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(71) Applicants: INSTITUT NATIONAL DE LA SANTE

ET DE LA RECHERCHE MEDICALE (INSERM)

[FR/FR]; 101, Rue de Tolbiac, 75013 PARIS (FR).

UNIVERSITE DE RENNES 1 [FR/FR]; 2 rue du Thabor

CS 46510, 35065 RENNES CEDEX (FR). UNIVERSITE

DE POITIERS [FR/FR]; 15, rue de l'Hôtel-Dieu,

86034 POITIERS CEDEX (FR). EHESP (ECOLE DES

HAUTES ÉTUDES EN SANTÉ PUBLIQUE) [FR/FR];

Avenue du Professeur Leon Bernard, 35000 RENNES

(FR). CENTRE HOSPITALIER UNIVERSITAIRE

DE POITIERS [FR/FR]; 2, rue de la Milétrie, 86000

POITIERS (FR). CENTRE HOSPITALIER UNIVERSI-

TAIRE DE RENNES [FR/FR]; Hôpital Ponchaillou 2, rue

Henri le Guilloux, 35033 RENNES CEDEX (FR).

(72) Inventors: YEFIMOVA, Marina; IRSET INSERM

U1085 CHU de Rennes 16 Bd de bulgarie, 35000 RENNES

(FR). BOURMEYSTER, Nicolas; STIM, ERL 7003 CN-

RS Pôle Biologie Santé 1 rue Georges BONNET, 86022

POITIERS cedex (FR). BECQ, Frédéric; Pôle biologie

santé, 1 rue Georges Bonnet TSA 51106, 86073 POITIERS

Cedex 9 (FR). RAVEL, Célia; CHU de Rennes 16 Bd de

Bulgarie, 35000 RENNES (FR). JEGOU, Bernard; IRSET

- INSERM U1085 9 Av. du Prof Léon Bernard, 35000 REN-

NES (FR).

(74) Agent: BLOT, Philippe et al.; 2, place d'Estienne d'Orves,

75441 PARIS CEDEX 09 (FR).

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(54) Title: MYELINOSOME AS A VECTOR FOR INTRACELLULAR DELIVERY OF MOLECULES OF INTEREST

(57) Abstract: The present invention relates to a new vector, the myelinosome, for intracellular delivery of molecules of interest, such as nucleic acids, proteins or chemical molecules. The Inventors for example showed that myelinosomes loaded with a protein or nucleic acid of interest are efficiently delivered into target cells. Besides, treatment of cells with myelinosomes carrying a functional protein (for example wild-type CFTR protein) rescued the phenotype of diseased cells carrying a nonfunctional protein (for example human cystic fibrosis bronchoepithelial cells carrying CFTR mutation). The present invention thus particularly relates to the use of myelinosomes for delivering at least one molecule of interest *in vitro* or *in vivo*, for example for the prevention, treatment and/or diagnostic of a disease. The present invention also relates to a method for producing myelinosomes, compositions suitable for *in vitro* use comprising said myelinosomes and pharmaceutical compositions comprising said myelinosomes. Figure: none



MYELINOSOME AS A VECTOR FOR INTRACELLULAR DELIVERY OF
MOLECULES OF INTEREST**Filed of the invention**

The present invention concerns vectors for intracellular delivery of molecules.

Technical background

Vectors for gene delivery are now essential tools, both for *in vitro* and *in vivo* applications. Such vectors for example include non-viral vectors (for example inorganic particles, cationic lipids, lipid nano-emulsions, solid lipid nanoparticles, peptide based vectors or polymer-based vectors), viral vectors and virus-like particles (VLP).

Gene therapy constitutes a promising treatment not only for genetic diseases, but also for cancer, inflammatory diseases and infectious diseases. However, gene therapy still presents safety issues, especially when using viral vectors. Efficient and safer vectors are thus still needed.

Vectors have been developed not only for gene therapy, but for the delivery of any kind of molecules, for example molecules useful for therapy and/or diagnostic applications. Such vectors may be used for a targeted delivery of a molecule, controlled and/or sustained release, increasing stability and/or bioavailability, etc..

Yefimova et al. (2016, Hum Mol Genet) disclosed the existence of organelles called myelinosomes that mediate elimination of mutant misfolded proteins in an unusual secretory process, thereby avoiding aggregation of these misfolded proteins in the cells. For example, myelinosomes secreted from testis somatic TM4 Sertoli cells provide the release of aggregate-prone Huntingtin (Htt) mutant involved in Huntington's disease (HD). Similarly, myelinosomes support the release of aggregate-prone F508delCFTR mutant protein responsible for cystic fibrosis (CF).

There is still a need for alternative vectors for intracellular delivery of molecules of interest, such as nucleic acids, proteins and/or chemical molecules, which vectors preferably present no or little toxicity and side effects.

Detailed description

The Inventors have surprisingly shown that molecules of interest, such as proteins or DNA and RNA molecules can be efficiently delivered in target cells using myelinosomes as a vector. In addition, the Inventors have shown that a DNA nucleic acid encoding a protein of interest delivered in targets cells using myelinosomes as a vector leads to the expression of the protein in the target cells. They also showed that treatment of cells with

myelinosomes carrying a functional protein (*for example wild-type CFTR protein*) rescued the phenotype of diseased cells carrying a nonfunctional protein (*for example human cystic fibrosis bronchoepithelial cells carrying CFTR mutation*).

Myelinosomes thus constitute a new kind of vectors for the intracellular delivery of molecules of interest.

Myelinosomes present the advantage of being natural vectors, thereby being intrinsically devoided of any toxicity or side effects.

Myelinosomes have unique properties, such as being able to deliver different kinds of molecules, such as DNA, RNA, proteins and chemical molecules, potentially in any kind of eukaryotic cell.

Advantageously, the molecules of interest can be efficiently loaded into the myelinosomes, for example simply by contacting said molecules with the myelinosomes.

A first object of the invention relates to the use of myelinosome as a vector for intracellular delivery *in vitro*, in particular for intracellular delivery of at least one molecule of interest.

The molecule of interest may be selected from the group consisting of a nucleic acid, a protein and a chemical molecule.

Another object of the invention is a myelinosome for use as a vector for delivering at least one molecule of interest in a subject, said molecule of interest being for example selected from the group consisting of a nucleic acid, a protein and a chemical molecule.

The invention particularly relates to a myelinosome for use as defined above as a vector for delivering at least one molecule of interest in a subject, for the prevention, treatment and/or diagnostic of a disease.

The myelinosome may be administered by the arterial, venous, intra-tissue, intracerebrospinal, intracerebral, intraventricular, intranasal, intrathecal, inhalation and/or intraperitoneal route.

The myelinosome may be used for delivering a functional CFTR protein and/or a nucleic acid encoding thereof into the cells, in particular the lung cells, of a patient suffering from Cystic Fibrosis or for delivering a functional Huntingtin protein and/or a nucleic acid encoding thereof in the cells, in particular the brain cells (for example after intracranial delivery, such as by administration via the intraventricular, intracerebral, intranasal and/or intrathecal route) of a patient suffering from Huntington Disease.

Another object of the invention is a method for producing myelinosomes, wherein said method comprises:

- a) culturing cells in a culture medium in pathological and/or stressful condition, for a time sufficient to induce the formation and secretion of myelinosomes,
- b) collecting the culture medium,
- c) removing cell debris from the culture medium collected in step b), to obtain a supernatant comprising myelinosomes,
- d) removing liquid fraction from the supernatant obtained in step c), to obtain a pellet comprising the myelinosomes, and
- e) optionally, separating myelinosomes from exosomes in the pellet obtained in step d), to obtain purified myelinosomes.

wherein said pathologic and/or stressful condition is preferably not the presence of a misfolded protein in the cells and wherein step a) preferably does not comprise transfecting the cells with a nucleic acid encoding a protein comprising (i) EGFP and (ii) a functional CFTR protein or functionall Htt protein.

The pathologic and/or stressful condition may be selected from the group consisting of the transfection of the cells with at least one nucleic acid, the overexpression of a protein, the presence of a chemical compound inducing the formation of myelinosomes (such as quinacrine), hypoxia, heat shock treatment and their combinations .

In the method as defined above, the cells in step a) may express at least one protein of interest.

In one embodiment, step a) as defined above may comprise:

- i. transfecting the cells with a nucleic acid encoding a protein of interest, to obtain transfected cells, and
- ii. culturing the transfected cells in a culture medium for a time sufficient to express the protein and induce the formation of myelinosomes.

The cells in step a) are for example eukaryotic cells, such as cells selected from the group consisting of TM4 cells, MIO-M1 cells, SH-SY5Y cells, COS cells and CFBE cells.

In the method as defined above, step c) may comprise centrifuging the culture medium at from 500 g to 700 g, for example at 600 g, preferably from 10 to 20 minutes, preferably 10 minutes, and/or step d) may comprise centrifuging the supernatant at from 16 000 g to 20 000 g, for example at 20 000 g, for example from 60 to 90 minutes, preferably 90 minutes.

