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(54) NANOGRAPHITE STRUCTURE/METAL NANOPARTICLE COMPOSITE

(57) The present invention makes it possible to efficiently recognize carbon nanotubes, carbon nanohorns or modifiers thereof and to support functional compounds by fusing the ability of ferritin molecules capable of forming nanoparticles of inorganic metal atoms or inorganic metal compounds. In addition, because ferritin molecules are capable of forming two-dimensional crystals at the interface, the present invention makes it possible to align carbon nanotubes, carbon nanohorns with the use of the molecular arrangement ability of ferritin fused with nanographite structure recognition peptides. A nanographite structure/metal nanoparticle composite, wherein a nanoparticle of an inorganic metal atom or an inorganic metal compound is retained in an interior space of a protein in which a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein such as ferritin, and wherein a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound are supported on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure, is constructed.

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Description

Technical Field

[0001] The present invention relates to a protein wherein a nanographite structure recognition peptide is fused or chemically bound to the surface of a cage protein such as ferritin, and a nanographite structure/metal nanoparticle composite constructed with the use of the protein, wherein a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound are supported on the nanographite structure, etc. For example, a nanographite structure/metal nanoparticle composite, wherein a plurality of nanoparticles are supported through a compound of graphite structure having a nanometer-scale fine structure and a cage protein such as fusion ferritin which recognizes the compound specifically, can be advantageously used for semiconductors, nanobiotechnology, etc.

Background Art

[0002] As a crystal structure of carbon, diamond and graphite have been known from long time ago, and (C60) was found by R. E. Smalley, R. F. Curl and H. W. Kroto et al., in 1985 (for example, Non-Patent Document 1). C60 has a soccer ball-like structure comprising 12 pentagons and 20 hexagons, and other than C60, there are large basket-like molecules such as C70 and C76, and this series of molecules is called "fullerene". Further, carbon compounds with new structures previously unknown, such as "carbon nanotube" (Non-Patent Document 2; Patent Document 1) and "carbon nanohorn" (Non-Patent Document 3; Patent Document 1) were successively discovered by Sumio Iijima, in 1991 and 1999, respectively. All of these fullerenes, carbon nanotubes and carbon nanohorns comprise six- and five-membered rings of carbon atoms, and form nanometer-scale fine structures, and therefore, they have attracted a lot of attention as "nanographite structure" recently.

[0003] The reasons why nanographite structures attract a lot of attention include: "carbon nanotubes can have both properties of metal and semiconductor due to the difference in their chirality" (Non-Patent Document 4), "metal-doped fullerene exhibits superconductivity" (Non-Patent Document 5), "selective gas storage capability shown by carbon nanohorns" (Non-Patent Document 6), "ability of carbon nanohorn for the support and sustained release of pharmaceutical compounds" (Patent Document 2; Non-Patent Document 7), and the like. With the use of these characteristic properties, nanographite structures are expected to be applied to new electrical materials, catalysts, optical materials, and other fields, more specifically, to wiring of semiconductors, fluorescent indicator tubes, fuel cells, gas storage, vectors for gene therapy, cosmetics, drug delivery systems, biosensors, etc.

[0004] One of the present inventors, Kiyotaka Shiba, and others have isolated a peptide motif which binds to a carbon nanohorn, one of nanographite structures, by the phage display technique (Patent Document 3; Non-Patent Document 8).

[0005] On the other hand, ferritin proteins have been known for long years as a protein which stores 'molecules of "iron" , which is an essential metal and is toxic at the same time' in living bodies. Ferritin exists universally, from animals and plants to bacteria, and is deeply involved in the homeostasis of iron element in living bodies or in cells. Ferritin from higher eucaryotes such as human and horse forms a spherical shell structure consisting of a 24-mer approximately 12 nm in diameter, formed from peptide chains whose molecular weight is about 20 kDa, and has an interior space of 7 to 8 nm. Ferritin stores ironmolecules in this interior space as amass of nanoparticulate iron oxide. With regard to 24 subunits which constitute a protein spherical shell (cage), there are two types (type H and type L), and the ratio of these types varies depending on organism species and tissues.

[0006] Ferritin stores iron nanoparticles inside it under natural circumstances. However, under artificial circumstances, it has been revealed that ferritin can store the following substances in addition to iron: oxides of beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, chromium, etc.; and nanoparticles of semiconductors, magnets such as cadmium selenide, zinc sulfide, iron sulfide and cadmium sulfide. Consequently, applied researches of ferritin in the fields of material engineering of semiconductors and health care have been actively conducted.

[0007] Patent Document 1: Japanese Laid-Open Patent Application No. 2001-64004
Patent Document 2: Japanese Patent Application No. 2004-139247
Patent Document 3: Japanese Laid-Open Patent Application No. 2004-121154
Non-Patent Document 1: Nature, 318: 162-163, 1985
Non-Patent Document 2: Nature, 354: 56-58, 1991
Non-Patent Document 3: Chem. Phys. Lett., 309: 165-170, 1999
Non-Patent Document 4: Nature, 391: 59-62
Non-Patent Document 5: Nature, 350: 600-601
Non-Patent Document 6: Nikkei Science, 42, August issue, 2002
Non-Patent Document 7: Mol Pharmaceutics 1: 399
Non-Patent Document 8: Langmuir, 20, 8939-8941, 2004

Disclosure of the Invention

Object to be Solved by the Invention

[0008] If it is possible to combine nanographite structures having excellent properties with metal-filled ferritin molecules, the development of composite materials having an unprecedented new function can be expected. In this case, a technique for making ferritin molecules efficiently recognize and bind to nanographite structures

such as carbon nanotubes and carbon nanohorns, is required. An object of the present invention is to make it possible to efficiently recognize carbon nanotubes, carbon nanohorns or modifiers thereof and to support functional compounds thereon by fusing the ability of ferritin molecules capable of forming nanoparticles of inorganic metal atoms or inorganic metal compounds with nanographite structure recognition peptides. In addition, because ferritin molecules are capable of forming two-dimensional crystals at the interface and have an ability for molecular arrangement, an object of the present invention is to make it possible to align carbon nanotubes , carbon nanohorns with the use of the molecular arrangement ability of ferritin fused with nanographite structure recognition peptides.

Means to Solve the Object

[0009] The present inventors have made a keen study for solving the above-mentioned object, and have confirmed that a plurality of nanoparticles can be supported on a nanographite structure by the process comprising the steps of: fusing cDNA encoding the amino-terminal of horse spleen-derived type L ferritin molecule with DNA encoding a peptide consisting of the amino acid sequence shown by SEQ ID NO: 1; expressing a protein having the amino acid sequence shown by SEQ ID NO: 26 with the use of E. coli; purifying the protein and retaining nanoparticles of metal oxide in the interior space of the fusion protein thus obtained. This led to the completion of the present invention.

