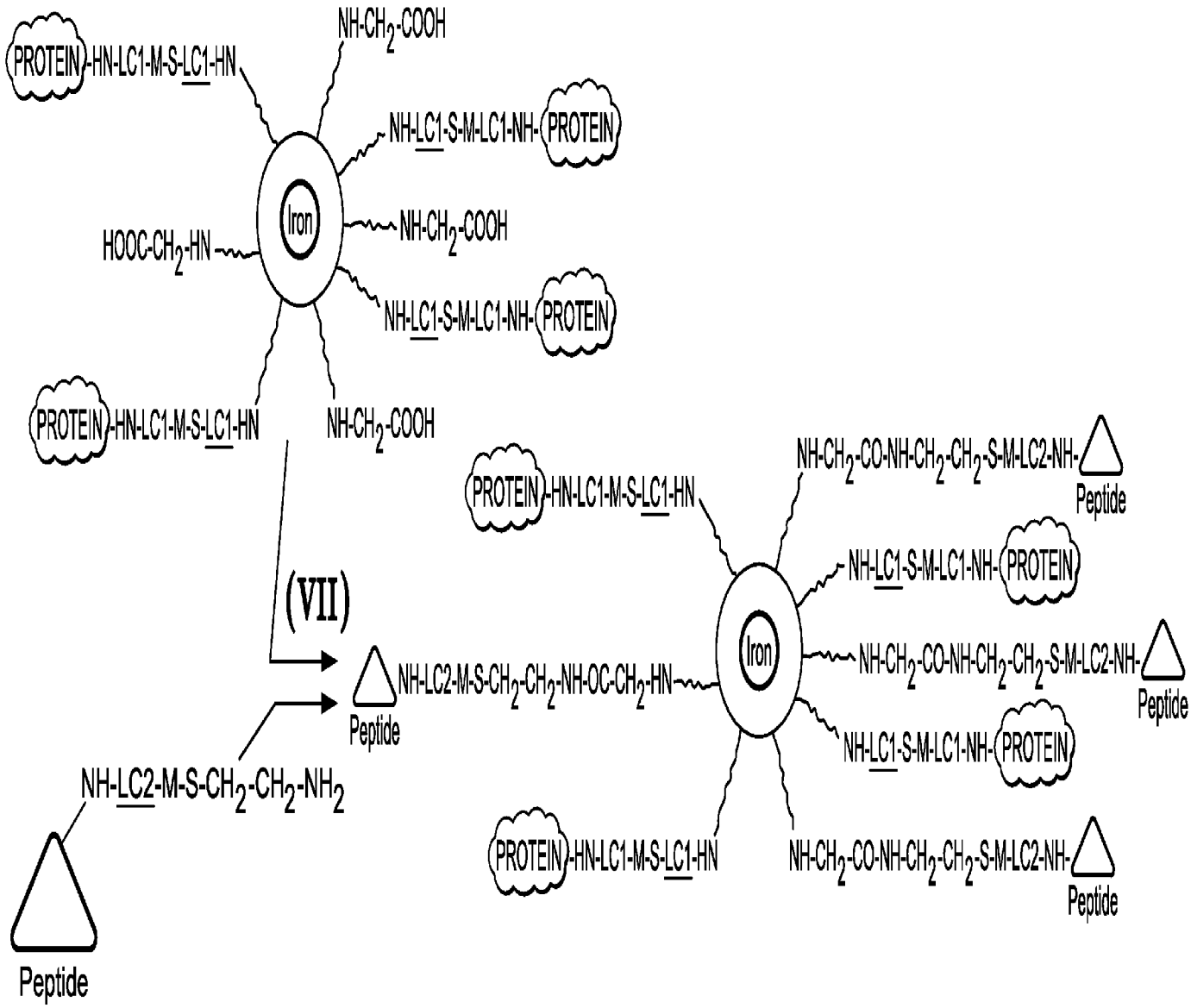

(V)
(VI)
NH-LC2-M
NH-LC2-M-S-CH2-CH2-NH2
NHS-LC2-SMCC
HS-CH2-CH2-NH2
Peptide
Peptide
Peptide

Fig. 2E

Fig. 2F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NH-CH2-COOH
NH-LC1-S-M-LC1-NH-PROTEIN
Iron
HOOC-CH2-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-PROTEIN
PROTEIN-HN-LC1-M-S-LC1-HN
NH-CH2-COOH
(VII)
NH-LC2-M-S-CH2-CH2-NH-OC-CH2-HN
Peptide
NH-LC2-M-S-CH2-CH2-NH2
Peptide
PROTEIN-HN-LC1-M-S-LC1-HN
NH-CH2-CO-NH-CH2-CH2-S-M-LC2-NH-
Peptide
NH-LC1-S-M-LC1-NH-PROTEIN
Iron
NH-CH2-CO-NH-CH2-CH2-S-M-LC2-NH-
Peptide
NH-LC1-S-M-LC1-NH-PROTEIN
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Peptide

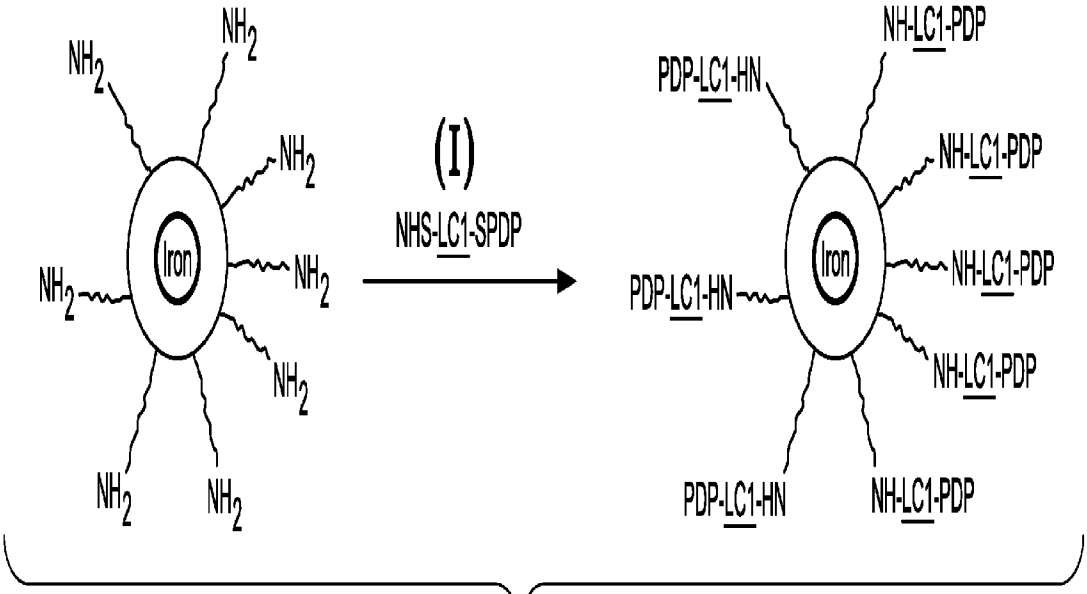
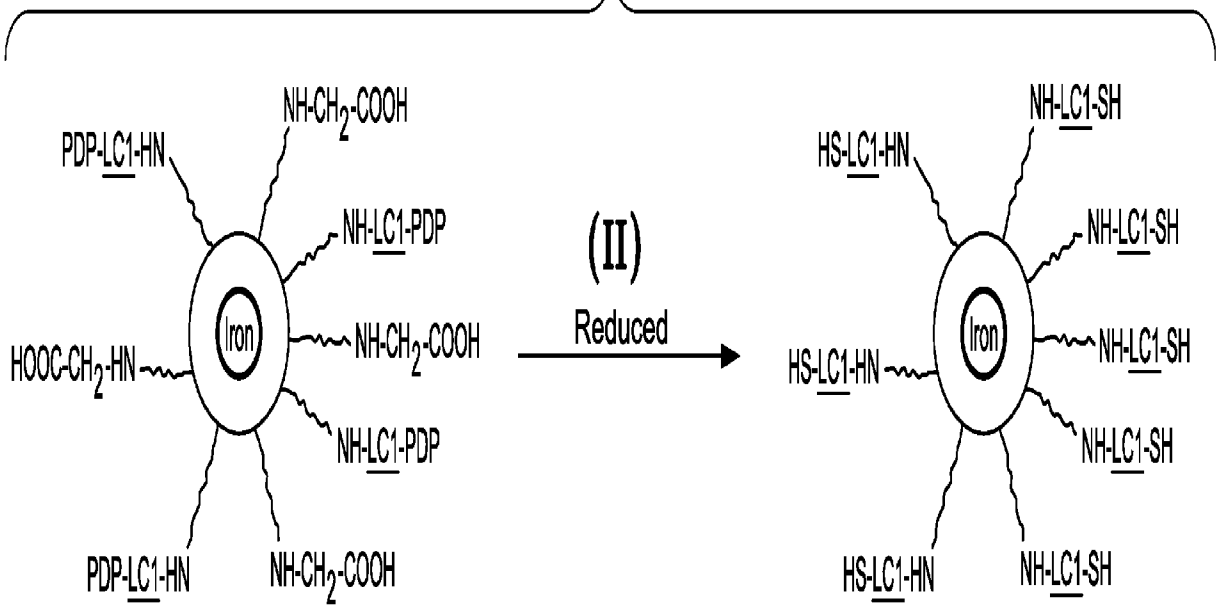

NH2
NH2
NH2
NH2
NH2
NH2
NH2
Iron
(I)
NHS-LC1-SPDP
NH-LC1-PDP
PDP-LC1-HN
NH-LC1-PDP
NH-LC1-PDP
PDP-LC1-HN
Iron
NH-LC1-PDP
PDP-LC1-HN
NH-LC1-PDP

Fig. 3A

Fig. 3B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PDP-LC1-HN
NH-LC1-PDP
HOOC-CH2-HN
Iron
NH-CH2-COOH
NH-LC1-PDP
PDP-LC1-HN
NH-CH2-COOH
(II)
Reduced
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH
HS-LC1-HN
Iron
NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH

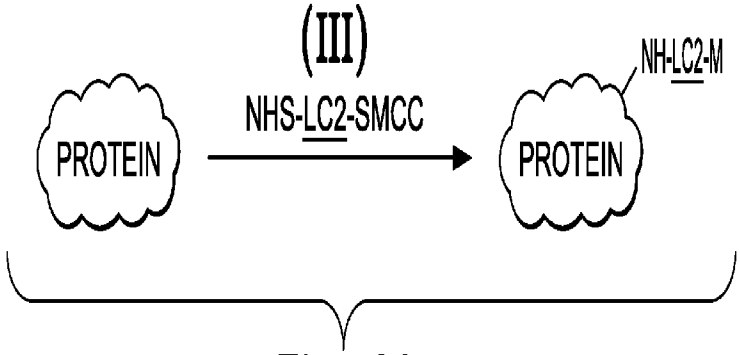
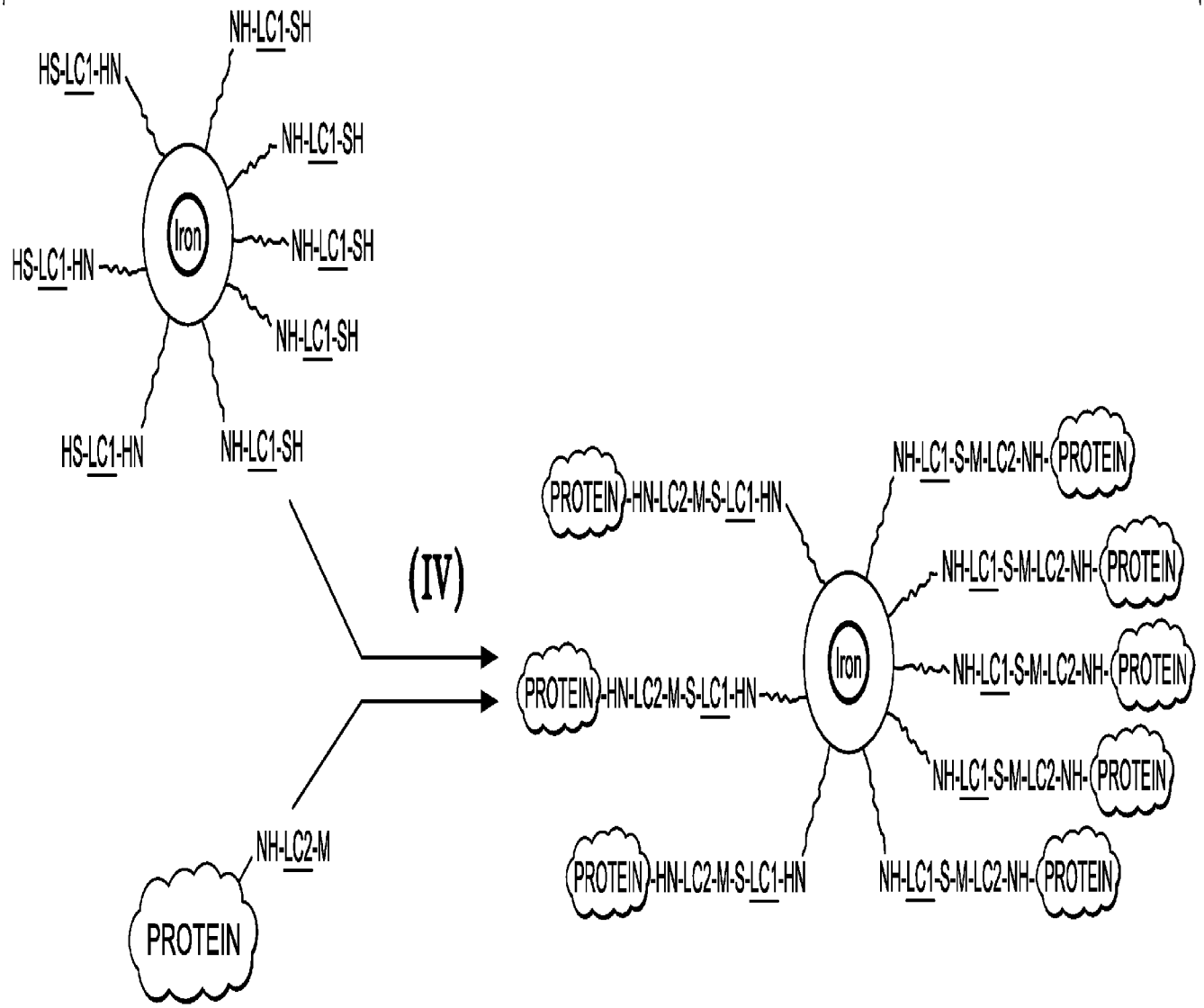

(III)
NHS-LC2-SMCC
PROTEIN
PROTEIN
NH-LC2-M

Fig. 3C

Fig. 3D


NH-LC1-SH
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NH-LC1-SH
Iron
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NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH
(IV)
NH-LC2-M
PROTEIN
PROTEIN-HN-LC2-M-S-LC1-HN
PROTEIN-HN-LC2-M-S-LC1-HN
PROTEIN-HN-LC2-M-S-LC1-HN
Iron
NH-LC1-S-M-LC2-NH-PROTEIN
NH-LC1-S-M-LC2-NH-PROTEIN
NH-LC1-S-M-LC2-NH-PROTEIN
NH-LC1-S-M-LC2-NH-PROTEIN
NH-LC1-S-M-LC2-NH-PROTEIN

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/61391

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53 (2012.01)
USPC - 435/7.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - G01N 33/53 (2012.01)
USPC - 435/7.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 530/400; 530/402 (keyword delimited))

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB, JPAB); PatBase and Google Scholar.
Search Terms Used: nanoparticle, nanobead, functionaliz$, protein, peptide, transduc$, translocat$, penetrat$, Tat, transcription factor, membrane, iron, dextran, polymer, polymer$, transpor$, fusion protein, Oct4, Sox2, Nanog, lin28, cMyc, Klf4, particle, nano$, induced,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0251726 A1 (Lin et al.) 09 November 2006 (09.11.2006) Fig 4-5; Table IV; para [0009], [0010], [0013], [0014], [0023], [0035], [0039], [0048]-[0049], [0076]-[0077], [0080], [0090], [0133]-[0146], [0181], [0187], [0245], [0270]-[0271], [0275], [0277], [0308]	1-4, 9-13 ---------- 5-8, 14
Y	US 2010/0298536 A1 (Park et al.) 25 November 2010 (25.11.2010) para [0028], [0032]	5-8
Y	US 2011/0190729 A1 (Kirkland et al.) 04 August 2011 (04.08.2011) para [0012], [0048], [0059]	8, 14
Y	US 2009/0226372 A1 (Ruoslahti et al.) 10 September 2009 (10.09.2009), para [0008]-[0034]	5-8, 14
A	US 2008/0213377 A1 (Bhatia et al.) 04 September 2008 (04.09.2008) Fig 10; para [0010]-[0012], [0189], [0208], [0388]-[0389]	4

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 December 2012 (04.12.2012)	28 DEC 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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(71)申请人 斯特姆詹尼克斯公司
地址 美国华盛顿
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(74)专利代理机构 北京中誉威圣知识产权代理有限公司 11279
代理人 王正茂 丛芳
(51)Int.Cl.
G01N 33/53(2006.01)

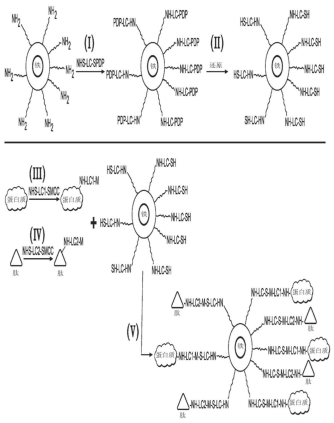
权利要求书1页 说明书10页 附图6页

(54)发明名称

用于胞内传递生物活性分子的官能化纳米粒子

(57)摘要

本发明公开了一种官能化生物相容性纳米粒子，其能够穿透哺乳动物细胞膜并胞内传递用于调节细胞功能的多个生物活性分子。该官能化生物相容性纳米粒子包括：中央纳米粒子，该中央纳米粒子的尺寸范围为约5nm至约50nm，并且在其上具有聚合物涂层，共价连接于所述聚合物涂层的多个官能团，其中多个生物活性分子连接于所述多个官能团，并且其中所述多个生物活性分子至少包括肽和蛋白质，其中所述肽能够穿透哺乳动物细胞膜并进入细胞，其中所述蛋白质能够在细胞内提供新功能。该蛋白质可以是选自Oct4、Sox2、Nanog、Lin28、cMyc和Klf4的转录因子。



CN 104094119 A

1. 一种官能化生物相容性纳米粒子，其能够穿透哺乳动物细胞膜并胞内传递用于调节细胞功能的多个生物活性分子，包括：

中央纳米粒子，其尺寸范围为 5 ～ 50nm，且在其上具有聚合物涂层，

共价连接于所述聚合物涂层的多个官能团，其中所述多个生物活性分子连接于所述多个官能团，并且其中所述多个生物活性分子至少包括肽和蛋白质，并且其中所述肽能够穿透哺乳动物细胞膜并进入细胞，并且其中所述蛋白质能够在细胞内提供新功能。

2. 根据权利要求 1 所述的官能化生物相容性纳米粒子，其中，所述纳米粒子包括铁。

3. 根据权利要求 2 所述的官能化生物相容性纳米粒子，其中，所述肽连接于所述蛋白质。

4. 根据权利要求 3 所述的官能化生物相容性纳米粒子，其中，所述肽和所述蛋白质各自通过一个或多个插入连接子分子而连接于所述纳米粒子。

5. 根据权利要求 1 所述的官能化生物相容性纳米粒子，其中，所述肽包含 5 ～ 9 个碱性氨基酸。

6. 根据权利要求 1 所述的官能化生物相容性纳米粒子，其中，所述肽包括 9 个以上碱性氨基酸。

7. 根据权利要求 5 所述的官能化生物相容性纳米粒子，其中，所述蛋白质是转录因子。

8. 根据权利要求 7 所述的官能化生物相容性纳米粒子，其中，所述转录因子选自由 Oct4、Sox2、Nanog、Lin28、cMyc 和 Klf4 组成的组。

9. 一种改变哺乳动物细胞内的细胞功能的方法，包括将有效量的权利要求 1 所述的官能化生物相容性纳米粒子给予细胞，并改变细胞内的细胞功能。

10. 根据权利要求 9 所述的改变哺乳动物细胞内的细胞功能的方法，其中，所述细胞功能的改变涉及细胞理化特性的改变。

11. 根据权利要求 9 所述的改变哺乳动物细胞内的细胞功能的方法，其中，所述细胞功能的改变涉及细胞增殖特性的改变。

12. 根据权利要求 9 所述的改变哺乳动物细胞内的细胞功能的方法，其中，所述细胞功能的改变涉及细胞存活能力的改变。

13. 根据权利要求 9 所述的改变哺乳动物细胞内的细胞功能的方法，其中，所述细胞功能的改变涉及细胞形态学表型特性的改变。

14. 根据权利要求 9 所述的改变哺乳动物细胞内的细胞功能的方法，其中，所述细胞功能的改变涉及细胞成为新细胞类型的获取能力，所述新细胞类型包括干细胞或更多特化细胞类型。