The method as defined above may further comprise a step f) of loading the myelinosomes with at least one molecule of interest.

The myelinosome obtained by the method as defined above may be used as a vector for intracellular delivery as defined above, for example *in vitro* or *in vivo*.

Another object of the invention is a modified myelinosome, preferably produced *in vitro*, for example by a method as defined above, wherein said myelinosome preferably does not comprise a protein comprising a mutant Htt protein, nor a protein comprising a mutant CFTR protein, nor a protein comprising EGFP and a functional Htt protein, nor a protein comprising EGFP and a functional CFTR protein. Said myelinosome may further comprise at least one molecule of interest as defined above. Said myelinosome may be in a frozen form.

Another object of the invention is a composition comprising (i) myelinosomes and (ii) a culture medium or buffer, wherein said myelinosomes preferably do not comprise a protein comprising a mutant Htt protein, nor a protein comprising a mutant CFTR protein, nor a protein comprising EGFP and a functional Htt protein, nor a protein comprising EGFP and a functional CFTR protein.

Another object of the invention is a pharmaceutical composition comprising myelinosomes and a pharmaceutically available vehicle.

Myelinosome

By the term “myelinosome”, it is herein meant a secretory organelle of nanometric scale (in particular from 200 nm to 700 nm) produced by a cell in a pathological and/or stressful condition, for example caused by genetic and/or environmental factors, which is then secreted by the cell.

Non-limitative examples of a pathological and/or stressful condition is the presence of a misfolded protein in a cell, the transfection of the cell with a nucleic acid, the presence of a chemical compound inducing the formation of myelinosomes in the cell (such as quinacrine), the seasonal regression of the testis for the testis cells.

Myelinosomes are characterized by markedly electron-dense osmiophile membranes and a stacked, reticulated or whorled arrangement, as can be observed by electronic microscopy.

The myelinosome may comprise up to 30 membrane layers. This number varies depending on the cell type. For example, a myelinosome produced by retinal glial Muller cells is rich of membrane layers.

The diameter of the central cavity of myelinosome generally varies from 100 nm to 500 nm. It does not depend on the number of membrane layers. Large myelinosomes can

indeed contain about 10 membrane layers, for example the myelinosomes produced by TM4 cells expressing mutant CFTR.

Some myelinosomes present a complex structure which seems to be resulted from the agglomeration of few myelinosomes.

5 Myelinosomes are different from exosomes and microvesicles, both structurally and functionally.

Exosomes and microvesicles for example do not have markedly electron-dense osmiophile membranes and a stacked, reticulated or whorled arrangement, contrary to myelinosomes.

10 Myelinosomes for example differ from exosomes by their size. The size of myelinosomes is generally from 200 nm to 700 nm, whereas exosomes have generally a size from 30 nm to 100 nm.

Myelinosomes also differ from exosomes and microvesicles by the presence of an endoplasmic-reticulum marker, the Calnexin protein.

15 Myelinosomes further differ from exosomes and microvesicles by the absence of CD63 marker.

As regard to their function, the Inventors have surprisingly shown that phagocytic cells have very limited capacity to ingest myelinosomes vesicles, unlike exosomes and microvesicles which are very easily ingested and degraded by the phagocytic system (*see Example 7*). Besides, unlike exosomes and microvesicles, myelinosomes are able to cross specific blood barriers, such as blood-retinal barrier (*see Example 6*).

20 The myelinosomes for use according to the invention are preferably produced *in vitro*, for example by (i) culturing cells in a pathological and/or stressful condition, in particular for a time sufficient to induce the formation and secretion of myelinosomes, said pathological and/or stressful condition being for example as defined above, and then (ii) purifying the myelinosomes.

A time sufficient to induce the formation and secretion of myelinosomes is for example at least 24h, preferably at least 48h, for example between 24h and 96h.

30 The myelinosome is preferably an isolated myelinosome, also referred to as a purified myelinosome.

The term "isolated" or "purified" in reference to a myelinosome herein refers to a myelinosome that has been substantially separated or purified away from the cells from which it originates and from the components of the medium wherein it has been secreted by the cell (said components including cell debris, exosomes and membrane-budding vesicles).

35

The myelinosome is preferably a modified myelinosome, i.e. a myelinosome modified so as to comprise at least one molecule of interest, preferably selected from the group consisting of a protein, nucleic acid and a chemical molecule.

The molecule of interest may be as defined below in the section of the same name.

5 In one embodiment, the molecule of interest may be loaded into the myelinosome produced *in vitro* by contacting said myelinosome with said molecule of interest. Loading of the molecule of interest into myelinosomes produced *in vitro* may thus be very simple and for example does not need to use specific conditions, such as for example electroporation.

10 In one embodiment, the myelinosome comprising a protein of interest as defined above may be obtained by transfecting cells with at least one nucleic acid encoding said protein of interest, culturing the cells, in particular for a time sufficient to express the protein of interest and induce the formation and secretion of myelinosomes, and purifying the myelinosomes.

15 The time sufficient to induce the expression of the protein and the formation and secretion of myelinosomes is for example at least 24h, preferably at least 48h, for example between 24h and 96h.

The myelinosome may be in a semi-dried form (for example the pellet obtained after centrifugation) or in a liquid form (for example a suspension).

20 The myelinosome in a semi-dried form may be frozen, for example at -20°C or below, for example -80°C, preferably without adding any culture medium.

The myelinosome in a liquid form may be provided in the form of a suspension of myelinosomes in a suitable liquid medium, such as for example a culture medium or a pharmaceutically acceptable vehicle.

25 By "culture medium", it is herein meant a cell culture medium.

Suitable cell culture media are well known by the skilled person.

The cell culture medium is for example DMEM medium (*Dulbecco's Modified Eagle Medium*), or DMEM/F12 medium (*Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12*).

30 The cell culture medium as defined above may be supplemented with FCS (Fetal Calf Serum) and/or antibiotics (such as gentamycin, penicillin and/or streptomycin) and/or antifungal (such as fungizone).

The pharmaceutically acceptable vehicle is for example as defined below.

35 The myelinosome in liquid form or in semi-dried form may be stored at 4°C or below, for example at -20°C or -80°C.

The myelinosome preferably does not comprise a protein comprising a mutant Htt protein, nor a protein comprising a mutant CFTR protein, nor a protein comprising EGFP and a functional Htt protein, nor a protein comprising EGFP and a functional CFTR protein.

5

The myelinosome is preferably obtained *in vitro*, for example by a method as defined below in the section "Method for producing myelinosomes".

The myelinosome may be provided in the form of a composition as defined below.

10 Molecule of interest

The molecule of interest is a molecule to be delivered in a cell, for example *in vitro* and/or *in vivo*.

The molecule of interest is preferably not a molecule present in naturally occurring myelinosomes.

15 In particular for *in vivo* use, the molecule of interest may be a therapeutic or diagnostic molecule.

The molecule of interest is preferably selected from the group consisting of a protein, a nucleic acid or a chemical molecule.

20 The protein of interest may for example be a therapeutic protein, a diagnostic protein, a protein whose effect and/or function is to be assessed and/or a protein useful for research or clinical purposes.

The protein of interest may for example be a cell surface receptor or channel, antibody, antibody fragment, cytokine, a protein presenting a particular intracellular localization (for example ER, mitochondria or nucleus) or any functional protein.

25 By "functional protein", it is herein meant a protein having the same activity as or a similar activity to a wild-type non mutated protein.

The functional protein may thus be a protein whose nonfunctional forms are involved in a genetic disease.

30 The functional protein may for example be a functional Huntingtin (Htt) protein or a functional CFTR (*Cystic Fibrosis Transmembrane conductance Regulator*) protein.

In one embodiment, the molecule of interest is not a misfolded mutant Htt protein, nor a misfolded mutant CFTR protein.

35 In one embodiment, the molecule of interest is not a protein comprising (i) EGFP and (ii) a protein selected from the group consisting of a functional Htt protein, mutant misfolded Htt protein, a functional CFTR protein and F508delCFTR protein.

By “misfolded protein”, it is herein meant a protein having an abnormal structure resulting in the deposition of insoluble aggregates.

In one embodiment, the molecule of interest is not a protein comprising EGFP.

An example of misfolded mutant Htt protein is the Htt protein encoded by a Htt gene carrying an expansion of CAG triplet.

An example of misfolded mutant CFTR protein is the F508delCFTR protein having a deletion of phenylalanine in the position 508 of the amino acid sequence of wild-type CFTR.

More generally, the molecule of interest is preferably not a misfolded protein involved in a disease, such as Huntington disease or the cystic fibrosis.

The nucleic acid of interest may be a single or double stranded DNA or RNA molecule.

The nucleic acid of interest may for example encode a protein of interest, for example as defined above, or a shRNA, siRNA or miRNA.