[0010] In other words, the present invention relates to: (1) a nanographite structure/metal nanoparticle composite, wherein a nanoparticle of an inorganic metal atom or an inorganic metal compound is retained in an interior space of a protein in which a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein, and wherein a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound are supported on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; (2) the nanographite structure/metal nanoparticle composite according to (1) mentioned above, wherein the cage protein belongs to a 0ferritin protein family; (3) the nanographite structure/metal nanoparticle composite according to (2) mentioned above, wherein the ferritin protein family is ferritin; (4) the nanographite structure/metal nanoparticle composite according to (3) mentioned above, wherein the ferritin is higher eucaryote-derived ferritin; (5) the nanographite structure/metal nanoparticle composite according to (4) mentioned above, wherein the higher eucaryote-derived ferritin is horse spleen-derived type L ferritin; (6) the nanographite structure/metal nanoparticle composite according to (1) mentioned above, wherein the cage protein is derived from a bacterium; (7) the nanographite structure/metal nanoparticle composite according to (1) mentioned above, wherein the cage protein is a viral particle; (8) the nanographite structure/metal nanoparticle composite according to any one of (1) to (7) mentioned above, wherein the nanographite structure recognition peptide is a peptide consisting of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20; (9) the nanographite structure/metal nanoparticle composite according to any one of (1) to (7) mentioned above, wherein the nanographite structure recognition peptide is a peptide containing whole or part of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 and capable of binding to a nanographite structure; (10) the nanographite structure/metal nanoparticle composite according to (8) or (9) mentioned above, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is DYFSSPYYEQLF (SEQ ID NO: 1); (11) the nanographite structure/metal nanoparticle composite according to (8) or (9) mentioned above, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is YDPFHII (SEQ ID NO: 2) ; (12) the nanographite structure/metal nanoparticle composite according to any one of (1) to (11) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal nanoparticle; (13) the nanographite structure/metal nanoparticle composite according to any one of (1) to (11) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal compound nanoparticle; (14) the nanographite structure/metal nanoparticle composite according to (13) mentioned above, wherein the metal compound nanoparticle is a metal oxide nanoparticle; (15) the nanographite structure/metal nanoparticle composite according to (13) mentioned above, wherein the metal compound nanoparticle is a magnetic material nanoparticle; (16) the nanographite structure/metal nanoparticle composite according to any one of (1) to (15) mentioned above, wherein the metal is iron, beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, zinc, cadmium or chromium; (17) the nanographite structure/metal nanoparticle composite according to any one of (1) to (11) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a nanoparticle of iron oxide, a nanoparticle of cadmium selenide, a nanoparticle of zinc selenide, a nanoparticle of zinc sulfide, or a nanoparticle of cadmium sulfide; (18) the nanographite structure/metal nanoparticle composite according to any one of (1) to (17) mentioned above, wherein the nanographite structure is a carbon nanotube or a carbon nanohorn; (19) the nanographite structure/metal nanoparticle composite according to (18) mentioned above, wherein the carbon nanotube or the carbon nanohorn is constituted of a carbon structure to which a functional group is added; (20) the nanographite structure/metal nanoparticle composite according to any one of (1) to (19) mentioned above, wherein the nanographite structure is two-dimensionally aligned on a substrate; (21) the nanographite structure/metal nanoparticle composite according to any one of (1) to (19) mentioned above, wherein the metal nanoparticle is two-dimension-

ally aligned on a substrate; and (22) the nanographite structure/metal nanoparticle composite according to (20) mentioned above, wherein the cage protein is removed.

[0011] The present invention also relates to: (23) a protein wherein a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein; (24) the protein according to (23) mentioned above, wherein the cage protein belongs to a ferritin protein family; (25) the protein according to (24) mentioned above, wherein the ferritin protein family is ferritin; (26) the protein according to (25) mentioned above, wherein the ferritin is higher eucaryote-derived ferritin; (27) the protein according to (26) mentioned above, wherein the higher eucaryote-derived ferritin is horse spleen-derived type L ferritin; (28) the protein according to (24) mentioned above, wherein the ferritin protein family is derived from a bacterium; (29) the protein according to (23) mentioned above, wherein the cage protein is a viral particle; (30) the protein according to any one of (23) to (29) mentioned above, wherein the nanographite structure recognition peptide is a peptide consisting of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20; (31) the protein according to any one of (23) to (29) mentioned above, wherein the nanographite structure recognition peptide is a peptide containing whole or part of an amino acid sequence shown by any one of SEQ ID NOs : 1 to 20 and capable of binding to a nanographite structure; (32) the protein according to (30) or (31) mentioned above, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is DYFSSPYYEQLF (SEQ ID NO: 1); (33) the protein according to (30) or (31) mentioned above, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is YDPFHII (SEQ ID NO: 2); (34) the protein according to any one of (23) to (33) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal nanoparticle; (35) the protein according to any one of (23) to (33) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal compound nanoparticle; (36) the protein according to (35) mentioned above, wherein the metal compound nanoparticle is a metal oxide nanoparticle; (37) the protein according to (35) mentioned above, wherein the metal compound nanoparticle is a magnetic material nanoparticle; (38) the protein according to any one of (22) to (36) mentioned above, wherein the metal is iron, beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, zinc, cadmium or chromium; (39) the protein according to (23) mentioned above, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a nanoparticle of iron oxide, a nanoparticleofcadmiumselenide, ananoparticleof zincselenide, a nanoparticle of zinc sulfide, or a nanoparticle of cadmium sulfide; (40) the protein according to any one of (23) to (39) mentioned above, wherein the nanographite structure is a carbon nanotube or a carbon nanohorn; and (41) the protein according to (40) mentioned above, wherein the carbon nanotube or the carbon nanohorn is constituted of a carbon structure to which a functional group is added.

[0012] The present invention further relates to: (42) a method for retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of (23) to (41) mentioned above, and supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; (43) a method for producing a composite of a nanographite structure and nanoparticles of an inorganic metal compound, comprising the steps of: retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of (23) to (41) mentioned above; supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; and removing a protein moiety by a heat treatment; (44) a method for producing a composite of a nanographite structure and nanoparticles of an inorganic metal compound, comprising the steps of: retaininganoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of (23) to (41) mentioned above; supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; and removing a protein moiety by an electron beam treatment; (45) a method for aligning a nanographite structure by binding the nanographite structure to the protein according to any one of (23) to (41) mentioned above which has formed a two-dimensional crystal; and (46) a method for aligning a nanographite structure by binding the nanographite structure to the protein according to any one of (23) to (41) mentioned above which has formed a two-dimensional array.

Brief Description of Drawings

[0013]

[Fig. 1] This is a view showing the crystal structure of horse spleen-derived type L ferritin (LF) and the presentation site of DYFSSPYYEQLF (SEQ ID NO: 1; N1 sequence). N-terminal site of the crystal structure of horse spleen-derived type L ferritin (LF0) is indicated in red. As shown in Fig. 1, because the N-terminal of LF0 is located outside of the molecule, a multiple number of N1 sequence can be presented by fusing the N-terminal with N1 sequence.