用于胞内传递生物活性分子的官能化纳米粒子

[0001] 相关申请的交叉参考

[0002] 本申请要求申请号为61/550,213、申请日为2011年10月21日的美国临时申请的优先权,该申请的全部内容通过引用并入本文。

技术领域

[0003] 本发明主要涉及有机合成和纳米生物技术,更具体而言,涉及用于将生物活性分子传递到细胞内以调节细胞功能的官能化纳米粒子以及与之相关的方法。

背景技术

[0004] 细胞正常增殖、迁移和分化成各种细胞类型的能力对胚胎形成和成熟细胞,包括但不限于各种遗传性或获得性疾病中造血细胞和/或心血管系统细胞的功能是至关重要的。干细胞和/或进一步分化的特化细胞类型的功能性能力在各种病理状态下会发生变化,但是可通过胞内引入生物活性成分而正常化。例如,观察到周期性中性粒细胞减少症或严重的先天性中性粒细胞减少症患者中有异常的细胞功能,例如骨髓干细胞/祖细胞的存活和/或分化为中性粒细胞的能力受损,此类患者可能患有严重的危及生命的感染,并可能发展成急性髓细胞性白血病或其他恶性肿瘤[Aprikyan等,Impaired survival of bone marrow hematopoietic progenitor cells in cyclic neutropenia(在周期性中性粒细胞减少症中受损的骨髓造血祖细胞的存活),Blood,97,147(2001);GoranCarlsson等,Kostmann syndrome:severe congenital neutropenia associated with defective expression of Bcl-2,constitutive mitochondrial release of cytochrome C,and excessive apoptosis of myeloid progenitor cells(科斯特曼综合征:与Bcl-2蛋白缺陷表达、细胞色素C的组成性线粒体释放及骨髓祖细胞的过度凋亡相关联的严重先天性中性粒细胞减少症),Blood,103,3355(2004)]。遗传性或获得性疾病,例如严重的先天性中性粒细胞减少症或巴特综合征是由各种基因突变引发的,并且因患者的血液和/或心肌细胞的制造和功能不足导致随后的中性粒细胞减少症、心肌梗塞和/或心力衰竭[Makaryan等,The cellular and molecular mechanisms for neutropenia in Barth syndrome(巴特综合征中中性粒细胞减少症的细胞和分子机制),Eur J Haematol,88:195-209(2012)]。严重的先天性中性粒细胞减少症的表型可由中性粒细胞弹性蛋白酶基因、HAX1基因或Wiskott-Aldrich综合征蛋白基因的不同取代、缺失、插入或截短突变引起[Dale等,Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia(先天性和周期性中性粒细胞减少症中编码中性粒细胞弹性蛋白酶的基因的突变),Blood,96:2317-2322(2000);Devriendt等,Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia(WASP中的组成性激活突变导致X-连锁的严重的先天性中性粒细胞减少症),Nat Genet.27:313-7(2001);Klein等,HAX1 deficiency causes autosomal recessive severe congenital neutropenia(Kostmann disease)(HAX1缺乏导致常染色体隐性遗传的严重先天性中性粒

细胞减少症（科斯特曼病）），Nat Genet，39:86-92(2007)]。

[0005] 其他遗传性疾病，如巴特综合征，是一种可能由线粒体 TAZ 基因的功能缺失突变所引发的多系统干细胞疾病，该类疾病与中性粒细胞减少症（血中性粒细胞水平下降）相关联，该类遗传性疾病可能会导致复发性的严重的、有时甚至危及生命的致命性感染和 / 或可能会导致心力衰竭的心肌梗塞，该心力衰竭可通过心脏移植来解决。在大多数情况下，与发病机制和遗传性或获得性人类疾病的发展相关联的该突变基因产物会影响不同的细胞内事件 (intracellular events)，这会导致异常的细胞功能和特定的疾病表型。

[0006] 用粒细胞集落刺激因子 (G-CSF) 治疗这些患者会诱导位于细胞表面的 G-CSF 受体分子发生构象变化，随之引发一系列的细胞内事件，最终将中性粒细胞的生产恢复至接近正常水平，并提高患者的生活质量 [Welte 和 Dale，Pathophysiology and treatment of severe chronic neutropenia（严重的慢性中性粒细胞减少症的病理生理学和治疗），Ann.Hematol.72,158(1996)]。然而，用 G-CSF 治疗的患者可能进化发展为白血病 [Aprikyan 等，Cellular and molecular abnormalities in severe congenital neutropenia predisposing to leukemia（严重的先天性中性粒细胞减少症中的细胞和分子异常易诱发白血病），Exp Hematol.31,372(2003) ;Philip Rosenberg 等，Neutrophil elastase mutations and risk of leukaemia in severe congenital neutropenia（严重的先天性中性粒细胞减少症中中性粒细胞弹性蛋白酶突变和白血病的风险），Br J Haematol.140,210(2008) ;Peter Newburger 等，Cyclic Neutropenia and Severe Congenital Neutropenia in Patients with a Shared ELANE Mutation and Paternal Haplotype:Evidence for Phenotype Determination by Modifying Genes（共有 ELANE 突变和父源单倍型患者的周期性中性粒细胞减少症和严重的先天性中性粒细胞减少症 :通过修饰基因测定表型的证据），Pediatr.Blood Cancer,55,314(2010)]，这正是探索新的替代方法的原因。

[0007] 可以通过使用截然不同的官能化纳米粒子胞内传递不同的生物活性分子，从而能够更有效地影响和调节细胞内事件。这些生物活性分子可以使细胞功能正常化或根据需要消除不需要的细胞。然而，细胞膜作为有效屏障保护细胞内事件的级联反应免受外源性刺激的影响。

[0008] 因此，在本领域中需要新型的生物活性分子，其能够穿透细胞膜并实现所关心的细胞内事件。本发明满足了这些需要，并提供了更多相关的优点。

发明内容

[0009] 在一些实施方案中，本发明涉及将蛋白质和 / 或肽连接于生物相容性纳米粒子以调节细胞功能的官能化方法。在一些实施方案中，本发明涉及官能化生物相容性纳米粒子本身。

[0010] 在一个实施方案中，一种能够穿透哺乳动物细胞膜并胞内传递用于调节细胞功能的多个生物活性分子的官能化生物相容性纳米粒子，包括 :中央纳米粒子，尺寸范围为 5 ～ 50nm，且在其上具有聚合物涂层 ;共价连接于所述聚合物涂层的多个官能团，其中所述多个生物活性分子连接于所述多个官能团，并且其中所述多个生物活性分子至少包括肽和蛋白质，并且其中所述肽能够穿透哺乳动物细胞膜并进入细胞，并且其中所述蛋白质能够在细

胞内提供新功能。

[0011] 中央纳米粒子可包括铁，并有磁性。本发明所述的肽可以连接于蛋白质（相反侧连接于纳米粒子）。肽和蛋白质可各自通过一个或多个插入连接子分子而连接于纳米粒子。在一些实施方式中，肽可以包括 5 ～ 9 个碱性氨基酸，而在其他实施方式中，肽包括 9 个以上碱性氨基酸。该蛋白质可以是选自由 Oct4、Sox2、Nanog、Lin28、cMyc 和 Klf4 组成的组的转录因子。

[0012] 另一方面，本发明涉及改变哺乳动物细胞内的细胞功能的方法。该新方法包括将有效量的官能化生物相容性纳米粒子给予细胞，并改变细胞内的细胞功能。细胞功能的改变涉及细胞理化特性的改变、细胞增殖特性的改变、细胞存活能力的改变或细胞形态学表型特性的改变。细胞功能的变化涉及细胞制造新细胞类型的获取能力，所述新细胞类型包括干细胞或更多特化细胞类型。

[0013] 本发明的上述及其他方面通过参考下面的详细描述和附图将变得更为清楚。但是，应当理解的是，可在不脱离本发明主旨和保护范围的前提下对本说明书中公开的实施方式进行各种改变、变更和替换。最后，本说明书中引用的各种参考文献都明确地通过引用并入本文。

附图说明

[0014] 图 1 示出根据本发明实施例的基于肽和蛋白质分子同时连接于纳米粒子的纳米粒子多步骤官能化方案。

[0015] 图 2A 示出根据本发明实施例的含胺基纳米粒子与等摩尔比的长链 LC1-SPDP 和碘乙酸纳米粒子的反应。

[0016] 图 2B 示出根据本发明实施例，还原 PDP 的二硫键以提供自由 SH 基纳米粒子。

[0017] 图 2C 示出根据本发明实施例的长链 LC1-SMCC 与蛋白质纳米粒子的赖氨酸基团的反应。

[0018] 图 2D 示出根据本发明实施例的多功能纳米粒子与蛋白质的反应，该蛋白质已经与 SMCC 反应并且包含末端反应性马来酰亚胺基纳米粒子。

[0019] 图 2E 示出肽的氨基与 LC2-SMCC 的反应。根据本发明实施例，该反应后随之进行与巯基乙醇的反应，将末端马来酰亚胺转化为醇纳米粒子。

[0020] 图 2F 示出根据本发明实施例的功能珠（和连接有蛋白质）与带修饰的肽的在纳米粒子上自由羧基端的反应。

[0021] 图 3 示出根据本发明实施例的含胺基的纳米粒子与 LC1-SPDP 纳米粒子的反应。

[0022] 图 3B 示出根据本发明实施例，还原 PDP 的二硫键以提供自由 SH 基纳米粒子。

[0023] 图 3C 示出根据本发明实施例的长链 LC2-SMCC 与蛋白质纳米粒子的赖氨酸基团的反应。

[0024] 图 3D 示出根据本发明实施例的多功能纳米粒子与蛋白质的反应，该蛋白质已经与 SMCC 反应并且包含了末端反应性马来酰亚胺基纳米粒子。

[0025] 对本领域普通技术人员来说，结合附图参考以下详细描述时，本发明的上述和其它方案将变得更加清楚。

具体实施方式

[0026] 为了胞内传递生物活性分子，本发明的发明人提出了一种基于带有共价连接的生物活性分子的细胞膜穿透性纳米粒子的通用手段。为此，本发明人在此提出一种新官能化方法，其确保了蛋白质和肽对纳米粒子的共价连接。本发明的经修饰了的细胞透过性纳米粒子为胞内传递用于调节细胞功能和/或细胞功能正常化的生物活性分子提供了通用机制。