The nucleic acid may be provided in the form of a vector comprising a nucleic acid encoding a protein of interest.

Alternatively, the nucleic acid of interest may for example be an antisense oligonucleotide, a siRNA, a shRNA (short hairpin RNA), a miRNA (MicroRNA), a dsRNA (double stranded RNA) or a RNA species that can be cleaved *in vivo* to form a siRNA.

The chemical molecule of interest may comprise or consist of a therapeutic molecule or a diagnostic molecule.

The therapeutic molecule may for example be an anti-cancer molecule, anti-inflammatory molecule, antibiotic, antifungal, analgesic, antipyretic and/or psychotropic.

The diagnostic molecule may comprise or consist of an imaging marker.

Composition comprising myelinosomes

The present invention also relates to a composition comprising myelinosomes, in particular myelinosomes as defined above.

The myelinosomes are preferably purified and/or modified myelinosomes, in particular comprising at least one molecule of interest.

The composition comprising myelinosomes may be a composition suitable for an *in vitro* use or suitable for an *in vivo* use.

The composition preferably comprises the myelinosomes in an amount effective to achieve the intended purpose.

The composition is preferably formulated as liquid, in particular as a suspension, or as an aerosol.

A composition suitable for an *in vitro* use for example comprises myelinosomes and a culture medium or a buffer. In one embodiment, the myelinosomes of said composition do not comprise a protein comprising a mutant Htt protein, nor a protein comprising a mutant CFTR protein, nor a protein comprising EGFP and a functional Htt protein, nor a protein comprising EGFP and a functional CFTR protein. In one embodiment, the myelinosomes of said composition do not comprise any mutant misfolded protein.

The culture medium is particularly as defined above.

The buffer may for example PBS (Phosphate Buffered Saline), Hepes, Tris, MOPS (3-(N-morpholino)propanesulfonic acid), for example at a pH between 6.8 and 7.6.

In one embodiment, the buffer is not PBS.

The amount of myelinosomes to be used in the composition suitable for an *in vitro* use depends, for example, on the protein of interest, the quantity of protein of interest loaded in the myelinosome and/or on the target cells.

A composition suitable for an *in vivo* use may be a pharmaceutical composition.

The present invention thus also relates to a pharmaceutical composition comprising myelinosomes and a pharmaceutically available vehicle.

The expression "pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the myelinosomes and of the molecule of interest, and that is preferably not toxic to the host to which is administered.

Pharmaceutically acceptable vehicles are well known by those skilled in the art.

The pharmaceutically available vehicle may for example be PBS (Phosphate Buffered Saline), Hepes, Tris, MOPS (3-(N-morpholino)propanesulfonic acid), for example at a pH between 6.8 and 7.6.

In one embodiment, the pharmaceutically acceptable vehicle is not PBS.

The myelinosomes of the pharmaceutical composition preferably comprise at least one molecule of interest, such as a therapeutic molecule or a molecule for diagnostic, for example a molecule of interest as defined above.

The amount of myelinosomes to be used in the pharmaceutical composition depends, for example, on the protein of interest, the quantity of protein of interest loaded in the myelinosome, the condition of the mammal intended for administration (e.g. weight, age, sex, health, concurrent treatment, if any, and frequency of treatment), the disease to

be diagnosed, prevented and/or treated, the mode of administration and/or the type of formulation.

Method for producing myelinosomes

5 The present invention also relates to a method for producing myelinosomes, wherein said method comprises:

- a) culturing cells in a culture medium in pathological and/or stressful condition, for a time sufficient to induce the formation and secretion of myelinosomes,
- b) collecting the culture medium,
- 10 c) removing cell debris from the culture medium, to obtain a supernatant comprising myelinosomes,
- d) removing liquid fraction from the supernatant obtained in step d), to obtain a pellet comprising the myelinosomes, and
- e) optionally, separating myelinosomes from exosomes in the pellet obtained
15 in step d), to obtain purified myelinosomes.

Step a)

20 The method comprises a step a) of culturing cells in a culture medium in pathological and/or stressful condition, for a time sufficient to induce the formation and secretion of myelinosomes.

The cells may be any eukaryotic cells, such as animal cells or plant cells.

The cells may also be prokaryotic cells.

The cells are preferably animal cells, more preferably mammalian cells.

25 The cells may for example be human cells, murine cell, Chinese Hamster cell, or monkey cells.

The cells may originate from a biological sample of a subject or from a cell line.

The culture is preferably carried out at a temperature suitable to the cells, for example at 37°C.

30 The culture medium is preferably a cell culture medium suitable to growth of the cells.

The culture medium may be as defined above.

The cells may be cells selected from the group consisting of TM4 cells, MIO-M1 cells, SH-SY5Y cells, COS cells and CFBE cells.

35 The time sufficient to induce the formation and secretion of myelinosomes may be determined by electronic microscopy in a sample of the culture medium.

A time sufficient to induce the formation and secretion of myelinosomes is for example at least 24h, preferably at least 48h, for example between 24h and 96h.

The pathologic and/or stressful condition may be selected from the group consisting of the transfection of the cells with at least one nucleic acid, the overexpression of a protein, the presence of a chemical compound inducing the formation of myelinosomes, hypoxia, heat shock treatment and their combinations.

By the expression "heat shock treatment", it is herein meant culturing the cells at a temperature greater than the optimal temperature range of function of these cells.

By the term "hypoxia", it is herein meant culturing the cells in conditions of hypoxia, such as for example hypoxia induced chemically, for example by adding a CoCl₂ solution, or hypoxia induced by reducing or removing oxygen supply.

When the pathologic and/or stressful condition is a chemical compound inducing the formation of myelinosomes, said compound is for example added in the culture medium.

The chemical compound inducing the formation of myelinosomes may be quinacrine, for example used at a concentration from 4 μ M to 400 μ M.

The pathologic and/or stressful condition is preferably not the presence of a mutant Htt protein or a mutant CFTR protein, or more generally is not the presence of a misfolded protein in the cells.

Step a) preferably does not comprise transfecting the cells with a nucleic acid encoding a protein comprising (i) EGFP and (ii) a functional CFTR protein or functional Htt protein

In one embodiment, the cells of step a) may express at least one protein of interest. Advantageously, said protein of interest is then found in the secreted myelinosomes.

For example, step a) may comprise:

- i. transfecting the cells with a nucleic acid encoding a protein of interest, to obtain transfected cells, and
- ii. culturing the transfected cells in the culture medium for a time sufficient to express the protein and induce the formation and secretion of myelinosomes.

Interestingly, the myelinosomes produced at the end of step ii) comprise the molecule of interest. Without wishing to be bound by theory, it is believed that the stressful condition due to the transfection and overexpression of the protein of interest in a cell results in the formation of myelinosomes, in order to remove the proteins of interest whose correct folding cannot be assessed by the cell because of their abundance.

Transfection in step i. thus allows both being in a pathological and/or stressful condition and the expression of the protein of interest by the cells.

Transfection of the cells may be performed by any method well known by the skilled person, such as for example electroporation, cationic lipid transfection or Ca/Phosphate precipitation.

The nucleic acid and the protein of interest are for example as defined above.

5 The time sufficient to express the protein may be optimized by assessing the expression of the protein at different interval of time post-transfection.

The time sufficient to express the protein and induce the formation and secretion of myelinosomes is for example at least 24h, preferably at least 48h, for example between 24h and 96h.

10

Steps b), c) and d) and optional step e) allow purifying the myelinosomes secreted in the culture medium.

Step b)

The method comprises a step b) of collecting the culture medium.

15

The myelinosomes produced in step a) are indeed secreted by the cells in the culture medium.

The culture medium is thus collected to purify the myelinosomes secreted by the cells.

20

Step c)

The method comprises a step c) of removing cell debris from the culture medium collected in step b), to obtain a supernatant comprising myelinosomes.

Removing cell debris may be performed by any suitable method of separation well known by the skilled person, such as centrifugation or membrane filtration.

25

Step c) may for example comprise centrifuging the culture medium at from 500g to 700g, for example at 600 g, for example from 10 minutes to 20 minutes, preferably for 10 minutes. The pellet is removed and a supernatant comprising myelinosomes is obtained.

Step c) may for example comprise passing the culture medium on a filtration membrane. In this case, the retentate is removed and the permeate corresponding to the supernatant comprising the myelinosomes is collected.

30

The filtration membrane used in step c) preferably retains particles of a size equal or greater to 1 μm .

Step d)

35

The method comprises a step d) of removing the liquid fraction from the supernatant obtained in step c), to obtain a pellet comprising the myelinosomes.

Removing the liquid fraction from the supernatant may be performed by any suitable method of separation well known by the skilled person, such as centrifugation or membrane filtration.

Step d) may for example comprise centrifuging the supernatant at from 16 000 g to 20 000 g, for example at 20 000 g, for example from 60 to 90 minutes, preferably for 90 minutes.