[Fig. 2] This is a frame format of the construction of N1-LF expression vector pKIS2. N1-LF recombinant ferritin expression vector pKIS2 was constructed by the process comprising the steps of: cutting pKITO,

a horse spleen-derived type L ferritin expression vector, with restriction enzymes BamHI and SacI; inserting synthetic DNAs of SEQ ID NOs : 22 and 23 which had been annealed, and then cutting the resultant product with BamHI; to that site, inserting a short DNA fragment produced when pKITO had been cut with BamHI.

[Fig. 3] This is a view showing the result of polyacrylamide gel electrophoresis of the final purified preparation of N1-LF. By polyacrylamide gel electrophoresis of 3 μg of the final purified preparation of N1-LF, the uniformity was evaluated. When the preparation was separated by using a concentration gradient gel (15 to 25%) and stained with Coomassie brilliant blue, a protein band was observed only at the position corresponding to the molecular weight of the desired N1-LF. Based on the observation, it was possible to confirm the preparation was highly pure. The left lane indicates molecular weight markers corresponding to 97.4, 66.3, 42.4, 30.0, 20.1, 14.4 kDa in descending order. The right lane indicates the final purified preparation of N1-LF.

[Fig. 4] This is a view showing the formation of nanoparticles of iron oxide in the interior space of N1-LF. It is an appearance of the solution at the time when nanoparticles of iron oxide were formed in the interior space of N1-LF. In control, no ferritin protein solution was contained. It can be seen from the color of the solution that nanoparticles of iron oxide were formed in the interior space of ferritin.

[Fig. 5] This is a photomicrograph taken by a transmission electron microscope showing the formation of nanoparticles of iron oxide in the interior space of N1-LF. The image of N1-LF stained with 1% aurothioglucose was observed with a JEOL1010, manufactured by JEOL Ltd., at 100 kV.

[Fig. 6] This is a photomicrograph taken by a transmission electron microscope showing the formation of nanoparticles of iron oxide in the interior space of LF0. The image of N1-LF stained with 1% aurothioglucose was observed with a JEOL1010, manufactured by JEOL Ltd., at 100 kV.

[Fig. 7] This is a view showing nanoparticles of metal oxide supported on a carbon nanohorn. By presenting a peptide capable of binding to carbon nanohorns to ferritin protein, N1-LF could specifically support a plurality of nanoparticles of metal oxide on the carbon nanohorn (left) . With horse spleen-derived type L ferritin (LF0), it was impossible to support nanoparticles of metal oxide on the carbon nanohorn (right).

[Fig. 8] This is a view showing nanoparticles of metal oxide supported on a single-wall carbon nanotube. By presenting a peptide capable of binding to carbon nanohorns to ferritin protein, N1-LF could support a plurality of nanoparticles of metal oxide on the single-wall carbon nanotube.

Best Mode of Carrying Out the Invention

[0014] The nanographite structure/metal nanoparticle composite of the present invention is not particularly limited as long as it is a composite wherein a nanoparticle of an inorganic metal atom or an inorganic metal compound is retained in an interior space of a protein in which a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein, and wherein a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound are supported on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure. In addition, the protein is not particularly limited as long as it is a protein wherein a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein. Here, the cage protein of the present invention means a protein having a space inside it and capable of containing a substance.

[0015] Examples of the above-mentioned cage protein include ferritin protein family, those derived from bacteria, and viral particles. As the ferritin protein family, ferritin and apoferritin are exemplified, and, for example, type L or type H ferritin derived from higher eucaryotes such as horse spleen-derived type L ferritin is preferably exemplified. Examples of the cage proteins derived from bacteria include DpsA protein and MrgA protein, and examples of the viral particles include viral particles of retrovirus, adenovirus, rotavirus, poliovirus, cytomegalovirus, cauliflower mosaic virus, etc.

[0016] Examples of the above-mentioned nanographite structure include carbon nonotubes and carbon nanohorns, and in addition, a modified nanographite structure wherein a carbon nanotube or a carbon nanohorn is constituted of a carbon structure to which a functional group such as an amino group, a hydroxyl group, and a carboxyl group is added.

[0017] As the above-mentioned nanographite structure recognition peptide, a peptide consisting of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 (see Patent Document 3; Non-Patent Document 8), and a peptide containing whole or part of an amino acid sequence shown by any one of SEQ ID NOs : 1 to 20 and capable of binding to a nanographite structure, are exemplified. Among them, a peptide of DYFSSPYYEQLF (SEQ ID NO: 1) and a peptide of YDPFHII (SEQ ID NO: 2) are preferably exemplified.

[0018] The site on the cage protein surface to which a nanographite structure recognition peptide is fused or chemically bound is not particularly limited as long as it is a site where the nanographite structure recognition peptide can be bound to a nanographite structure. For example, in the case of ferritin, a loop structure site exposed on the ferritin surface, are exemplified in addition to amino terminals.

[0019] With regard to the method for fusing a nanographite structure recognition peptide to the surface of a cage protein such as ferritin, as described in Examples,

the method can be conducted in accordance with the methods described in "Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989", "Current Protocols in Molecular Biology, Supplement 1-38, John Wiley & Sons (1987-1997)", etc. Further, as the method for chemically binding a nanographite structure recognition peptide to the surface of a cage protein such as ferritin, the methods described in literatures (Proteins second edition, T. E. Creighton, W. H. Freeman and Company, New York, 1993; and G. T. Hermanson, in Bioconjugate Techniques, ed. G. T. Hermanson, Academic Press, San Diego CA, 1996, pp. 169-186.) are exemplified.

[0020] Examples of the above-mentioned nanoparticles of an inorganic metal atom or an inorganic metal compound include: nanoparticles of metals such as iron, beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, zinc, cadmium or chromium; and nanoparticles of metal compounds such as nanoparticles of oxides, hydroxides, carbonate, etc. of these metals and nanoparticles of magnetic materials. Preferable examples include nanoparticles of iron oxide, nanoparticles of cadmium selenide, nanoparticles of zinc selenide, nanoparticles of zinc sulfide, and nanoparticles of cadmium sulfide.

[0021] By further fusing or chemically binding a functional peptide, for example, a peptide capable of binding to titanium, to the above-mentioned protein wherein a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein, it is also possible to align nanographite structures on a titanium substrate, and moreover, to support a plurality of nanoparticles on the nanographite structures.