[0027] 细胞正常增殖、迁移和分化成各种细胞类型的能力对胚胎发育和成熟细胞，包括但不限于各种遗传性或获得性疾病中造血细胞和心血管系统的干细胞/祖细胞和进一步分化的细胞的功能是至关重要的。干细胞和/或进一步分化的特化细胞类型的这种功能性能力在细胞内事件中的异常改变所导致的各种病理状态下会发生改变，但可通过胞内引入生物活性成分而正常化。例如，在患有严重的危及生命的感染并可能发展成白血病的周期性中性粒细胞减少症或严重的先天性中性粒细胞减少症患者中观察到人骨髓祖细胞的存活和/或分化为中性粒细胞的能力受损，其可通过中性粒细胞弹性蛋白酶的细胞膜穿透性小分子抑制剂而正常化，它干扰异常的细胞内事件并明显地恢复正常表型。然而，这种小分子对导致疾病的目标突变产物基本无效，这正是需要用于胞内传递能够调节细胞功能的生物活性分子的可替代性的有效的细胞膜穿透手段的原因。

[0028] 本说明书中公开的方法利用生物相容性纳米粒子，包括例如类似于前文在科学文献中描述的超顺磁性氧化铁粒子。这种类型的纳米粒子在临床环境中可被用于骨髓细胞、淋巴结、脾脏和肝脏的磁共振成像[参见例如 Shen 等，Monocrystalline iron oxide nanocompounds(MION)(单晶氧化铁纳米复合物 (MION));physicochemical properties. Magn.Reson.Med.,29,599(1993);Harisinghani 等，MR lymphangiography using ultrasmallsuperparamagnetic iron oxide in patients with primary abdominal and pelvic malignancies(对原发性腹腔和盆腔恶性肿瘤患者利用超小超顺磁氧化铁的 MR 淋巴管造影)，Am.J.Roentgenol.172,1347(1999)]。这些磁性氧化铁纳米粒子包含 5nm 左右的涂覆有交联葡聚糖的核，并具有大约 45nm 的总粒子尺寸。重要的是，已经证明这些包含交联的细胞膜可透过性 Tat- 衍生肽的纳米粒子，以每个细胞高达 30pg 的超顺磁氧化铁纳米粒子的量，有效地内在化到造血细胞和神经祖细胞中 [Lewin 等，Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells(Tat 肽衍生磁性纳米粒子实现在体内祖细胞的跟踪和恢复)，Nat. Biotechnol.18,410(2000)]。此外，纳米粒子的插入不影响骨髓衍生 $CD34^+$ 原始祖细胞的增殖和分化特性或细胞存活能力 [MaiteLewin 等，Nat.Biotechnol.18,410(2000)]。这些纳米粒子可用于在体内跟踪标记细胞。

[0029] 标记细胞保持其分化能力，还可以使用磁共振成像在组织样品中被检测到。本发明人在此提出基于新纳米粒子的手段，其被功能化以携带肽和蛋白质，所述肽和蛋白质可以作为优良的载体胞内传递生物活性分子用于目标细胞内事件的细胞重编程解决方案并且调节细胞功能和特性。

[0030] 纳米粒子 - 肽/蛋白质耦合物的概述：

[0031] 纳米粒子基于铁或带有生物相容性涂层（例如葡聚糖多糖）的其它材料，其具有连接有不同长度的连接子的 X/Y 官能团，反过来通过它们的 X/Y 官能团共价连接于蛋白质

和 / 或肽（或其它小分子）。

[0032] 可用于交联的官能团包括：

[0033] $-NH_2$（例如，赖氨酸，α $-NH_2$）；

[0034] -SH，

[0035] -COOH，

[0036] $-NH-C(NH)(NH_2)$，

[0037] 碳水化合物，

[0038] - 羟基 (OH)，

[0039] - 通过连接子上的叠氮基的光化学反应连接。

[0040] 交联试剂包括：

[0041] SMCC[4-(N- 马来酰亚胺基 - 甲基）环己烷 -1- 羧酸琥珀酰亚胺酯］也可以是磺基 -SMCC，该磺基琥珀酰亚胺基衍生物用于交联氨基和巯基。

[0042] LC-SMCC（长链 SMCC）。也可以是磺基 -LC-SMCC。

[0043] SPDP[N- 琥珀酰亚胺基 -3-（吡啶基二硫代）- 丙酸酯］也可以是磺基 -SPDP。与胺反应提供巯基。

[0044] LC-SPDP（长链 SPDP）。也可以是磺基 -LC-SPDP。

[0045] EDC[1- 乙基 -3-(3- 二甲基氨基丙基）碳二亚胺盐酸盐］用于将 -COOH 基团与 $-NH_2$ 基团连接的试剂。

[0046] SM(PEG)n，其中 n ＝ 1,2,3,4……24 乙二醇单位。也可以是磺基 -SM(PEG)n 衍生物。

[0047] SPDP(PEG)n，其中 n ＝ 1,2,3,4……12 乙二醇单位。也可以是磺基 -SPDP(PEG)n 衍生物。

[0048] 同时含有羧基和胺基的 PEG 分子。

[0049] 含有羧基和巯基的 PEG 分子。

[0050] 封端剂和阻断剂包括：

[0051] 柠康酸酐 - 对 NH 特异性

[0052] 乙基马来酰亚胺 - 对 SH 特异性

[0053] 巯基乙醇 - 对马来酰亚胺特异性

[0054] 鉴于上述情况，本发明人处理了生物相容性纳米粒子以在表面上产生功能性胺，其反过来被用于化学键合蛋白质和短肽。

[0055] 在将蛋白质，例如绿色荧光蛋白或转录因子，连接于超顺磁性纳米粒子或可替代的纳米粒子的情况下，可采用以下方法：外部包含氨基官能团的超顺磁珠可以通过商业渠道从不同的制造商处购得。它们的尺寸范围可以是 20-50nm，每毫升具有 10^{15}-10^{20} 个纳米粒子，每个纳米粒子具有 10 个以上胺基团。将纳米粒子通过使用截留分子量为 10,000 道尔顿分子的 Amicon 离心过滤单元（微柱）放置到适当的反应缓冲液 (0.1M 磷酸盐缓冲液，pH7.2) 中。通常要求清洗大约四次以确保适合的缓冲系统。按照制造商所推荐的那样，将纳米粒子从过滤器单元中移出（通过低速旋转翻转柱 / 过滤装置）。

[0056] 将 SMCC（来自 Thermo Fisher) 以 1mg/ml 的浓度溶解于由 ACROS（密封小瓶且无水）得到的二甲基甲酰胺 (DMF)。样品密封且几乎立即使用。

[0057] 将 10μl 溶液加入到纳米粒子中至体积为 200μl。这提供了相对于存在的可用胺基团远远过量的 SMCC，使反应进行 1 小时。可以用截留分子量为 3000 道尔顿分子的 Amicon 离心过滤柱移除过量的 SM 和 DMF。通常要求 5 次体积更换以确保适当的缓冲交换。重要的是过量的 SMCC 在这个阶段被除去。

[0058] 将任何肽基分子，例如市售可得的绿色荧光蛋白 (GFP) 或纯化的重组绿色荧光蛋白或其它蛋白质加入到含有一定量的乙二醇的溶液中，在 -30℃冷冻。边剧烈搅拌边加入在 14μl、10μl 新鲜制备的 DTT（二硫苏糖醇，克莱兰氏试剂）中含 3μg 蛋白质的 PBS 溶液。因为蛋白质通常含有一个以上的半胱氨酸，倾向于交联不同的 GFP 分子。因此，过量的 DTT 还原二硫键并释放绿色荧光蛋白。使反应在 4℃进行 2 小时，然后通过截留分子量为 3000 的分子的 Amicon 离心过滤单元除去过量的试剂。

[0059] 将活化的纳米粒子和蛋白质溶液混合，并使其反应 2 小时，之后将未反应的蛋白质通过具有适当的截留分子量（在使用 GFP 的本例中截留分子量为 50,000 道尔顿）的 Amicon 离心过滤装置除去。样品储存于 -80℃。也可以代替使用 Amicon 旋转过滤柱，使用含有固定尺寸过滤组件的小旋转柱、例如 Bio Rad P 柱。它们都是尺寸排阻柱。还应当指出的是 SMCC 也可以作为磺基衍生物（磺基 -SMCC）购得，使其更溶于水。也可以用 DMSO 代替 DMF 作为标记试剂的溶剂载体，另外它应该是无水的。

[0060] 所有的其他交联试剂都可以以类似的方式被使用。SPDP 也是以与 SMCC 相同的方式被施用于蛋白质 / 适当的肽。它易溶于 DMF。二硫键通过与 DTT 反应 1 小时或 1 小时以上而被切断。除去副产物和未反应的材料后，通过使用截留分子量为 3,000 的 Amicon 离心过滤柱将其纯化。

[0061] 其他更直接可控的用肽和蛋白质标记纳米粒子的手段是使用两种不同的双官能耦合剂。反应顺序有些类似于图 1。碘乙酸用于将选定数量的“羧基”基团引入到纳米粒子表面。

[0062] 用氨基巯基乙醇处理含 LC-SMCC 的肽。这能够通过巯基成键，并提供自由氨基。然后通过使用 EDC 将此氨基与纳米粒子上的羧基耦合。已知 EDC 为 1- 乙基 -3[3- 二甲基氨基丙基] 碳二亚胺盐酸盐。该耦合步骤在反应方案的最后执行。

[0063] 图 1 示出磁性纳米粒子 - 蛋白质 / 肽加成物的一般性描述。磁性纳米粒子涂覆有多糖，然后被官能化。其表面可以被胺取代。也可以改变 / 变形成任何其他的功能形式。扩展子 / 连接子将两个单元物理性连接在一起。