Step d) may for example comprise passing the supernatant obtained at step c) on a filtration membrane. In this case, the permeate is removed and the retentate corresponding to the pellet comprising the myelinosomes is collected.

The filtration membrane used in step d) preferably retains particles of a size equal or greater to 200 nm.

The removed liquid fraction particularly comprises light vesicles.

By "light vesicles", it is herein meant the major part of vesicles described as "exosomes" and/or membrane-budding microvesicles.

Step d) may comprise the use of a combination of at least two filtration membranes.

For example, step d) may comprise:

- passing the supernatant obtained at step c) on a filtration membrane retaining particles of a size equal or greater to 700 nm and collecting the permeate, and
- passing the obtained permeate on a filtration membrane retaining particles of a size equal or greater to 200 nm and collecting the retentate corresponding to the pellet comprising the myelinosomes.

Step e)

The method may further comprise a step e) of separating myelinosomes from exosomes, in the pellet obtained in step c).

Separating myelinosomes from exosomes may be performed by any method allowing a separation based on a difference of size, such as flow cytometry, for example using FACS (*Fluorescence Activated Cell Sorting*) technique, or density gradient ultracentrifugation.

For that purpose, the pellet comprising myelinosomes obtained in step d) may be suspended in any suitable buffer.

In the vesicle sorting protocol, small exosome vesicles are eliminated and myelinosomes are for example sorted from 200 nm to 700 nm.

The samples comprising the purified myelinosomes obtained after separation may be further characterized, for example by transmission electron microscopy (TEM) to

assess the purity of fractionation and/or by WB (Western-Blot) analysis for assessing the presence/absence of CD63 and calnexin protein.

Steps c), d) and e) / size exclusion chromatography

Alternatively, steps c), d) and e) may be performed in a single step, by size exclusion chromatography.

Size exclusion chromatography indeed allows myelinosome purification, including separation from exosomes, directly from the culture medium collected in step b).

Alternatively, size exclusion chromatography may also be performed additionally to steps c) and d) and, optionally, to step e).

Because of the large size of myelinosomes by comparison with exosomes and microvesicles, myelinosomes vesicles are obtained in the eluent fraction releasing immediately after those corresponding to "void volume" of size-exclusion column. Exosomes have a retention time much longer than those of myelinosomes.

Step f)

The method as defined above may further comprise a step f) of loading the myelinosomes with at least one molecule of interest.

Step e) being optional, step f) may be performed after steps a), b), c) and d) or after steps a), b), c), d) and e).

The molecule of interest may be as defined above in the section of the same name.

The myelinosomes may be loaded with one kind of molecule of interest or different kinds of molecules of interest, for example at least two or at least three different kinds of molecules of interest.

A molecule of interest may be loaded into the myelinosome by contacting the myelinosome with said molecule of interest.

Loading of the molecule(s) of interest into the myelinosomes may thus be very simple and may not need to use specific conditions, such as for example electroporation.

The myelinosome obtained by the method as defined above may be used as a vector for intracellular delivery, for example *in vitro* or *in vivo*, for example in a patient.

Use of myelinosome as a vector for intracellular delivery *in vitro*

The invention also relates to the use of myelinosome as a vector for intracellular delivery *in vitro*, in particular for intracellular delivery of at least one molecule of interest.

Myelinosomes thus constitute an alternative vector for transfecting cells with a nucleic acid and can advantageously also be used for delivering any compound loaded in the myelinosomes, such as a protein or a chemical molecule.

In the above-defined use, the myelinosome preferably comprises said at least one molecule of interest to be delivered intracellularly.

In the above-defined use, the molecule of interest may be delivered in the cells by contacting the myelinosomes comprising said molecule of interest with the cells.

The myelinosome may be provided in the form of a composition as defined above, in particular a composition suitable for an *in vitro* use as defined above.

The molecule of interest is as defined above in the section of the same name.

The molecule of interest may be selected from the group consisting of a nucleic acid, a protein and a chemical molecule.

The myelinosome comprising said at least one molecule of interest is for example as defined above and/or produced by the method as defined above.

The cells may be cells in suspension or adherent cells.

The cells are preferably in a culture medium, for example a suitable culture medium as defined above.

The cells may be eukaryotic cells, such as animal cells or plant cells, or prokaryotic cells.

The cells are preferably animal cells, more preferably mammalian cells.

The cells may for example be human cells, murine cell, or monkey cells.

The cells may originate from a biological sample of a subject or from a cell line.

Myelinosome for use as a vector for delivering a molecule of interest

The invention also relates to a myelinosome for use as a vector for delivering at least one molecule of interest in a subject.

The invention also relates to a method for delivering at least one molecule of interest in a subject in need thereof, comprising administering at least one myelinosome comprising said molecule of interest in said patient.

The use of myelinosome as a vector for delivering at least one molecule of interest in a subject is particularly advantageous, because phagocytic cells have very limited capacity to ingest myelinosomes. Myelinosomes are therefore not degraded by the phagocytic cells, contrary for example to exosomes and microvesicles.

The molecule of interest is administered in an effective amount to achieve the intended purpose, for example diagnosing, preventing and/or treating a disease.

The molecule of interest is for example as defined above.

The molecule of interest is particularly useful for diagnosing a disease, preventing a disease and/or treating a disease in said subject. The molecule of interest is for example a therapeutic molecule or a diagnostic molecule.

5 The molecule of interest is for example selected from the group consisting of a nucleic acid, a protein and a chemical molecule.

The myelinosome is preferably as defined above and may be provided in the form of a pharmaceutical composition, for example as defined above.

The pharmaceutical composition may for example be a liquid composition, such as a suspension, or an aerosol.

10 Myelinosomes thus constitute a safer alternative vector for gene therapy and can advantageously also be used for delivering any compound loaded in said myelinosomes, such as a protein or a chemical molecule, in the cells of a subject.

The subject may be a human being or a non-human animal, preferably a non-human mammal.

15 A non-human mammal is for example a mouse, rat, cat, dog, rabbit or primate.

The subject is preferably a human being, also referred to as individual or patient.

The subject may be of any age, for example an infant, child, adolescent, adult, elderly people, and of any sex.

20 The invention also relates to a myelinosome for use as defined above as a vector for delivering at least one molecule of interest in a subject, for the prevention, treatment and/or diagnostic of a disease, said molecule of interest being particularly useful for the prevention, treatment and/or diagnostic of said disease.

25 The invention also relates to a method for diagnosing a disease, preventing a disease and/or treating a disease in a subject in need thereof, comprising administering to said subject at least one myelinosome comprising at least one molecule of interest useful for diagnosing, preventing and/or treating said disease.

The myelinosome may be administered by the arterial, venous, intra-tissue, intra-cerebrospinal, intracerebral, intraventricular, intranasal, intrathecal, inhalation and/or intraperitoneal route.

30 Advantageously, the myelinosome may be used or administered in a patient to deliver at least one molecule of interest across a blood barrier.

The blood barrier may be the blood-retinal barrier, blood testicular barrier, blood follicular and/or the blood-brain barrier.

35 The myelinosome may be used for delivering a functional protein or a nucleic acid encoding said functional protein into the cells of a patient suffering from a genetic disease,

wherein said genetic disease particularly results from or involves a non functional form of said protein or its absence.

The myelinosome may for example be used for delivering a functional CFTR protein and/or a nucleic acid encoding a functional CFTR protein into the cells, particularly into the lung cells, of a patient suffering from Cystic Fibrosis or for delivering a functional Huntingtin protein and/or a nucleic acid encoding a functional Huntingtin protein in the cells, in particular the brain cells (for example by intracranial delivery, such as by administration via the intraventricular, intracerebral, intranasal and/or intrathecal route), of a patient suffering from Huntington Disease.

The invention also relates to a method for preventing and/or treating a subject suffering from a genetic disease resulting from or involving a nonfunctional form of a protein or its absence, wherein said method comprised administering to said subject at least one myelinosome comprising (i) at least one nucleic acid encoding a functional form of said protein and/or (ii) a functional form of said protein.

The invention particularly relates to a method for preventing and/or treating a subject suffering from Cystic Fibrosis, wherein said method comprises administering to said subject at least one myelinosome comprising (i) a nucleic acid encoding a functional CFTR protein and/or (ii) a functional CFTR protein.

The invention particularly relates to a method for preventing and/or treating a subject suffering from Huntington Disease, wherein said method comprises administering to said subject at least one myelinosome comprising a nucleic acid encoding a functional Htt protein and/or (ii) a functional Htt protein.

The invention will be further illustrated by the following figure and examples. However, these examples and figure should not be interpreted in any way as limiting the scope of the present invention.

Figures

Figure 1: Pattern of distribution of green LC3-EGFP puncta in COS7 cells transfected with LC3-EGFP plasmid vector (a) and myelinosomes carrying LC3-EGFP (b).