[0022] In the case of cage proteins such as ferritin capable of forming two-dimensional crystals, nanometer-sized pattern making are made possible by two-dimensional crystallization of the cage proteins. For instance, a nanographite structure/metal nanoparticle composite wherein nanographite structures are two-dimensionally aligned on a substrate, and a nanographite structure/metal nanoparticle composite wherein metal nanoparticles are two-dimensionally aligned on a substrate can be obtained by the process comprising the steps of: retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of a protein wherein a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein; supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; and removing a protein moiety by a heat treatment or an electron beam treatment. The composite thus obtained can be the basic technique for high integration of memory devices, etc., in the field of semiconductors including memory devices.

[0023] In addition, a nanographite structure/metal nanoparticle composite wherein nanographite structures are two-dimensionally aligned on a substrate, and a nanographite structure/metal nanoparticle composite wherein metal nanoparticles are two-dimensionally aligned on a substrate can be obtained also by constituting cage proteins into a two-dimensional array, for example, by the method for immobilizing proteins on a substrate by forming a cross-linking between proteins or proteins and the substrate with the use of reactivity between a compound having a plurality of functional groups such as glutaraldehyde and a side chain of an amino acid which constitutes the protein: or by the method for immobilizing proteins by placing SAM (molecules capable of self-assembling into membranes) having a functional group on a substrate and forming a linkage between the functional group and a side chain of an amino acid which constitutes the protein. The composite thus obtained can be the basic technique for high integration of memory devices, etc. , in the field of semiconductors including memory devices.

[0024] Hereinafter, the present invention is described more specifically with reference to Examples, however, the technical scope of the present invention is not limited to these exemplifications.

Example 1

[0025] The preparation of DNA (pKIS2) for expressing the fusion ferritinprotein (N1-LF, Fig. 1) wherein a nanographite structure recognition peptide consisting of the amino acid sequence shown by SEQ ID NO: 1 (N1) is fused with horse spleen-derived type L ferritin (LF) was conducted in accordance with the following procedure. In brief, an annealing reaction was conducted by: mixing 100 pmole/μl each of synthetic DNAs of SEQ ID NOs: 22 and 23 which are complementary to each other and encode Met, an initiation codon, and subsequently the amino acid sequence shown by SEQ ID NO: 21, and have a restriction enzyme BamHI linker sequence on the initiation codon side, and a restriction enzyme SalI linker sequence on the opposite side, in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$; heating the resultant mixture at 70° C for 10 minutes; and then slowly cooling the mixture to room temperature. Then, cDNA of horse spleen-derived type L ferritin digested a plasmid pKITO which had been cloned into downstream of tac promoter (Okuda et al. 2003, Biotechnology and Bioengineering, Vol84, No. 2, p187-194) with restriction enzymes BamHI and SalI; a large DNA fragment, about 6 kb, separated by 1% agarose gel electrophoresis was purified with Gene Clean II kit (BIO101); the purified substance was mixed with the aforementioned annealed DNA, and bound by using T4 DNA ligase.

[0026] Next, this DNA and pKITO were digested by BamHI respectively and DNA fragments separated by 1% agarose gel electrophoresis, the former was a fragment of about 6 kb, and the latter was a fragment of about 300 bp, were purified with Gene Clean II kit (BIO101), the purified substances were bound by using T4 DNA

ligase. The bound DNA was cloned into E. coli XLI-bluestrain(hsdR17, supE44, recA1, endA1, gyrA46, thi, relA1, lac/F' [proAB+, lacI^q△ (lacZ) M15;;Tn10 (tetR)]) in accordance with an ordinary method (Molecular Cloning Third Edition, Cold Spring Harbor Laboratory Press), and a clone into which a BamHI fragment of about 300 bp was inserted in the desired direction was determined by a dideoxy termination method (CEQ DTCS Quick start kit, Beckman, California), through DNA sequencing with the use of a primer (SEQ ID NO: 24) in a BamHI fragment of about 300 bp from pKITO. For the migration and data analysis of the reactant, an automated capillary sequencer (CEQ2000, Beckman) was used (Fig. 2).

[0027] The fusion ferritin protein wherein a nanographite structure recognition peptide is fused with horsespleen-derived type L ferritin was expressed and purified as follows.

[0028] The E. coli XLI-blue strain was transformed with pKIS2 in accordance with an ordinary method, and a colony was picked up with a sterilized pick and shaking-cultured in 5 ml of LB medium at 37° C for 16 to 18 hours. Then this culture solution was transplanted to 1 litter of LB medium and shaking-culture was conducted at 37° C for another 16 to 18 hours. The E. coli was collected by centrifugation (Beckman J2-21M, JA-14 rotor, 5000 rpm, 5 minutes). The E. coli thus collected was washed with 80 ml of 50 mM Tris-HCl, pH 8.0, and collected by centrifugation (Kubota, 5922, RA410M2 rotor, 4000 rpm, 10 minutes) again. The collected E. coli was suspended in 30 ml of 50 mM Tris-HCl, pH 8.0, and an ultrasonic disruptor (BRANSON, SONIFIER 250, micro tip, output level maximum, duty cycle 50%, 2 minutes; this procedure was repeated 3 to 4 times) was used to obtain a solution of disrupted E. coli cells. The solution of disrupted E. coli cells was subjected to centrifugation (Kubota, 5922, RA410M2 rotor, 8000 rpm, 30 minutes) to collect soluble fractions. By putting the fractions into a warm bath at 65° C for 20 minutes, coexisting proteins were denatured. The denatured coexisting proteins which formed precipitates were removed by centrifugation (Kubota, 5922, RA410M2 rotor, 8000 rpm, 30 minutes), and the supernatant was collected.

[0029] To the collected supernatant, 5 M NaCl was added such that the final concentration was adjusted to 0.5 M, and the resultant mixture was stirred and allowed to stand still at room temperature for 5 to 10 minutes, and then a precipitate was collected by centrifugation (Kubota, 5922, RA410M2 rotor, 5000 rpm, 10 minutes). The precipitate was dissolved in 20 ml of 50 mM Tris-HCl (pH 8.0), and to this mixture, 5 M NaCl was added again such that the final concentration was adjusted to 0.5 M, and the resultant mixture was stirred and allowed to stand still at room temperature for 5 to 10 minutes, and then a precipitate was collected by centrifugation (Kubota, 5922, RA410M2 rotor, 5000 rpm, 10 minutes). The precipitate was further dissolved in 20 ml of 50 mM Tris-HCl (pH 8.0), and to this mixture, 5 M NaCl was added again such that the final concentration was adjusted to 0.375 M this time, and the resultant mixture was stirred and allowed to stand still at room temperature for 5 to 10 minutes, and then a precipitate was collected by centrifugation (Kubota, 5922, RA410M2 rotor, 5000 rpm, 10 minutes). The collected precipitate was dissolved in 10 ml of 50 mM Tris-HCl (pH 8.0).