[0064] 各种各样的官能团可被用于通过交联反应将纳米粒子化学连接于蛋白质。可用的官能团的变化允许每次一个步骤地将大量的蛋白质 / 肽连接于纳米粒子。

[0065] 类似地，各种交联试剂或反应催化剂可被用于通过异型双官能试剂将纳米粒子与蛋白质 / 肽交联。还应当指出的是这些交联试剂有不同的长度。例如许多包含代表扩展子或“长链”的 LC 符号。聚乙二醇化的化合物也可为不同的长度。这样不同长度的连接子可以被加入到该纳米粒子中，并对较大的分子（例如蛋白质）和小分子（例如肽）提供不同的连接长度。

[0066] 通常，不同蛋白质可能会含有相同的官能团，因此很难用各种蛋白质来标记纳米粒子。因为有使官能团发生变化的试剂，所以，能够改变蛋白质的官能团，从而阶段性地获得选择性而不会受到其他蛋白质的干扰。这需要改变蛋白质上的官能团。

[0067] 各种试剂可以用于改变蛋白质，以便不同的化学作用可以用于由所希望的官能团连接蛋白质。例如，化合物（如 SPDP）可以用于将胺转换为巯基，然后其将与马来酰亚胺部分反应。

[0068] 在逐步地将蛋白质连接于珠（纳米粒子）时，先前连接的蛋白质的残基和活性基团可能干扰耦合化学作用。因而可使用永久性或可逆的封端剂阻断这些活性部分以免受将要用于连接第二个或第三个蛋白质到纳米粒子上的试剂的干扰。

[0069] 可使用大量不同的封端化合物阻断未反应部分。因为封端化合物也可能会干扰蛋白质活性，所以需要谨慎使用。通常在第二个化学连接步骤是必需的，并且该官能团可能会干扰时使用。

[0070] 为了表明蛋白质可以使用上面提到的化学作用连接于珠（纳米粒子），给出了磁性纳米粒子的合成，其包含源自水母的绿色荧光蛋白质。LCC-SMCC 被用于该合成方案。

[0071] N- 羟基琥珀酰亚胺与纳米粒子上的自由胺基团发生化学反应以形成化学键。提供能与 GFP 反应的马来酰亚胺端基。已知 GFP 具有两个半胱氨酸，并且来自不同的 GFP 分子的半胱氨酸会反应形成二硫键。为了消除这种干扰，首先用克莱兰氏试剂 (Cleland’s reagent) 还原该分子。

[0072] 将该蛋白质纯化，然后使其与含有 LC- 马来酰亚胺基团的珠反应。使反应进行 1 小时，用 Amicon 旋转过滤器（截留分子量 50K）纯化反应。用荧光电子显微镜拍摄照片。

[0073] 可以在纳米粒子上设置多种类型的官能团。使其能够连接三个以上不同的蛋白质。

[0074] 首先是与表面上的胺。

[0075] 特劳特试剂 (Traut’s reagent) 可用于将这些胺中的一部分转化为巯基。除此之外，碘乙酸可以用于将部分胺转化为羧酸。

[0076] 对于蛋白质和肽，胺都被转化为如下详细描述的具有不同连接子长度的官能团。其将作为通用基团来连接蛋白质和肽。

[0077] 图 1 示出纳米粒子官能化、肽类和蛋白质与纳米粒子的连接的示意性代表例。

[0078] 如下所述地进行合成和涂覆 :通过 Thermo Fisher 在市售可得的 NHS-LC-SPDP 是长链扩展子，两端带有对胺特异性的双官能耦合剂，并且具有可被转化为硫化物的二硫键。

[0079] 扩展子的一端有 N- 羟基琥珀酰亚胺酯，而另一端具有吡啶基二硫代基团。这个二硫代基团可以被还原而生成巯基。使 NHS-LC-SPDP 与纳米粒子反应，反应可以从未被掺入的 NHS-LC-SPD 中清除。然后如图 1 所示将耦合纳米粒子还原。

[0080] 制造耦合蛋白质 :用亲和层析柱纯化的生物活性蛋白质含有来自羧基末端赖氨酸残基的自由 ε- 胺基团，羧基末端赖氨酸残基是为了促进与纳米粒子连接而加入的。NHS-LC-SMCC 用作双官能耦合剂。该分子具有 LC1 链扩展子。一端具有胺特异性的 N- 羟基琥珀酰亚胺试剂。另一端包含马来酰亚胺基团，具体为巯基。一旦材料与蛋白质连接，就从反应混合物中分离出来，马来酰亚胺耦合蛋白质将被添加到该含巯基的纳米粒子中。所得材料通过凝胶过滤而分离。

[0081] 肽耦合到纳米粒子 :在这种情况下，肽还包含羧基末端赖氨酸，其作为 NHS 酯 -LC- 马来酰亚胺耦合的基体发挥作用。该分子具有 LC2 链扩展子。所有流程都类似于上述对蛋白质的描述。

[0082] 在最佳实施方式中，膜透过性肽和蛋白质将被以不同的比例混合，以实现最大数目的分子连接于纳米粒子。根据之前公布的研究，每个纳米粒子具有 3 ～ 4 个分子的表面结合细胞透过性肽就足以进行有效的超顺磁性纳米粒子的胞内传递。

[0083] 利用 LC2- 扩展臂提供了增加肽基分子键合量的重要手段。使用不同浓度的 NHS-LC-SPDP 使锚定在纳米粒子表面的肽和蛋白质分子的数量增加，因此，能够实现更有效的渗透以及更可靠的细胞重编程活性。

[0084] 肽和蛋白质连接于纳米粒子 :可以使用图 1 所示的流程来实现。在这种情况下，将 SMCC 标记的蛋白质和肽按比例加入到珠中并使其反应。

[0085] 用肽和蛋白质标记纳米粒子的另一种更直接可控的手段是使用两种不同的双官能耦合试剂 (图 2A-F)。反应顺序有些类似于图 1，一些改进之处在下文中描述。

[0086] 碘乙酸用于将选定数量的“羧基”基团引入到纳米粒子表面。这是在步骤 I 中进行的 (参见图 2A-F，步骤 (I-VII))。

[0087] 用氨基乙醇处理含有 NH-LC-SMCC 的肽。这能够通过巯基成键，并提供自由氨基基团。然后使用 EDAC(EDC) 将此氨基耦合于纳米粒子上的羧基。已知 EDAC 为 1- 乙基 -3[3- 二甲基氨基丙基] 碳二亚胺盐酸盐。该耦合步骤在反应方案的最后执行。

[0088] 另一方面，本发明还涉及用于调节胞内活性的连接于官能化纳米粒子的生物活性分子的传递方法。例如，首先将人体细胞、成纤维细胞或者市售可得的或使用标准或改良实验步骤可获得的其它类型的细胞在无菌条件下平铺 (plate) 到具有或不具有细胞粘附底物 (饲养细胞，明胶、基质胶、纤连蛋白等) 的固体表面。将平铺细胞 (plated cell) 与特定因子组合培养一段时间，使细胞分裂 / 增殖或维持可接受的细胞生存能力。实例是血清和 / 或各种生长因子，它可以在之后被除去或更新，而继续进行培养。在官能化生物相容性细胞透过性纳米粒子的存在下培养平铺细胞，该官能化生物相容性细胞透过性纳米粒子在存在或不存在磁场的条件下使用此处描述的各种方法连接有生物活性分子。在超顺磁性纳米粒子的情况下使用磁铁使得细胞和纳米粒子之间的接触表面积呈现重要的增长，从而进一步增强官能化纳米粒子对细胞膜的穿透。根据需要，用官能化纳米粒子反复处理细胞群，以胞内传递生物活性分子。

[0089] 将细胞悬浮在培养基中，通过离心或细胞分离除去未掺入纳米粒子，留下作为集群存在的细胞。然后，将集群细胞再悬浮，并在新鲜的培养基中再培养一段适宜的时间。可以通过分离、再悬浮和再培养的多次循环，直至观察到胞内传递的特定生物活性分子引起随之发生的生物效应后收集细胞。

[0090] 本发明的一个用途是筛选对细胞重编程有效的一个化合物 (或多个化合物)。该化合物使用本说明书中公开的一个或多个方法以所希望的细胞群连接于纳米粒子，培养适当时间，然后测定由化合物产生的任何调节作用。可以包括 :细胞重编程的开始和多功能干细胞的产生，细胞分化或转分化成进一步特化的或不同特化的细胞类型，检查细胞毒性，代谢变化或对收缩活动的效果等功能。

[0091] 本发明的另一种用途是生成作为药物的特化细胞或在传递装置中用以治疗人体或动物体。这使得临床医生能够将细胞施用到受损组织 (不论是心脏、肌肉、肝脏等) 中或周围，该施用是经脉管系统施用或直接施用到肌肉或器官壁内，从而植入特化细胞，控制损伤，并参与组织的肌肉组织再生和特化功能恢复。

[0092] 本发明的一种用途涉及用其他蛋白质（如 Oct4 和 Sox2 转录因子）官能化的纳米粒子，从而确保具有保存完好的基因组的干细胞或进一步分化的细胞类型的细胞重编程或产生。

[0093] 本发明的另一种用途是筛选对细胞重编程有效的一个化合物（或多个化合物）。该化合物使用本说明书中公开的多个方法以所希望的细胞群连接于纳米粒子，培养适当时间，然后测定由化合物产生的任何调节作用。可以包括：细胞重编程的开始和多功能干细胞的产生，细胞分化或转分化成进一步特化的或不同特化的细胞类型，检查细胞毒性，代谢变化或对收缩活动的效果等功能。

[0094] 本发明还有一种用途，就是生成作为药物的特化细胞或在传递装置中用以治疗人体或动物体。这使得临床医生能够将细胞施用到受损组织（不论是心脏、肌肉、肝脏等）中或周围，该施用经脉管系统施用或直接施用到肌肉或器官壁内，从而植入特化细胞，控制损伤，并参与组织的肌肉组织再生和特化功能恢复。

[0095] 作为进一步的说明（但并非是限制），下面的实施例公开了本发明的其它方面。

[0096] 实施例

[0097] 实施例 1

[0098] 使用 LC-SMM 作为交联剂将 GFP 连接到超顺磁性粒子（连接于珠的胺基团），然后将其直接耦合于 GFP 上的巯基。将 LC-SMCC（来自 Thermo Fisher）以 1mg/μl 的浓度溶解在由 ACROS（密封小瓶且无水）得到的二甲基甲酰胺 (DMF)。样品密封且几乎立即使用。