Figure 2: Pattern of distribution of green EGFP in COS7 cells transfected by DNase-treated myelinosomes carrying mHtt-exon1-EGFP.

Figure 3: Images of human seminal plasma in TEM (*Transmission Electron Microscopy*) (A) and cryo TEM (B and C). Figure A shows the presence of myelinosomes in human seminal plasma.

Figure 4: mHtt142Q protein delivery to cells by myelinosomes isolated from the culture media of TM4 Sertoli cells transfected with mHtt142Q-EGFP. Myelinosomes are integrated into the cytoplasmic part (cap) of spermatozoa. B shows enlargement of the part delimited by the square in A.

Figure 5: Plasma membrane localization of wild-type CFTR 48h after incubating CFBE cells bearing mutant CFTR with myelinosomes produced by TM4 cells transfected with WT CFTR-EGFP vector. Arrows show EGFP staining. Nuclei were stained with DAPI.

Figure 6: Myelinosome vesicles crossing blood-retinal barrier. EM micrograph of retinal section from 23 week-old R6/1 mice processed through immunogold staining using monoclonal mouse 2B4 anti Huntingtin antibody. Secondary anti-mouse antibody was conjugated with 10 nm gold granules. EM micrograph shows that the antibodies against ubiquitous Huntingtin protein stain cell cytoplasm and cell organelles, as confirmed by the presence of gold granules. Myelinosome vesicles (Mye) carrying gold granules (dashed arrows) are present in intercellular spaces between 2 neighboring RPE cells, separated by tight junctions (black arrows). Abbreviations: mt – mitochondria, MEL – melanin granules; RPE – retinal pigmented epithelium.

Figure 7: Myelinosome vesicles express endoplasmic reticulum (ER) marker calnexin protein. EM micrograph of myelinosome vesicles isolated from the culture media of TM4 Sertoli cells transfected with plasmid vector carrying F508delCFTR-EGFP protein. Immunogold staining using rabbit polyclonal anti-calnexin protein. Secondary anti-rabbit antibody was conjugated with 15 nm gold granules. Gold granules concentrate on myelinosome vesicles.

EXAMPLES

Example 1 – *In vitro* delivery of proteins in target cells using myelinosomes as a vector

Material and Methods

(i) Cells and cell culture

Human MIO-M1 cells were from University College (London, Great Britain); human neuroblastoma SH-SY5Y cells were from ATCC Cell Biology Collection (Molsheim, France), murine TM4 Sertoli cells and monkey COS7 fibroblast cells were from IGBMC (Illkirch, France) cell culture service collection. CFBE cells stably expressing F508del-CFTR were from Dr. J.P. Clancy (University of Alabama at Birmingham, Birmingham, Alabama). MIO-M1, SH-SY5Y and COS7 cells were cultured in DMEM (GIBCO, 31966) medium supplemented with 10% FCS and 50 µg/ml gentamycin (PAN Biotech, P06-03025P) and 2 µg/ml fungizone (Sigma, number A2411). TM4 Sertoli cells were cultured

in DMEM/F12 medium (Gibco, number 31330), containing 2.5% FBS, 5% HS, and supplemented with 1% antibiotic mix (10 000 U/ml penicillin and 50 mg/ml streptomycin, GIBCO 15140). CFBE cells were cultured in DMEM (GIBCO, 31966) medium supplemented with 10% FCS. In all cases 0.5x10⁶ cells were plated into 6-well dishes or in 3.5 cm² Petri dishes and cultured at 37°C with 5% CO₂. All cell lines were tested for mycoplasma contamination using mycoplasma detection kit (MycoAlert, LONZA LT07-418).

(ii) Chemicals and plasmids

Dulbecco's phosphate buffered saline (number D5652), bovine serum albumin (BSA) (number A9647). Polyclonal rabbit primary antibodies were: anti-EGFP (Molecular Probes, A6455). The monoclonal mouse antibodies used were anti-GFP (B-2) (Santa-Cruz Biotechnology, sc-9996), 2B4 and 1C2 (IGBMC antibody platform, Illkirch). The other antibodies were: secondary anti-rabbit (Santa-Cruz Biotechnology, sc-2004), anti-mouse (GE Healthcare, LNA 931) antibodies conjugated to horseradish peroxidase; for immunostaining: anti-rabbit and anti-mouse antibodies conjugated to Alexa-488 (CAR-A21441), to Alexa-594 (GAM-A21422, DAM-A31570) from Molecular Probes (Invitrogen) or to FP-647 (GAR-FPGARBTTG0642) from Interchim. For immunoelectron microscopy, we used anti-rabbit antibodies conjugated with 15 nm gold beads (Auroprobe TM/EM, LRPN422V/AA/G15) and anti-mouse antibodies conjugated with 10 nm gold beads (Sigma-Aldrich, G7777). Vectors used for Htt and mHtt expression were pEGFP-C2-trHtt-17Q and pEGFP-C2-trHtt-142Q generated at IGBMC (Strasbourg, France), the vector for the expression of CFTR-EGFP was from Dr. Stanton BA (University of Alabama). We also used transfection vector encoding autophagy protein LC3 pSELECT-GFP-LC3 (InvivoGen; Catalog # psetz-gfplc3).

(iii) Transient transfection

Myelinosomes were produced in transiently transfected cells as described below. Cells were transfected *in vitro* with a transfection vector comprising a nucleic acid encoding a protein of interest, for example mCFTR or mHTT-exon1, or their normal counterparts (CFTR, HTT exon1). To be easily detected, all proteins were expressed as fusion proteins with EGFP.

Transfection of MIO-M1, SH-SY5Y and TM4 cells with expression vectors encoding mHTT, HTT, mCFTR or CFTR were performed using Lipofectamine 2000 (Invitrogen) reagent in 6-well plates or in 3.5 cm² Petri dishes, containing 0.5x10⁶ cells following the manufacturer's instructions. The ratio of plasmid/Lipofectamine in 2.0 ml of serum-free incubation mix was 4.0 µg/10 µl for MIO-M1, SH-SY5Y cells and 4.4 µg/16 µl for TM4 cells.

Cells were cultured 48h further to the transfection in a culture medium.

(iv) Differential centrifugation

48h post-transfection, the culture medium comprising the myelinosomes was collected and some fresh medium was added to the transfected cells. 48h after adding of the fresh medium, a second harvesting of myelinosomes from the culture medium was performed. Myelinosomes were still present in the culture medium of the second harvesting, but in lesser quantity comparing to the first one. 96h after the beginning of transfection (or 48 h after the replacement of culture media), no myelinosomes were detected in the culture media.

The myelinosomes were purified from the collected culture medium as follows: the cultured medium was centrifuged for 10 min at 600g. The pellet was discarded and the supernatant centrifuged for 90 min at 20 000g. The supernatant was discarded and the pellet obtained contains the fraction enriched in myelinosomes. In TM4-derived preparation, 20 000g myelinosome-enriched fraction also contained 100-120 nm microvesicles; while 20 000g fractions from Muller cells and SH-SY5Y cells were free of microvesicles.

For immediate use of the myelinosomes, the pellet was suspended in a culture media (the media can be supplemented with 2-10% of inactivated serum, or not). For long term storage (2-3 months), the pellet was frozen at -20°C “in dry form”, i.e. without adding any media.

(v) Delivery to target cells

The myelinosomes were added to non-transfected SH-SY5Y or MIO-M1 cells or to CFBE cells. Routinely, myelinosomes were collected from 2 wells of transfected cells and the purified myelinosomes were added into 1 well of naïve cells. Naïve cells were incubated with myelinosomes at 37°C with 5% CO₂. For long-term (5-7 days) incubation with myelinosomes, SH-SY5Y cells were cultivated in their cultivation media (see above) containing 2% of FCS to slow cell growth.

Results

Few minutes after adding the myelinosomes carrying EGFP-fused proteins to naïve cells, said cells become green-colored.

For SH-SY5Y cells

Starting from 24 h of incubation with myelinosomes carrying mHtt-exon1-EGFP, the isolated green points can be detected in cell cytoplasm by IF (Immunofluorescence). The number of green points increased progressively over time, so that by 72 h the points seemed to agglomerate, forming aggregate-like structures.

WB (Western Blot) analysis revealed the emergence of high MW aggregated forms of mHtt in naïve SH-SY5Y cells incubated with myelinosomes produced by TM4 or MIO-MI cells, expressing mHTT-exon1-EGFP.

For COS7 cells

In this experiment, the pattern of transfection with plasmid vector was compared to those with myelinosomes carrying normal protein. For this purpose autophagy marker LC3 protein was chosen.

Myelinosomes were collected from culture media of TM4 Sertoli cells transfected with LC3-EGFP plasmid, and then added to naïve COS7 cells. After 48 h of incubation the pattern of distribution of green staining in myelinosome-transfected cells was compared with the cells transfected with plasmid vector carrying LC3-EGFP.