[0030] In addition, the purification by gel filtration chromatography was conducted as needed. In other words, 200 to 500 μl of the purified preparation mentioned above was poured into an SW4000XL column (TOSOH) equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM NaN3, purification and separation were conducted by chromatography at a flow rate of 1 ml/min, and a fraction corresponding to a ferritin 24-mer was collected (Fig. 3).

Example 2

[0031] It was confirmed by the following procedure that as in the case of recombinant apoferritin, the N1-LF obtained in Example 1 has an ability to form nanoparticles of iron oxide in its interior space.

To a solution comprising 50 mM HEPES-NaOH (pH 7.0) and 0.5 mg/ml N1-LF, 50 mM ammonium iron (II) sulfate hexahydrate was added at an amount, 1/10 of the volume of the solution (final concentration 5 mM), and the resultant mixture was allowed to stand still at room temperature overnight (Fig. 4). Subsequently, a procedure to precipitate excessive iron oxides by centrifugation (Kubota, 5922, RA410M2 rotor, 3000 rpm, 10 minutes) and remove them was repeated twice. Next, by a centrifugal operation with the use of an ultracentrifuge (Beckman, TLA 100.4 rotor, 50,000 rpm, 1 hour), N1-LF was precipitated. This precipitate was dissolved in 50 mM Tris-HCl (pH 8.0), overlaid on an equal amount of 15% sucrose solution, and N1-LF present in sucrose fractions was collected by conducting a centrifugal operation again with the use of the ultracentrifuge (Beckman, TLA 100.4 rotor, 50,000 rpm, 1 hour). With regard to the collected N1-LF, the formation of nanoparticles of iron oxide was confirmed by a transmission electron microscope (JEOL1010, 100 kV, stained with 1% aurothioglucose, Fig. 5). The collected N1-LF was dialyzed against 50 mM Tris-HCl (pH 8.0), then quantitated by BioRad Protein Assay (BioRad), and used for other experiments.

(Comparative example 1)

[0032] With regard to the horse spleen-derived type L ferritin (LF0), a recombinant was used as in the case of Example 1. The recombinant was prepared as follows. The E. coli XLI-blue strain was transformed with pKITO in accordance with an ordinary method, and a colony was picked up with a sterilized pick and shaking-cultured in 5 ml of LB medium at 37° C for 16 to 18 hours. Then this culture solution was transplanted to 1 litter of LB medium and shaking-culture was conducted at 37°C for another 16 to 18 hours. The E. coli was collected by centrifugation

(Beckman J2-21M, JA-14 rotor, 5000 rpm, 5 minutes). The E. coli thus collected was washed with 80 ml of 50 mM Tris-HCl (pH 8.0), and collected by centrifugation (Kubota, 5922, RA410M2 rotor, 4000 rpm, 10 minutes) again. The collected E. coli was suspended in 30 ml of 50 mM Tris-HCl (pH 8.0), and then an ultrasonic disruptor (BRANSON, SONIFIER 250, micro tip, output level maximum, duty cycle 50%, 2 minutes ; this procedure was repeated 3 to 4 times) was used to obtain a solution of disrupted E. coli cells. The solution of disrupted E. coli cells was subjected to centrifugation (Kubota, 5922, RA410M2 rotor, 8000 rpm, 30 minutes) to collect soluble fractions. By putting the fractions into a warm bath at 65°C for 20 minutes, coexisting proteins were denatured. The denatured coexisting proteins which formed precipitates were removed by centrifugation (Kubota, 5922, RA410M2 rotor, 8000 rpm, 30 minutes), and the supernatant was collected.

[0033] The supernatant was poured into Q-sepharose HP (Amersham), which is a carrier for anion exchange chromatography, equilibrated with 50 mM Tris-HCl (pH 8.0), and the elution was conducted with 100 ml of 100 to 500 mM sodium chloride concentration gradient (3 ml/min). About 40 ml of fractions containing LF0 was concentrated by Centriprep 10 (Amicon) to 2.5 to 3 ml, and the resultant was poured into a 60 cm-long gel filtration chromatograph Sephacryl S-400 equilibrated with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl (herein after referred to as TBS), and chromatography was conducted at a flow rate of 1.5 ml/min. Up to 100 μl of each fraction containing LFO was poured into an SW4000XL column equilibrated with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM NaN3, and analyzed by chromatography at a flow rate of 1 ml/min, and a fraction corresponding to a ferritin 24-mer was confirmed and used for the experiment described below.

(Comparative example 2)

[0034] The formation of nanoparticles of iron oxide in the interior space of LFO obtained in Comparative example 1 was conducted in a same procedure as described in Example 2. The formation of nanoparticles was confirmed in a same manner as described in Example 2, as well (Fig. 6).

Example 3

[0035] The following experiment was conducted in order to show that: though the N1-LF (SEQ ID NO: 26) having nanoparticles of iron oxide in its interior space obtained in Example 2 specifically binds to a nanographite structure, the LFO (SEQ ID NO: 25) having nanoparticles of iron oxide in its interior space obtained in Comparative example 2 cannot bind to a nanographite structure.

[0036] High-power CO_2 gas laser beam (output power 100 W, pulse width 20 ms, continuous wave) was emitted over the surface of carbon in a form of a sintered round bar in ambient pressure of 6×10^4 Pa of Ar gas, and the resultant soot-like substance was suspended in ethanol, then ultrasonic agitation (frequency 40 kHz, 60 minutes) and decantation were repeated 4 times to obtain single-wall carbon nanohorns. About 200 mg of the single-wall carbon nanohorns was put into 40 ml of nitric acid at a concentration of about 70%, and reflux was conducted for 1 hour at 130°C. After the reflux, the resultant was neutralized and washed by repeating dilution with ion-exchange water, centrifugation, and disposal of the supernatant, and water-soluble single-wall carbon nanohorns having a functional group (including a carboxyl group) were prepared.

[0037] The carbon nanohorns were dissolved in 0.1% fetal bovine serum albumin, 0.05% polyoxyethylenesorbitan monolaurate [hereinafter referred to as Tween 20 (Sigma, St. Louis)] contained in TBS (hereinafter referred to as TBS-BT) such that the concentration was adjusted to 1 mg/ml. The carbon nanohorns were precipitated by a centrifugal operation (Kubota, 5922, AT-2018M rotor, 15000 rpm, 15 minutes), and the precipitate was suspended in TBS-BT such that the concentration was adjusted to 1 mg/ml. This operation was repeated 3 times , and subsequently the precipitated carbon nanohorns were suspended in TBS-BT containing N1-LF or LFO having 0.1 mg/ml of nanoparticles of iron oxide in its core, such that the concentration was adjusted to 1 mg/ml. The suspension was rotated and stirred for 12 hours at room temperature with a rotator RT-50 manufactured by Taitec. In order to remove ferritin molecules which had not bound, the carbon nanohorns were precipitated by a centrifugal operation (Kubota, 5922, AT-2018M rotor, 15000 rpm, 15 minutes), and the precipitate was washed 5 times with 400 μl of TBS containing 0.05% Tween 20, and then the solution was substituted with sterilized water for the demineralization of the precipitate. Thus treated precipitate was observed under a transmission electron microscope (TOPCON EM-002B, accelerating voltage 120 kV), and it was observed that a plurality of nanoparticles of iron oxide were supported on a carbon nanohon when N1-LF was mixed with carbon nanohorns, on the other hand in case of LF0, nanoparticles of iron oxide were not observed on carbon nanohorns. Based on the observation, it was confirmed that N1-LF has an ability to specifically bind to carbon nanohorns, and that the method for supporting nanoparticles on nanographite structures utilizing this ability is effective (Fig. 7).