[0099] 将 10μl 溶液加入到纳米粒子中至体积为 200μl。这样提供了相对于存在的可用的胺基团远远过量的 SMCC，使反应进行 1 小时。可以用截留分子量为 3000 道尔顿的分子的 Amicon 旋转过滤器移除过量的 SMCC 和 DMF。通常要求 5 次体积更换以确保适当的缓冲交换。重要的是过量的 SMCC 在这个阶段被除去。

[0100] 将任何肽基分子、例如市售可得的绿色荧光蛋白 (GFP) 或纯化的重组绿色荧光蛋白或其它蛋白质加入到含有一定量的乙二醇的溶液中，在 -30℃冷冻。边剧烈搅拌边加入在 14μl、10μl 新鲜制备的 DTT（二硫苏糖醇，克莱兰氏试剂 (Cleland's reagent)）中含 3μg 蛋白质的 PBS 溶液。因为蛋白质通常含有一个以上的半胱氨酸，倾向于交联不同的 GFP 分子。因此，过量的 DTT 还原二硫键并释放绿色荧光蛋白。使反应在 4℃进行 2 小时，然后通过截留分子量为 3,000 的 Amicon 离心过滤单元除去过量的试剂。

[0101] 将活化的纳米粒子和蛋白质溶液混合，并使其反应 2 小时，之后将未反应的蛋白质通过具有适当的截留分子量（在使用 GFP 的本例中截留分子量为 50,000 道尔顿）的 Amicon 离心过滤单元除去。样品储存于 -80℃。还应当指出的是也可以使用 SMCC 的磺基衍生物（磺基 -SMCC），其更溶于水。也可以用 DMSO 代替 DMF 作为标记试剂的溶剂载体，另外它应该是无水的。

[0102] 实施例 2

[0103] 在该方法中，使用赖氨酸的氨基对珠上的巯基进行耦合反应。在这些研究中使用刚刚以 pH7.2 的 0.1M 磷酸缓冲液平衡过的珠。新鲜配制 1mg/ml(DMF 中）的 LC-SPDP。在剧烈搅拌下将 10μl 的 SPDP 溶液加入到珠悬浮液中，并使其反应 1 小时。接着，将未反应的材料通过离心去除，并使用截留分子量为 10K 的 Amicon 旋转过滤器以磷酸盐缓冲液清洗纳米粒子。使用克莱兰氏试剂切断 SPDP 的二硫键，将 1mg 加入到溶液中，并使其反应 1 小

时。将副产物和未反应的克莱兰氏试剂通过截留分子量为 10K 的 Amicon 旋转过滤器除去。

[0104] 在上述反应的进行中，使用 N- 乙基马来酰亚胺阻断 GFP。将过量的乙基马来酰亚胺加入到 GFP 溶液中。反应在室温下进行 1 小时，用截留分子量为 3K 的 Amicon 旋转过滤器除去不需要的材料。然后，使 GFP 与过量的 SMCC 反应 1 小时。随后，将 GFP 用旋转柱纯化，然后与 PDP- 纳米粒子反应。反应进行 1 小时，并用截留分子量为 50K 的 Amicon 旋转过滤器纯化终产物。

[0105] 实施例 3

[0106] 将从商业渠道获得或使用所描述的标准试验方法 [Moretti 等，Mouse and human induced pluripotent stem cells as a source for multipotent Isll cardiovascular progenitors(小鼠和人类诱导多能干细胞作为多能 Isll 心血管祖细胞的来源)。FASEB J.24:700(2010)] 获得的人体成纤维细胞在无菌条件下以 150,000 个细胞的密度平铺在 6 孔板中的固体表面，该固体表面有或没有以 150,000-200,000 的密度预先平铺的饲养细胞。饲养细胞可通过商业渠道或用标准的实验室方法获得。将平铺细胞与特定因子组合培养一段时间，使细胞分裂 / 增殖或维持在含血清培养基中可接受的细胞存活能力，从而可以在之后除去或再生，并在湿润的培养箱 (5％ CO_2 和环境 O_2) 中在无菌条件下持续培养。

[0107] 用 50μl 含有官能化生物相容性细胞透过性纳米粒子的悬浮液处理在锥形管底部收集到的细胞或平铺细胞，该官能化生物相容性细胞透过性纳米粒子在存在或不存在磁场的情况下使用本说明书中公开的各种方法连接有生物活性分子。

[0108] 在超顺磁性纳米粒子的情况下使用磁场使得细胞和纳米粒子之间的接触表面积呈现重要的增长，从而确保官能化纳米粒子对细胞膜的穿透进一步增强。重要的是，类似于连接有 PEG 的聚 (乙二醇)PEG- 介导保护的几种以蛋白质为基础的药物 (PEG-GCSF，Amgen，CA ；PEG- 干扰素，Schering-Plough/Merck，NJ)，用于与耦合肽连接的纳米粒子增加了多肽的尺寸并遮住蛋白质的表面，从而减少蛋白质被蛋白水解酶降解，从而获得所使用的蛋白质分子更长期的稳定性。根据需要，用官能化纳米粒子反复处理细胞群，以胞内传递生物活性分子

[0109] 将细胞悬浮在培养基中，通过以约 1200×g 离心 10 分钟除去未掺入的纳米粒子，留下作为集群存在的细胞。然后，将集群细胞再悬浮，再使用类似的流程清洗，并在新鲜的培养基中再培养适当的时间。可以通过分离、再悬浮和在培养基中再培养的多次循环直至观察到胞内传递的特定生物活性分子引起随之发生的生物效应，收集细胞。

[0110] 在使用绿色荧光蛋白的特定例中，细胞透过性纳米粒子将蛋白质传递到细胞内，通过靶细胞获得新的绿色荧光。该新获得的能力允许后续的分选，以及将带有胞内传递的蛋白质的细胞以高度均质化进行分离，其可进一步用于各种应用。重要的是，连接有蛋白质的细胞透过性官能化纳米粒子不会以任何形式整合到细胞基因组中，从而确保每个具有新 (在这种情况下为荧光) 特性的细胞保持完整的基因组，并保留细胞 DNA 的完整性。

[0111] 在不脱离其主旨或本质特征的情况下，本发明可以以其他具体形式实施。因此，上述实施方式应被认为是说明性的，而不是限制本说明书中描述的发明。因此，本发明的范围由所附的权利要求书而不是由前面的说明书来表示，并且在与权利要求等同的含义和范围内的所有改变都涵盖于此。

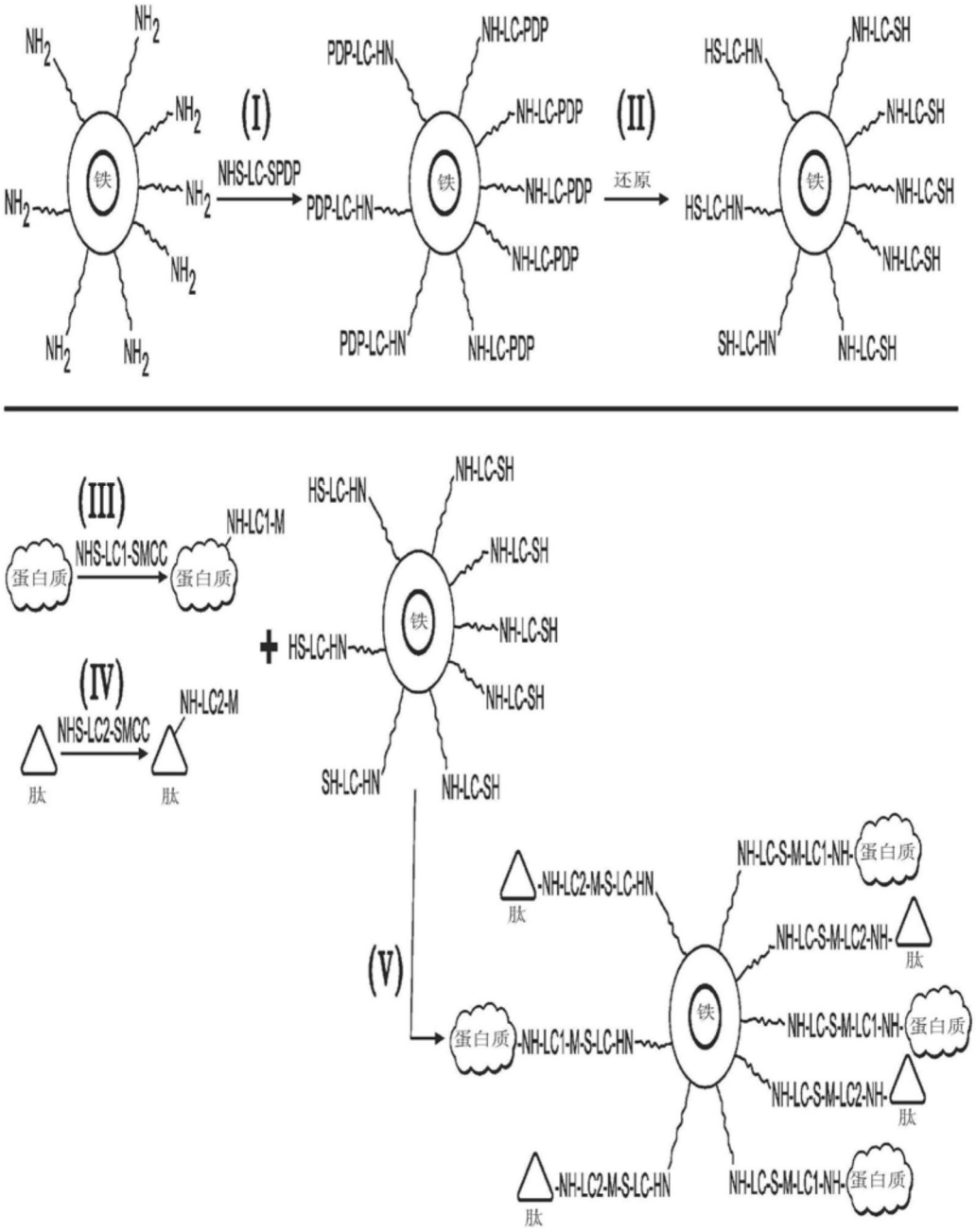

铁
NH2
(I)
NHS-LC-SPDP
PDP-LC-HN
NH-LC-PDP
(II)
还原
HS-LC-HN
NH-LC-SH
(III)
NHS-LC1-SMCC
蛋白质
NH-LC1-M
(IV)
NHS-LC2-SMCC
肽
NH-LC2-M
SH-LC-HN
(V)
NH-LC2-M-S-LC-HN
NH-LC-S-M-LC1-NH-
NH-LC-S-M-LC2-NH-
NH-LC1-M-S-LC-HN