Figure 1 shows the identical pattern of distribution of green LC3-EGFP puncta in COS7 cells transfected with LC3-EGFP plasmid vector (a) and myelinosomes, carrying LC3-EGFP (b).

To exclude the involvement of plasmid transfection vector in the phenomenon observed, two sets of control experiments have been performed.

Control experiments:

1. Incubation of cells with plasmid in absence of transfection reagent

COS7 cells were incubated with plasmid in serum-free incubation mix likewise as described above (transient transfection), except that Lipofectamine was omitted from incubation mixture. 48 h later the cells were washed with fresh culture media, and then analysed for the presence of green fluorescence. No green fluorescent signal was observed (data not shown).

2. DNase treatment

Myelinosomes collected from 6 wells of 6-well dish containing TM4 or MIO-MI cells expressing mHtt exon-1-EGFP were suspended in 90 µl of serum-free media used for cell cultivation. Then 10 µl of DNase solution (50 µg/1 ml) in DNase reaction buffer containing 10mM Tris-HCl, 2,5 mM MgCl₂, 0.5mM CaCl₂, pH 7.6 was added. The mixture was incubated 15 min at 37°C; then reaction was stopped by adding of 1 ml of ice-cold serum-free DMEM. After that, the mixture was centrifuged, myelinosome pellet was washed twice, suspended in serum -free DMEM media and added to untransfected COS7 cells at the same ratio as above.

48 h later the cells were analyzed for the presence of green fluorescent staining. Figure 2 shows that cells, exposed to myelinosomes pre-treated with DNase exhibit green fluorescent staining. Thus, treatment with DNase did not affect the “transfecting” ability of myelinosomes.

Example 2 – *In vitro* delivery of GFP in spermatozoids using myelinosomes as a vector

Material and Methods

Results

Myelinosomes were found in human seminal plasma, evidenced by TEM (Figure 3 A).

The fusion of myelinosomes with human gametes was shown using cryopreserved spermatozoa stored under liquid nitrogen for IVF (*in vitro* fertilization). Sperm collection has been performed through routine clinical procedure. After collection, live sperm was proceeded on Percoll gradient. Mobile spermatozoa were suspended in 1 ml of Fercult medium (FertiPro, France).

The cells were then incubated overnight at 37° with myelinosomes, isolated from the culture media of TM4 Sertoli cells, transfected with mHtt142Q-EGFP. Next day the preparation was centrifuged at 2000 rpm for 10 min. The supernatant was discarded and the pellet was fixed in 2.5% glutaraldehyde/PBS, then proceeded for electron microscopy. It was observed the integration of myelinosomes into the cytoplasmic part (cap) of spermatozoa (Figure 4).

Example 3 – *In vitro* delivery of DNA and RNA using myelinosomes as a vector

EGFP-mHtt-exon1-loaded myelinosomes were used to transfect COS7 cells (or SH-SY5Y cells) and evolution of fluorescence in target cells was followed during a week. In fact, the fluorescence did not fade away (indicating that at least the proteins were not degraded), but also that sister cells were also fluorescent, and to the same level that parent cells. This is consistent with the presence of DNA coding for EGFP-mHtt-exon1 into these cells that was transported by myelinosomes.

Example 4 – *In vitro* delivery of a chemical molecule using myelinosomes as a vector

a) Quinacrine

Treatment with quinacrine has been shown to result in massive emergence of intracellular myelinosomes in hepatocyte cells (Prince et al., 1993, Tissue and Cell, Vol. 25, p 103-110).

Secretory Sertoli or Muller cells are treated with fluorescent xenobiotic quinacrine (red emission spectra, 525nm) to induce the formation of myelinosomes. The

myelinosomes are then purified by differential centrifugation as disclosed in Example 1. The purified myelinosomes are loaded with a molecule of interest conjugated with a fluorescent marker.

Then, the purified myelinosomes loaded with said molecule of interest are contacted with target cells and fluorescence is analyzed in the target cells.

b) DNase

As described above in Example 1, "Control experiments", "DNase treatment", incubation of myelinosome vesicles isolated from cells, expressing mHtt exon-1-EGFP with DNase did not affect "transfection ability" of myelinosome vesicles. At the same time, the incubation of "transfected" COS7 cells with nuclear marker DAPI (4',6 diamidino-2-phenylindole; 0.5 lg/ml) did not reveal a nuclear staining in COS7 cells. This indicates the insertion of functional DNase protein into myelinosome vesicle, and its further delivery into COS7 cells.

Example 5 – Effect of CFTR protein delivery in cells carrying mutant CFTR using myelinosomes as a vector

CFBE cells (human cystic fibrosis bronchoepithelial cells with CFTR mutation) do not express a mature form of CFTR on the plasma membrane. CFBE cells were incubated with myelinosomes, produced by TM4 cells transfected with WT CFTR-EGFP vector.

After 48 h of incubation, the cells were washed with PBS and fixed with 4% of PAF/PBS for 5 min. EGFP-green staining was amplified using anti-EGFP antibodies (Molecular Probes, A6455). Secondary antibody was Alexa-488 (CAR-A21441), the nuclei were stained with DAPI (D9542, Sigma).

Figure 5 shows that treatment with myelinosomes carrying WT CFTR rescues the phenotype of CFBE cells. After treatment, CFTR protein is indeed found on the plasma membrane (see arrows).

Example 6 - Unlike exosomes and microvesicles, myelinosomes are able to cross specific blood barriers (namely blood-retinal barrier)

Material and Methods

Eyes from 23 week old transgenic R6/1 Huntington disease (HD) mice were fixed overnight at 4°C by immersion in 2.5% glutaraldehyde (Sigma Aldrich, G5882) and 2.5% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4), then washed in cacodylate buffer. The samples were postfixed in 1% osmium tetroxide (Sigma Aldrich, 201030) for 1 h at 4 C, then dehydrated through graded alcohols and embedded in Epon 812 resin.

For immunolabeling 100 nm sections adherent onto nickel grids were used. Sections were oxydated for 30 min in 5% sodium metaperiodate/bidistilled H₂O; then washed 3-5 min in bidistilled H₂O and blocked in 3% BSA/PBS for 30 min. The incubation with primary antibodies was performed overnight at 4°C. Then the sections were washed 3-5 min in 0.05% Tween/PBS and incubated with secondary anti-mouse, anti-goat and anti-rabbit antibodies, respectively (dilution 1/50-1/100) conjugated to 10 nm or 15 nm colloidal gold. After washing, the sections were contrasted with uranyl acetate and lead citrate and examined at 80 kV with a JEM1010, Jeol Electron Microscope equipped with Quemesa, Olympus Systems Imaging Solution Camera.

Results

EM micrograph of Figure 6 shows that the antibodies against ubiquitous Huntingtin protein stain cell cytoplasm and cell organelles, as confirmed by the presence of gold granules. Myelinosome vesicles (Mye) carrying gold granules (dashed arrows) are present in intercellular spaces between 2 neighboring RPE cells, separated by tight junctions (black arrows).

Example 7 - Unlike exosomes and microvesicles, which are very easily ingested and degraded by the phagocytic system, phagocytic cells have very limited capacity to ingest myelinosomes vesicles

Material and Methods

Myelinosome vesicles, collected from the culture media of TM4 Sertoli or MIO-M1 Muller cells transfected with a plasmid vector coding for mutant Huntingtin-exon1-EGFP protein (protocols for cell transfection and myelinosome isolation are the same as described earlier) were added to non-transfected TM4 Sertoli cells and MIO-M1 Muller cells. Routinely, myelinosomes were collected from 2 wells of transfected cells and added the purified myelinosomes into 1 well of naïve cells. Naïve cells were incubated with myelinosomes at 37°C with 5% CO₂ in appropriated cultivation media for 2- 6 hours. Then, the cells were washed in PBS and fixed in paraformaldehyde/PBS (pH 7.4) for 3 min, after that the cells were analysed by confocal laser scanning microscopy using a confocal FV-1000 station installed on an inverted microscope IX-81 (Olympus, France).

Results

Confocal analysis revealed rare green-colored vesicles inside the cytoplasm of TM4 Sertoli cells and MIO-M1 Muller cells (data not shown). Because the degradation of ingested substrates by rodent Sertoli cells takes 29 hours (Chemes, 1986; Russell et al.,

1990; Griswold, 2014) the absence of green-colored suggests the very limited ability of phagocytic Sertoli cells to ingest myelinosome vesicles.

Example 8 - Myelinosomes can be distinguished from exosomes by the presence of Calnexin protein and the absence of CD63 marker

As shows Figure 7, myelinosome vesicles are stained with antibody against endoplasmic-reticulum marker calnexin protein by immune-electron microscopy (unlike exosomes and microvesicles, which are calnexin-negative).