Example 4

[0038] Hipco (Carbon Nanotechnologies Inc., Texas), a single-wall carbon nanotube synthesized by chemical vapor deposition, was treated with 1×10^{-5} Torr for 5 hours at 1750° C, and then reflux was conducted for 30 minutes at about 130° C in nitric acid at a concentration of about 70%. After that, neutralization with sodium hydroxide and washing with distilled water were conducted,

and single-wall carbon nanotubes having a functional group (including a carboxyl group) were prepared.

[0039] The single-wall carbon nanotubes were dissolved in TBS-BT in a same manner as described in Example 3. The single-wall carbon nanotubes were precipitated by a centrifugal operation (Kubota, 5922, AT-2018M rotor, 15000 rpm, 15 minutes), and the resultant precipitate was suspended in TBS-BT again. This operationwas repeated 3 times, and subsequently the precipitated single-wall carbon nanotubes were suspended, in a same manner as described in Example 3, in TBS-BT containing N1-LF having nanoparticles of iron oxide in its core. The suspension was rotated and stirred for 12 hours at room temperature with a rotator RT-50 manufactured by Taitec. In order to remove ferritin molecules which had not bound, the single-wall carbon nanotubes were precipitated by a centrifugal operation (Kubota, 5922, AT-2018M rotor, 15000 rpm, 15 minutes), and the precipitate was washed 5 times with TBS containing 0.05% Tween 20, and then the solution was substituted with sterilized water for the demineralization of the precipitate. Thus treated precipitate was observed under a transmission electron microscope (TOPCON EM-002B, 120 kV), and it was confirmed that a plurality of nanoparticles of iron oxide was supported on a carbon nanohorn when N1-LF was mixed with single-wall carbon nanotubes (Fig. 8).

Claims

1. A nanographite structure/metal nanoparticle composite, wherein a nanoparticle of an inorganic metal atom or an inorganic metal compound is retained in an interior space of a protein in which a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein, and wherein a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound are supported on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure.

2. The nanographite structure/metal nanoparticle composite according to claim 1, wherein the cage protein belongs to a ferritin protein family.

3. The nanographite structure/metal nanoparticle composite according to claim 2, wherein the ferritin protein family is ferritin.

4. The nanographite structure/metal nanoparticle composite according to claim 3, wherein the ferritin is higher eucaryote-derived ferritin.

5. The nanographite structure/metal nanoparticle composite according to claim 4, wherein the higher eucaryote-derived ferritin is horse spleen-derived type L ferritin.

6. The nanographite structure/metal nanoparticle composite according to claim 1, wherein the cage protein is derived from a bacterium.

7. The nanographite structure/metal nanoparticle composite according to claim 1, wherein the cage protein is a viral particle.

8. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 7, wherein the nanographite structure recognition peptide is a peptide consisting of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20.

9. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 7, wherein the nanographite structure recognition peptide is a peptide containing whole or part of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 and capable of binding to a nanographite structure.

10. The nanographite structure/metal nanoparticle composite according to claim 8 or 9, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is DYFSSPYYEQLF (SEQ ID NO: 1).

11. The nanographite structure/metal nanoparticle composite according to claim 8 or 9, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is YDPFHII (SEQ ID NO: 2).

12. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 11, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal nanoparticle.

13. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 11, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal compound nanoparticle.

14. The nanographite structure/metal nanoparticle composite according to claim 13, wherein the metal compound nanoparticle is a metal oxide nanoparticle.

15. The nanographite structure/metal nanoparticle composite according to claim 13, wherein the metal compound nanoparticle is a magnetic material nanoparticle.

16. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 15, wherein the metal is iron, beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, zinc, cad-

mium or chromium.

17. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 11, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a nanoparticle of iron oxide, a nanoparticle of cadmium selenide, a nanoparticle of zinc selenide, a nanoparticle of zinc sulfide, or a nanoparticle of cadmium sulfide.

18. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 17, wherein the nanographite structure is a carbon nanotube or a carbon nanohorn.

19. The nanographite structure/metal nanoparticle composite according to claim 18 , wherein the carbon nanotube or the carbon nanohorn is constituted of a carbon structure to which a functional group is added.

20. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 19 , wherein the nanographite structure is two-dimensionally aligned on a substrate.

21. The nanographite structure/metal nanoparticle composite according to any one of claims 1 to 19, wherein the metal nanoparticle is two-dimensionally aligned on a substrate.

22. The nanographite structure/metal nanoparticle composite according to claim 20, wherein the cage protein is removed.

23. A protein wherein a nanographite structure recognition peptide is fused or chemically bound to a surface of a cage protein .

24. The protein according to claim 23, wherein the cage protein belongs to a ferritin protein family.

25. The protein according to claim 24, wherein the ferritin protein family is ferritin.

26. The protein according to claim 25, wherein the ferritin is higher eucaryote-derived ferritin.

27. The protein according to claim 26, wherein the higher eucaryote-derived ferritin is horse spleen-derived type L ferritin.

28. The protein according to claim 24, wherein the ferritin protein family is derived from a bacterium.

29. The protein according to claim 23, wherein the cage protein is a viral particle.

30. The protein according to any one of claims 23 to 29, wherein the nanographite structure recognition peptide is a peptide consisting of an amino acid sequence shown by any one of SEQ ID NOs: 1 to 20.

31. The protein according to any one of claims 23 to 29, wherein the nanographite structure recognition peptide is a peptide containing whole or part of an amino acid sequence shown by any one of SEQ ID NOs : 1 to 20 and capable of binding to a nanographite structure.

32. The protein according to claim 30 or 31, wherein the amino acid sequence shown by any one of SEQ ID NOs : 1 to 20 is DYFSSPYYEQLF (SEQ ID NO: 1).

33. The protein according to claim 30 or 31, wherein the amino acid sequence shown by any one of SEQ ID NOs: 1 to 20 is YDPFHII (SEQ ID NO: 2).

34. The protein according to any one of claims 23 to 33, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal nanoparticle.

35. The protein according to any one of claims 23 to 33, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a metal compound nanoparticle.