图 1

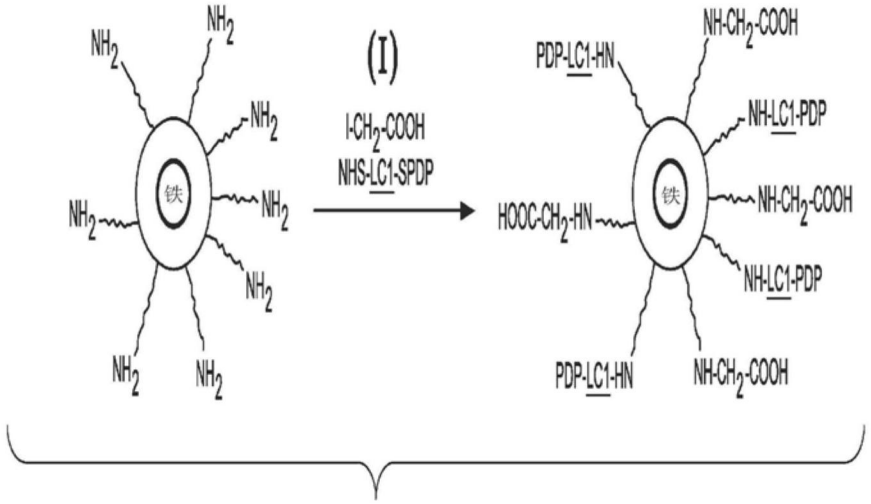

(I)
I-CH2-COOH
NHS-LC1-SPDP
铁
NH2
NH-CH2-COOH
PDP-LC1-HN
NH-LC1-PDP
HOOC-CH2-HN

图 2A

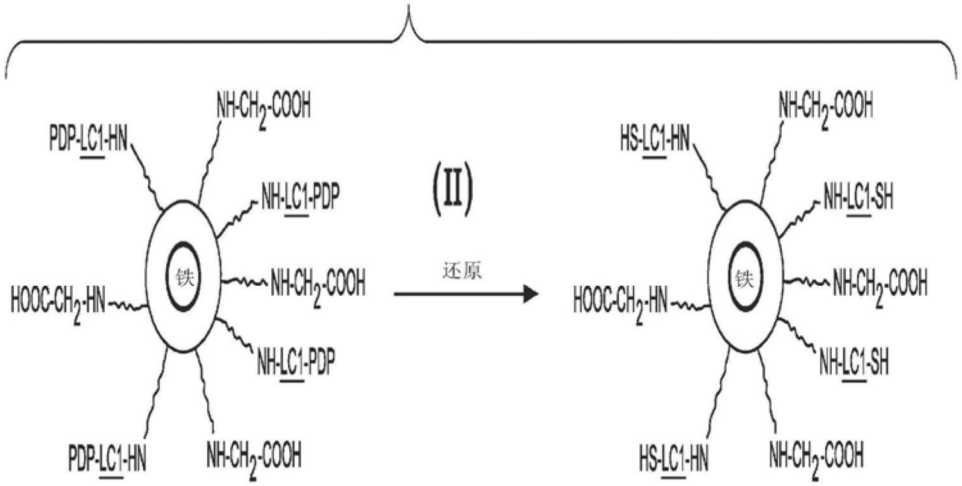

(II)
还原
铁
PDP-LC1-HN
NH-CH2-COOH
NH-LC1-PDP
HOOC-CH2-HN
HS-LC1-HN
NH-LC1-SH

图 2B

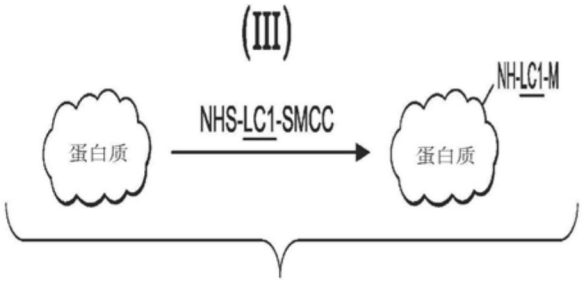

(III)
NHS-LC1-SMCC
蛋白质
NH-LC1-M

图 2C

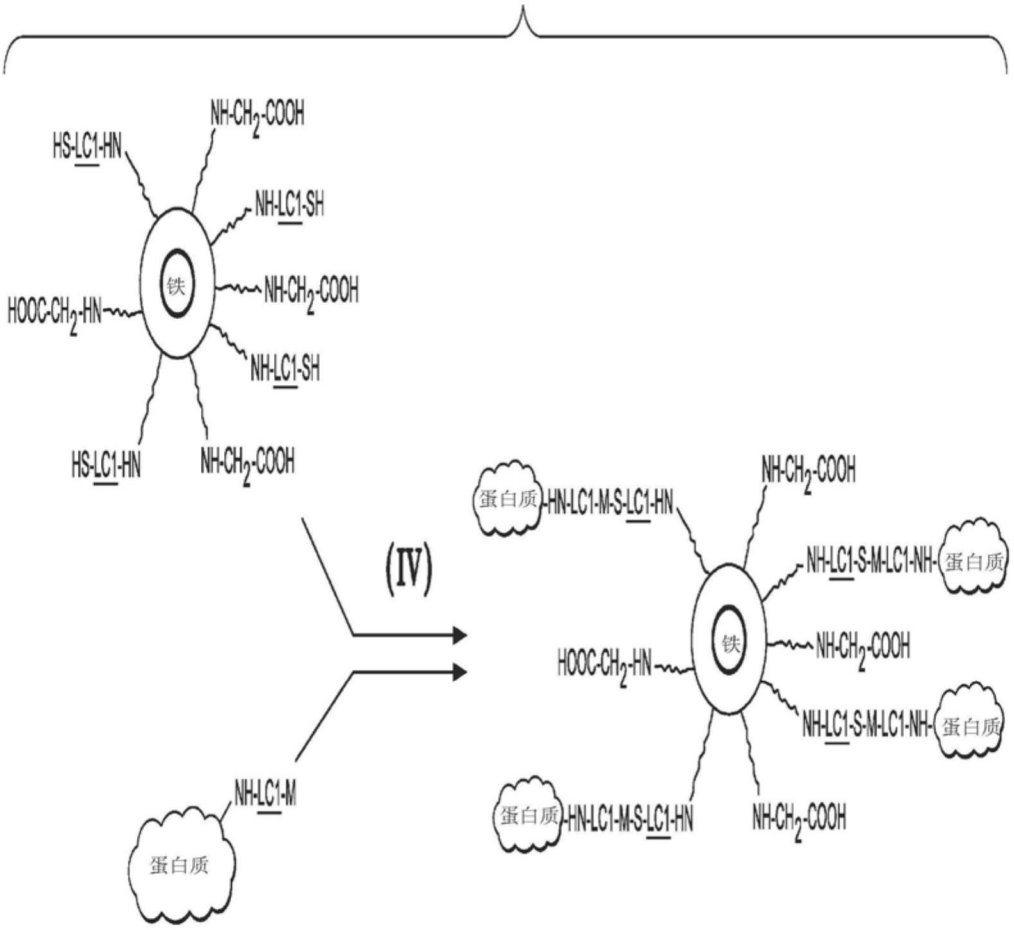

NH-CH2-COOH
HS-LC1-HN
NH-LC1-SH
铁
NH-CH2-COOH
HOOC-CH2-HN
NH-LC1-SH
HS-LC1-HN
NH-CH2-COOH
(IV)
NH-LC1-M
蛋白质
蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-蛋白质
铁
NH-CH2-COOH
HOOC-CH2-HN
NH-LC1-S-M-LC1-NH-蛋白质
蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-COOH