Besides, myelinosome vesicles are free of anti CD63 immunostaining by immuno-electron microscopy, unlike exosomes and microvesicles, as shown in Yefimova et al. (2016, Hum Mol Genet. 1;25(19):4170-4185). .

Example 9 – Delivery of a functional protein LC3

Myelinosomes were purified from cells transfected with a plasmid encoding LC3 protein fused to GFP. COS7 cells were then transfected with these myelinosomes. Protein LC3 fused to GFP was then observed in the cytoplasm of the COS7 cells (data not shown).

CLAIMS

1. Use of myelinosome as a vector for intracellular delivery *in vitro*.
2. The use according to claim 1 for intracellular delivery of at least one molecule of interest selected from the group consisting of a nucleic acid, a protein and a chemical molecule.
3. A myelinosome for use as a vector for delivering at least one molecule of interest in a subject.
4. The myelinosome for use according to claim 3, wherein said molecule of interest is selected from the group consisting of a nucleic acid, a protein and a chemical molecule.
5. The myelinosome for use according to claim 3 or 4, in the prevention, treatment and/or diagnostic of a disease.
6. A method for producing myelinosomes, wherein said method comprises:
 - a) culturing cells in a culture medium in pathological and/or stressful condition, for a time sufficient to induce the formation and secretion of myelinosomes,
 - b) collecting the culture medium,
 - c) removing cell debris from the culture medium collected in step b), to obtain a supernatant comprising myelinosomes,
 - d) removing liquid fraction from the supernatant obtained in step c), to obtain a pellet comprising the myelinosomes, and
 - e) optionally, separating myelinosomes from exosomes in the pellet obtained in step d), to obtain purified myelinosomes,wherein the pathological and/or stressful condition is not the presence of a misfolded protein in the cell and wherein step a) does not comprise transfecting the cells with a nucleic acid encoding a protein comprising (i) EGFP and (ii) a functional CFTR protein or functional Htt protein.
7. The method according to claim 6, wherein the pathologic and/or stressful condition is selected from the group consisting of the transfection of the cells with a nucleic acid, the overexpression of a protein, the presence of a chemical

compound inducing the formation of myelinosomes, hypoxia, heat shock treatment and their combinations.

- 5 **8.** The method according to claim 7, wherein the chemical compound inducing the formation of myelinosomes is quinacrine.
- 9.** The method according to any one of claims 6 to 8, wherein step a) comprises:
- 10 i. transfecting the cells with a nucleic acid encoding a protein of interest, to obtain a transfected cell, and
- ii. culturing the transfected cells in a culture medium for a time sufficient to express the protein and induce the formation of myelinosomes.
- 10.** The method according to any one of claims 6 to 9, wherein step c) comprises centrifuging the culture medium at from 500 g to 700 g, preferably from 10
- 15 minutes to 20 minutes and/or step d) comprises centrifuging the supernatant at from 16 000 g to 20 000 g, preferably from 60 to 90 minutes.
- 11.** The method according to any one of claims 6 to 10, further comprising a step f) of loading the myelinosomes with at least one molecule of interest.
- 20 **12.** The use of myelinosome according to claim 1 or 2 or a myelinosome for use according to any one of claims 3 to 5, wherein said myelinosome is obtained by the method according to any one of claims 6 to 11.
- 25 **13.** A modified myelinosome comprising at least one molecule of interest, wherein said myelinosome does not comprise a protein comprising a mutant Htt protein, nor a protein comprising a mutant CFTR protein, nor a protein comprising EGFP and a functional Htt protein, nor a protein comprising EGFP and a functional CFTR protein.
- 30 **14.** A composition comprising (i) purified myelinosomes and (ii) a culture medium or a buffer.
- 35 **15.** A pharmaceutical composition comprising myelinosomes and a pharmaceutically available vehicle.

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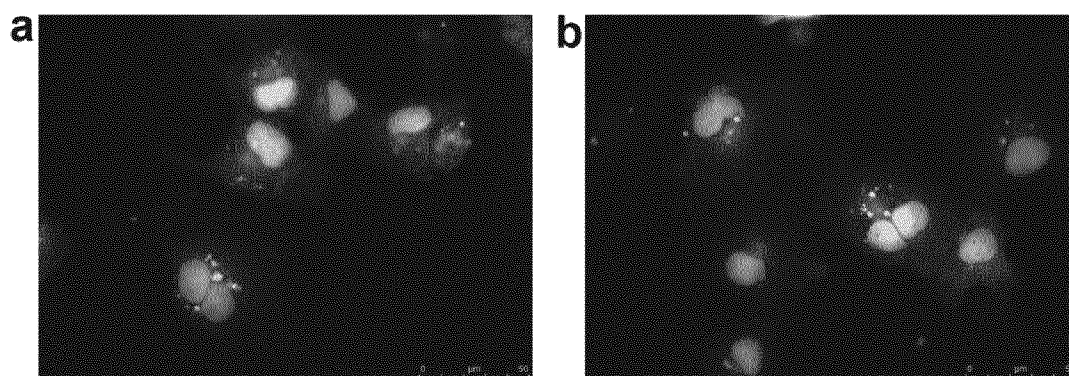


FIG.1

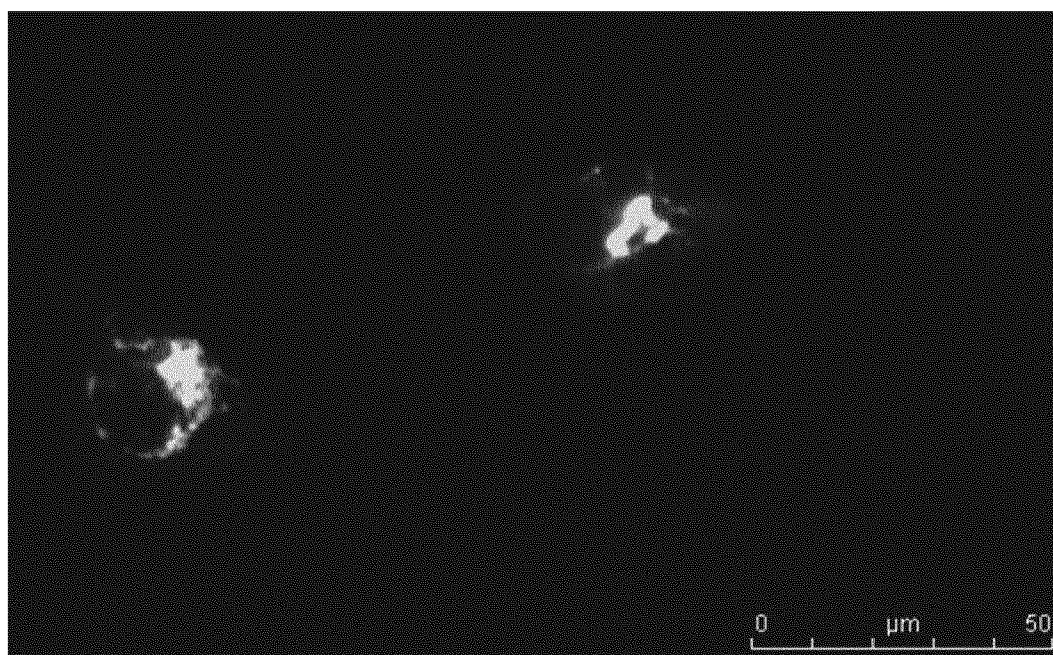


FIG.2

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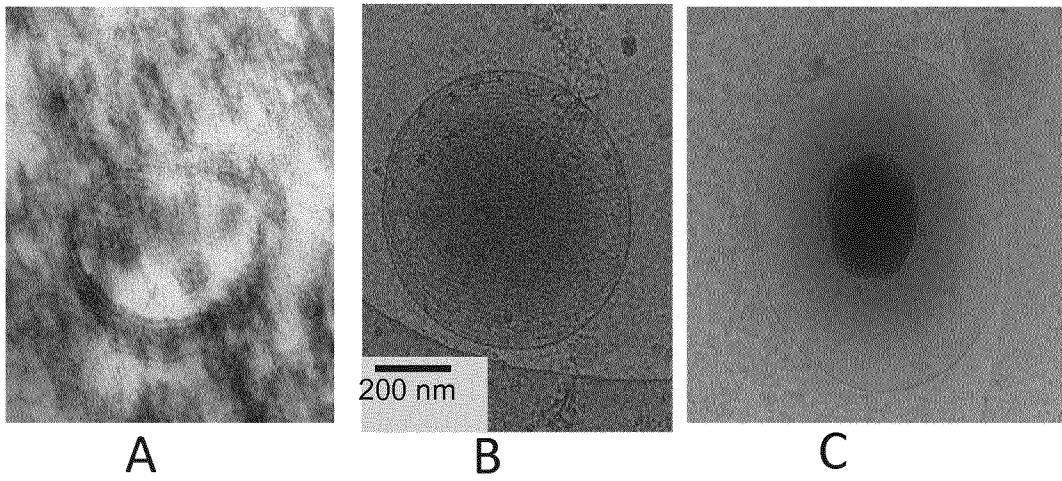