36. The protein according to claim 35, wherein the metal compound nanoparticle is a metal oxide nanoparticle.

37. The protein according to claim 35, wherein the metal compound nanoparticle is a magnetic material nanoparticle.

38. The protein according to any one of claims 22 to 36, wherein the metal is iron, beryllium, gallium, manganese, phosphorus, uranium, lead, cobalt, nickel, zinc, cadmium or chromium.

39. The protein according to claim 23, wherein the nanoparticle of an inorganic metal atom or an inorganic metal compound is a nanoparticle of iron oxide, a nanoparticle of cadmium selenide, a nanoparticle of zinc selenide, a nanoparticle of zinc sulfide, or a nanoparticle of cadmium sulfide.

40. The protein according to any one of claims 23 to 39, wherein the nanographite structure is a carbon nanotube or a carbon nanohorn.

41. The protein according to claim 40, wherein the carbon nanotube or the carbon nanohorn is constituted of a carbon structure to which a functional group is added.

42. A method for retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of claims 23 to 41, and supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure.

43. A method for producing a composite of a nanographite structure and nanoparticles of an inorganic metal compound, comprising the steps of: retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of claims 23 to 41; supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; and removing a protein moiety by a heat treatment.

44. A method for producing a composite of a nanographite structure and nanoparticles of an inorganic metal compound, comprising the steps of: retaining a nanoparticle of an inorganic metal atom or an inorganic metal compound in an interior space of the protein according to any one of claims 23 to 41; supporting a plurality of nanoparticles of an inorganic metal atom or an inorganic metal compound on a nanographite structure with the use of affinity of the nanographite structure recognition peptide to the nanographite structure; and removing a protein moiety by an electron beam treatment.

45. A method for aligning a nanographite structure by binding the nanographite structure to the protein according to any one of claims 23 to 41 which has formed a two-dimensional crystal.

46. A method for aligning a nanographite structure by binding the nanographite structure to the protein according to any one of claims 23 to 41 which has formed a two-dimensional array.

Fig. 1

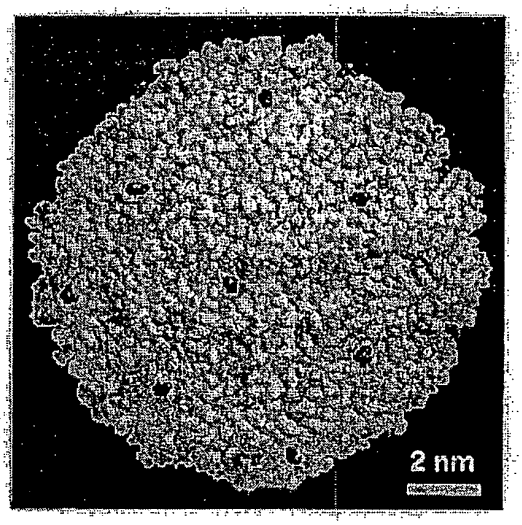
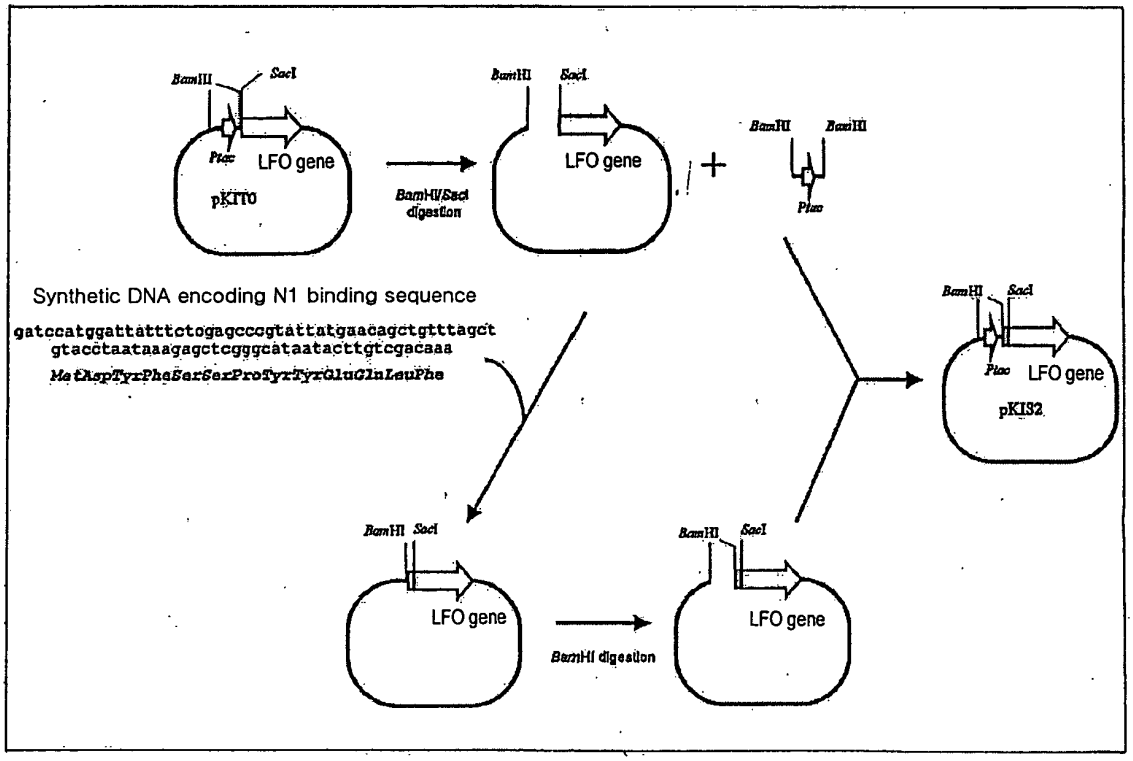

2 nm

Fig. 2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SacI
Ptac
LFO gene
pKIT0
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LFO gene
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Ptac
Synthetic DNA encoding N1 binding sequence
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MetAspTyrPheSerSerProTyrTyrGluGlnLeuPhe
BamHI
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BamHI digestion