图 2D

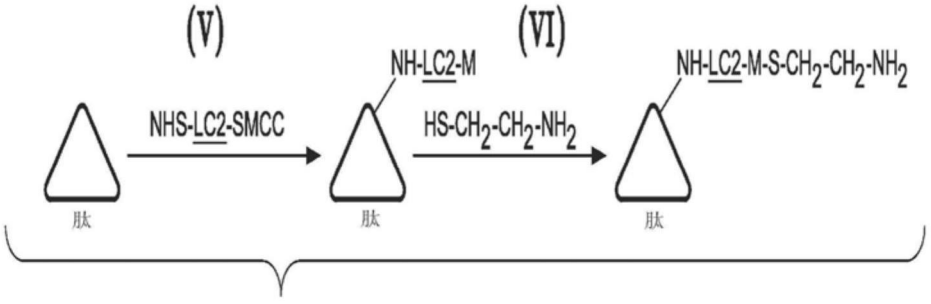

(V)
(VI)
NH-LC2-M
NH-LC2-M-S-CH2-CH2-NH2
NHS-LC2-SMCC
HS-CH2-CH2-NH2
肽
肽
肽

图 2E

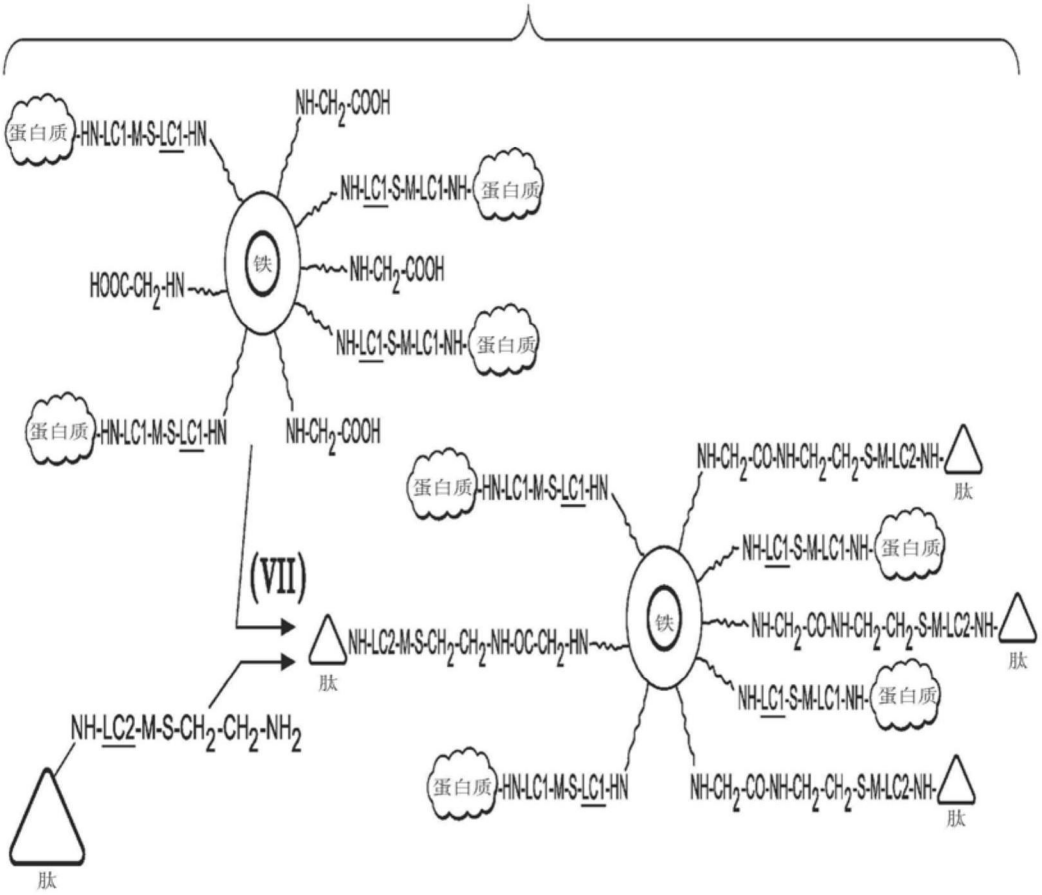

蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-蛋白质
铁
HOOC-CH2-HN
NH-CH2-COOH
NH-LC1-S-M-LC1-NH-蛋白质
蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-COOH
(VII)
NH-LC2-M-S-CH2-CH2-NH2
肽
NH-LC2-M-S-CH2-CH2-NH-OC-CH2-HN
肽
蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-CO-NH-CH2-CH2-S-M-LC2-NH-
肽
NH-LC1-S-M-LC1-NH-蛋白质
铁
NH-CH2-CO-NH-CH2-CH2-S-M-LC2-NH-
肽
NH-LC1-S-M-LC1-NH-蛋白质
蛋白质-HN-LC1-M-S-LC1-HN
NH-CH2-CO-NH-CH2-CH2-S-M-LC2-NH-
肽

图 2F

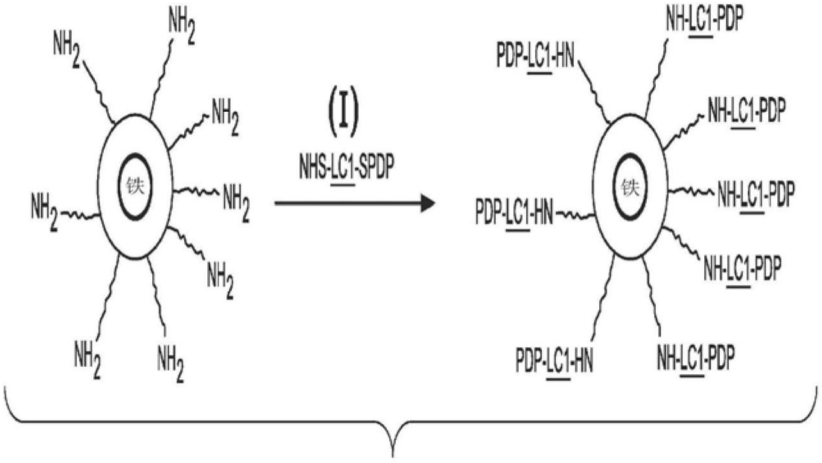

NH2
NH2
NH2
NH2
铁
NH2
NH2
NH2
NH2
(I)
NHS-LC1-SPDP
NH-LC1-PDP
PDP-LC1-HN
NH-LC1-PDP
铁
PDP-LC1-HN
NH-LC1-PDP
NH-LC1-PDP
PDP-LC1-HN
NH-LC1-PDP

图 3A

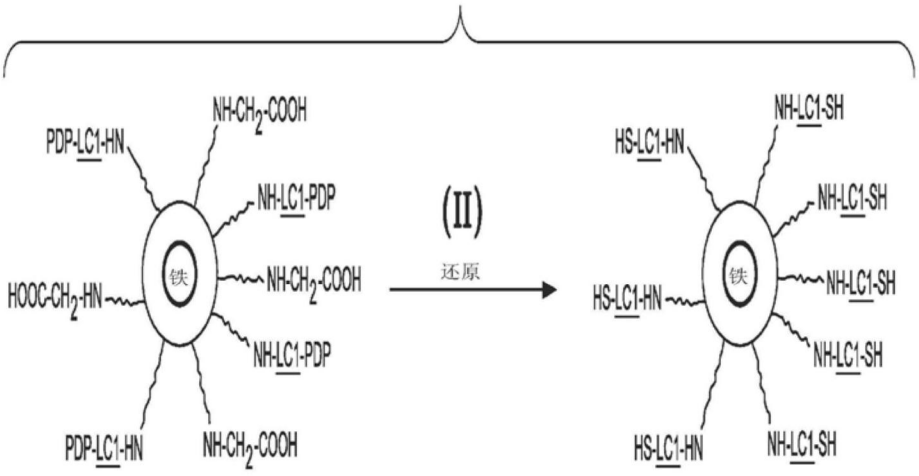

PDP-LC1-HN
NH-CH2-COOH
NH-LC1-PDP
HOOC-CH2-HN
铁
NH-CH2-COOH
NH-LC1-PDP
PDP-LC1-HN
NH-CH2-COOH
(II)
还原
HS-LC1-HN
NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
铁
NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH

图 3B

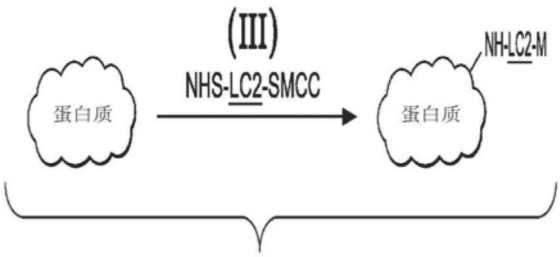

(III)
NHS-LC2-SMCC
蛋白质
蛋白质
NH-LC2-M

图 3C

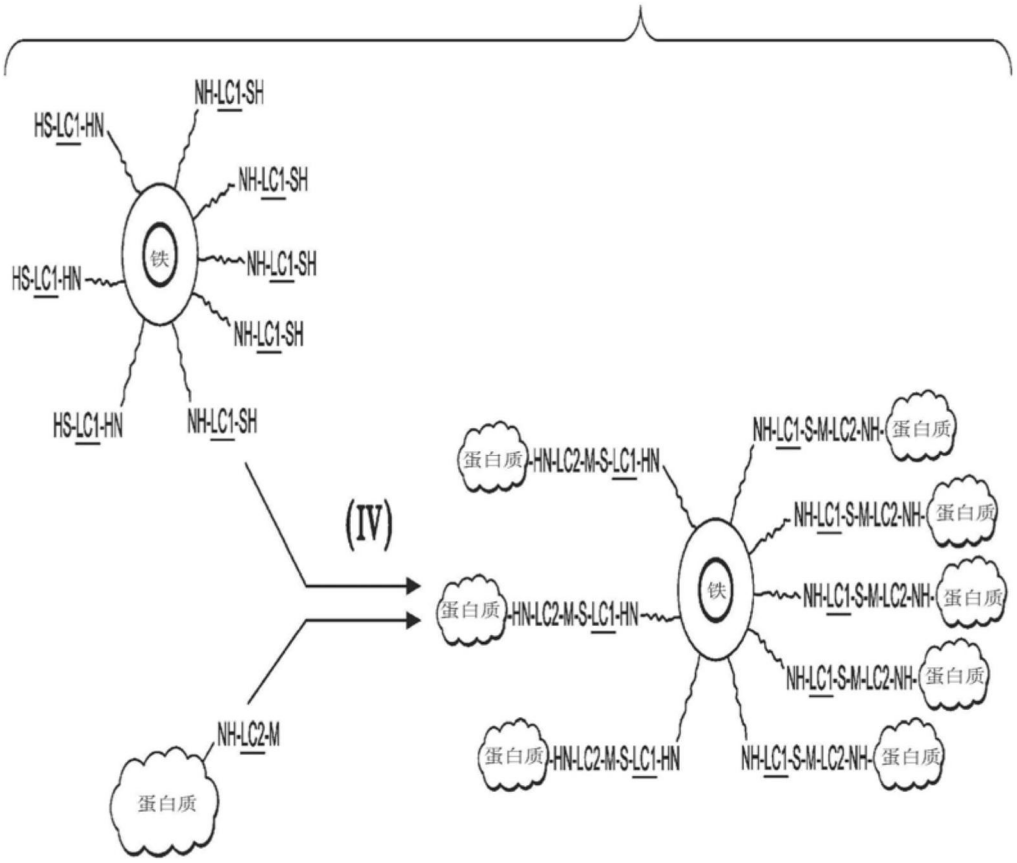

铁
HS-LC1-HN
NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH
NH-LC1-SH
HS-LC1-HN
NH-LC1-SH
(IV)
NH-LC2-M
蛋白质
蛋白质-HN-LC2-M-S-LC1-HN
蛋白质-HN-LC2-M-S-LC1-HN
蛋白质-HN-LC2-M-S-LC1-HN
铁
NH-LC1-S-M-LC2-NH-蛋白质
NH-LC1-S-M-LC2-NH-蛋白质
NH-LC1-S-M-LC2-NH-蛋白质
NH-LC1-S-M-LC2-NH-蛋白质
NH-LC1-S-M-LC2-NH-蛋白质

图 3D

Abstract

Functionalized biocompatible nanoparticles capable of penetrating through a mammalian cell membrane and delivering intracellularly a plurality of bioactive molecules for modulating a cellular function are disclosed herein The functionalized biocompatible nanoparticles comprise: a central nanoparticle ranging in size from about 5 to about 50 nm and having a polymer coating thereon, a plurality of functional groups covalently attached to the polymer coating, wherein the plurality of bioactive molecules are attached to the plurality of the functional groups, and wherein the plurality of bioactive molecules include at least a peptide and a protein, and wherein the peptide is capable of penetrating through the mammalian cell membrane and entering into the cell, and wherein the protein is capable of providing a new functionality within the cell. The protein may be a transcription factor selected from the group consisting of Oct4, Sox2, Nanog, Lin28, cMyc, and Klf4.