Fig. 3

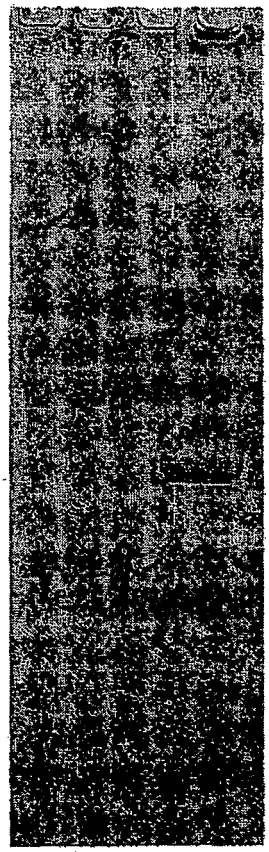


Fig. 4

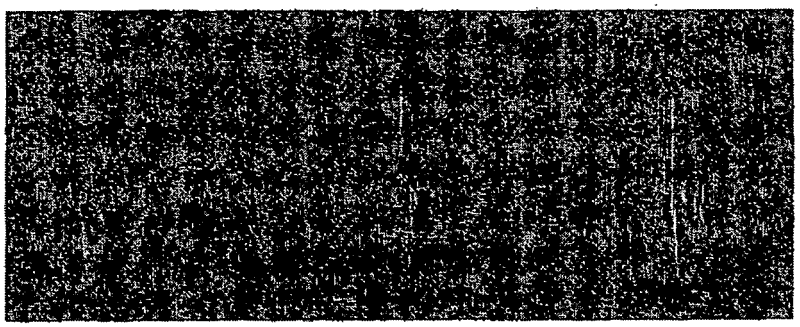


Fig. 5

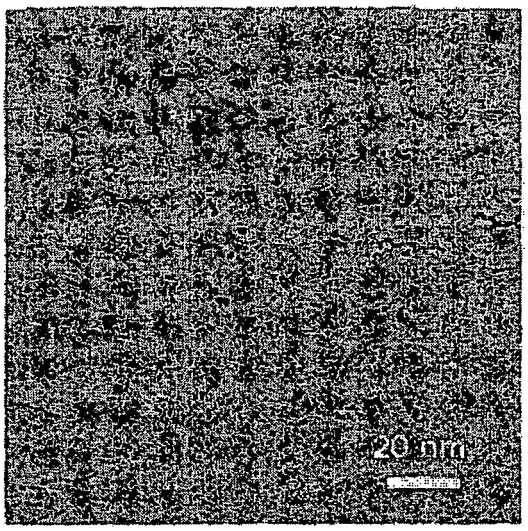


Fig. 6

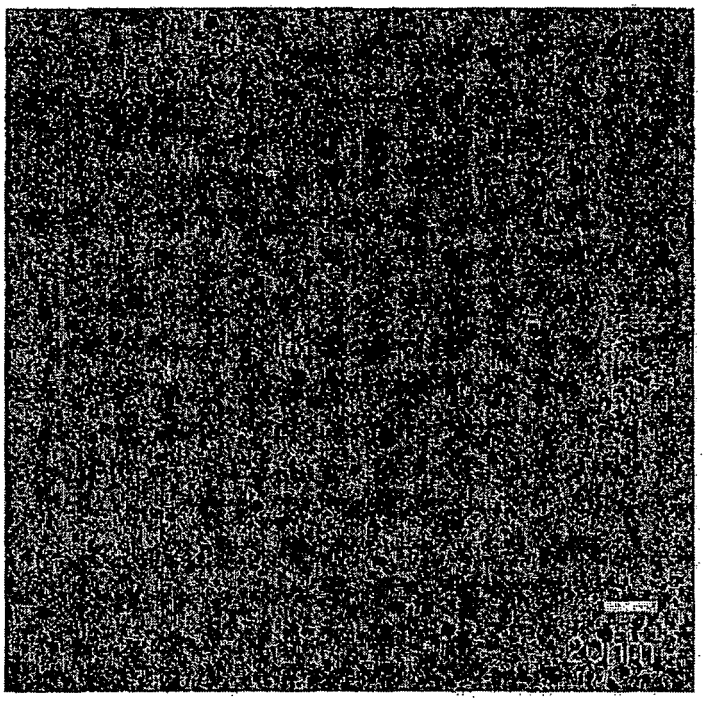


Fig. 7

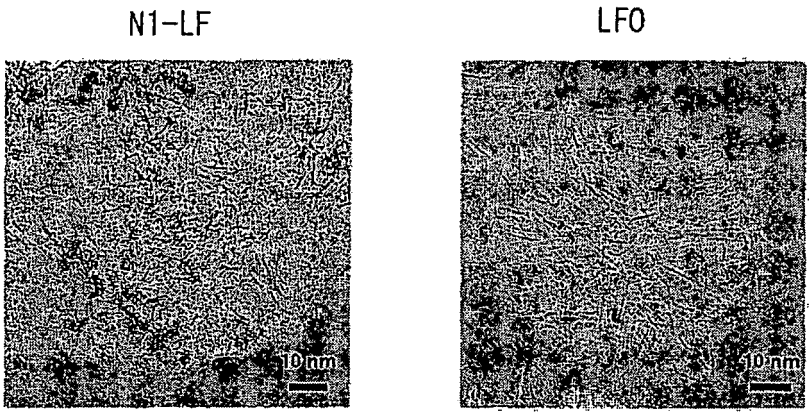
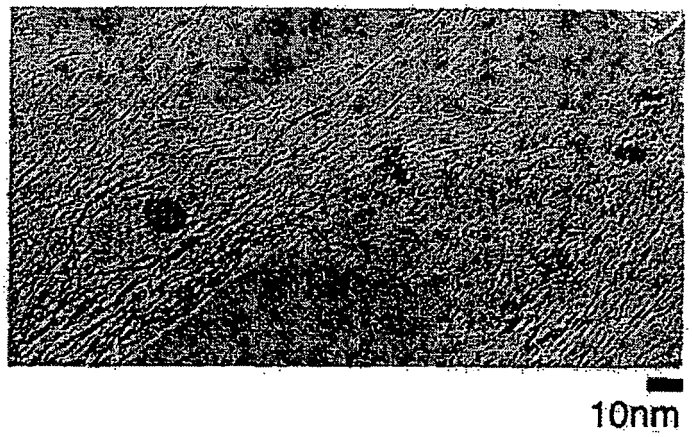


Fig. 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2005/023675

A. CLASSIFICATION OF SUBJECT MATTER
C01B31/02(2006.01), ***C07K7/06***(2006.01), ***C07K7/08***(2006.01), ***C07K14/195***(2006.01), ***C07K14/47***(2006.01), ***C12N15/00***(2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C01B31/00-31/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2006
Kokai Jitsuyo Shinan Koho 1971-2006 Toroku Jitsuyo Shinan Koho 1994-2006

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus(JOIS)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 2004-121154 A (Japanese Foundation For Cancer Research), 22 April, 2004 (22.04.04), Claims; Par. No. [0017] & WO 2004/031381 A1 & US 2005/277160 A1	1-46
A	JP 2001-181842 A (Matsushita Electric Industrial Co., Ltd.), 03 July, 2001 (03.07.01), Claims (Family: none)	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 March, 2006 (02.03.06)	14 March, 2006 (14.03.06)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (April 2005)

REFERENCES CITED IN THE DESCRIPTION

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- *Chem. Phys. Lett.,* 1999, vol. 309, 165-170 **[0007]**
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- **OKUDA et al.** *Biotechnology and Bioengineering,* 2003, vol. 84 (2), 187-194 **[0025]**
- Molecular Cloning. Cold Spring Harbor Laboratory Press **[0026]**