FIG.3

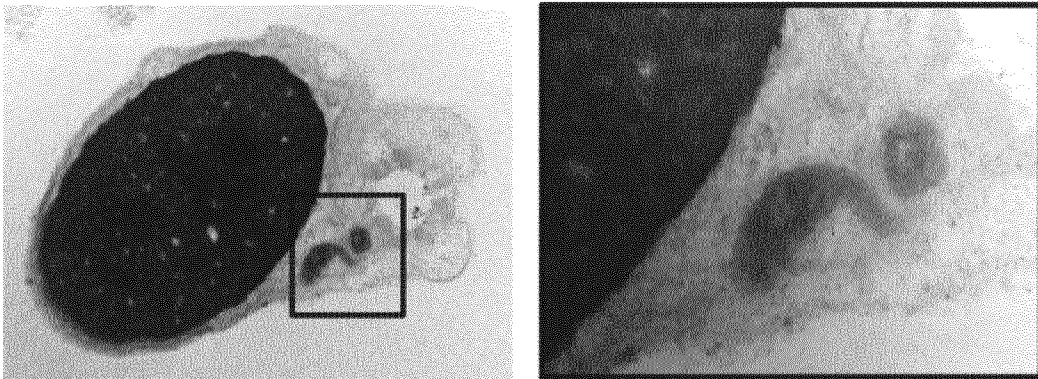


FIG.4

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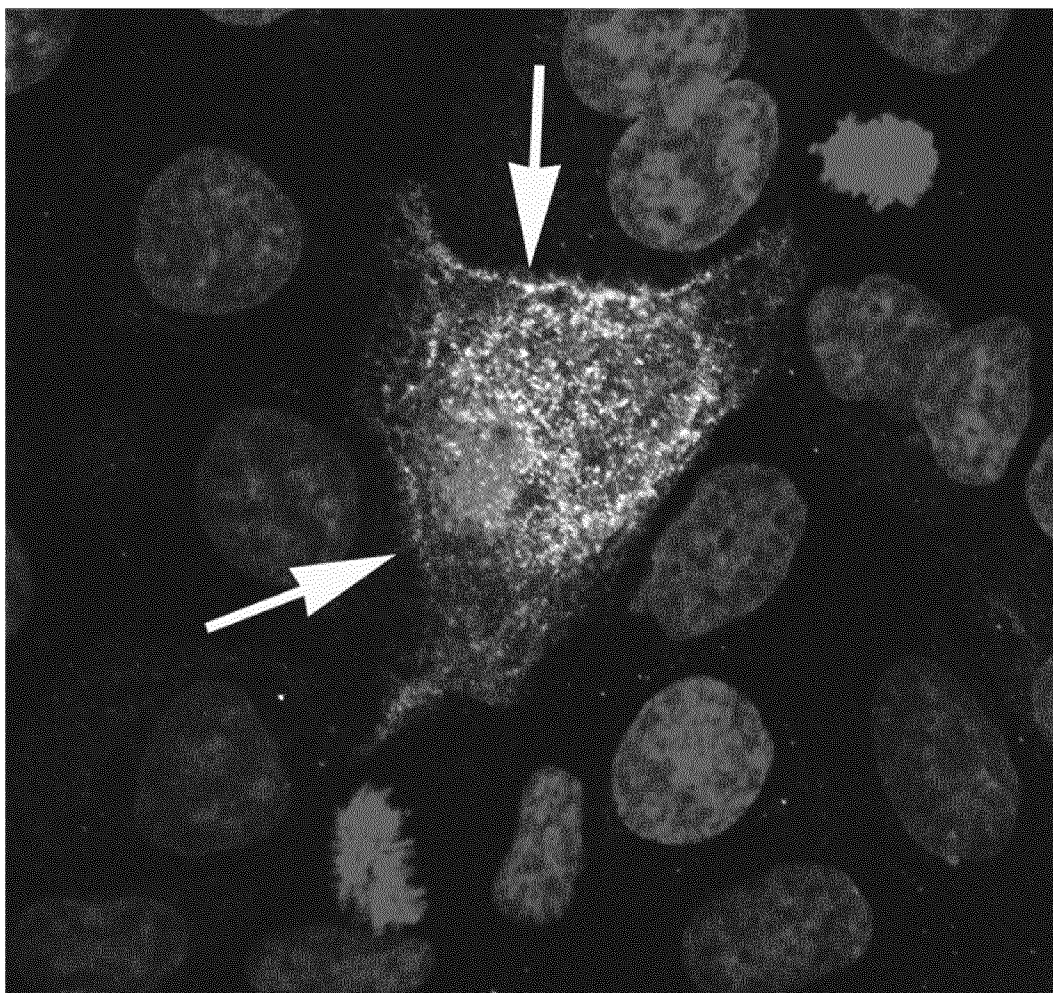


FIG.5

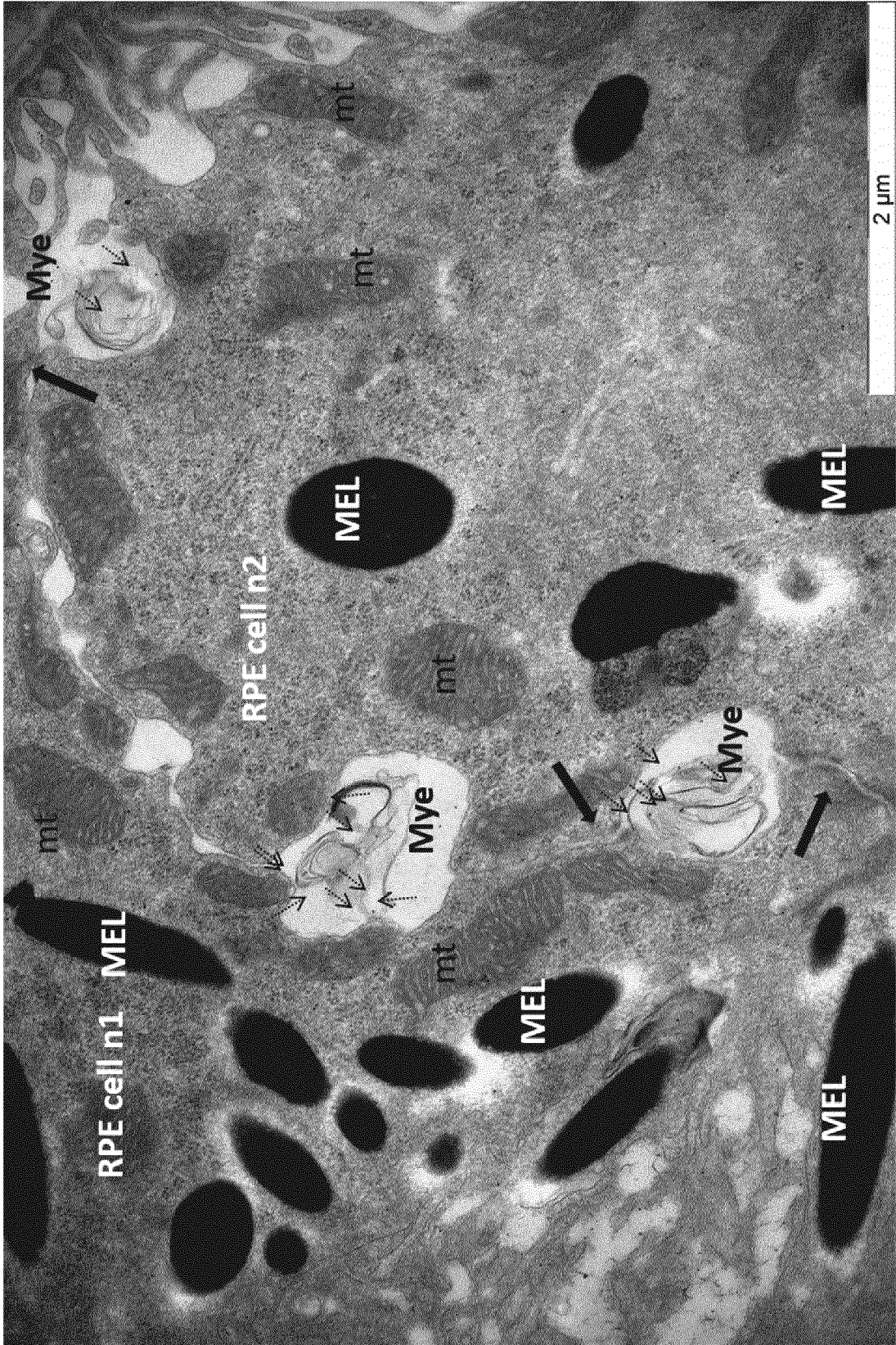


FIG.6

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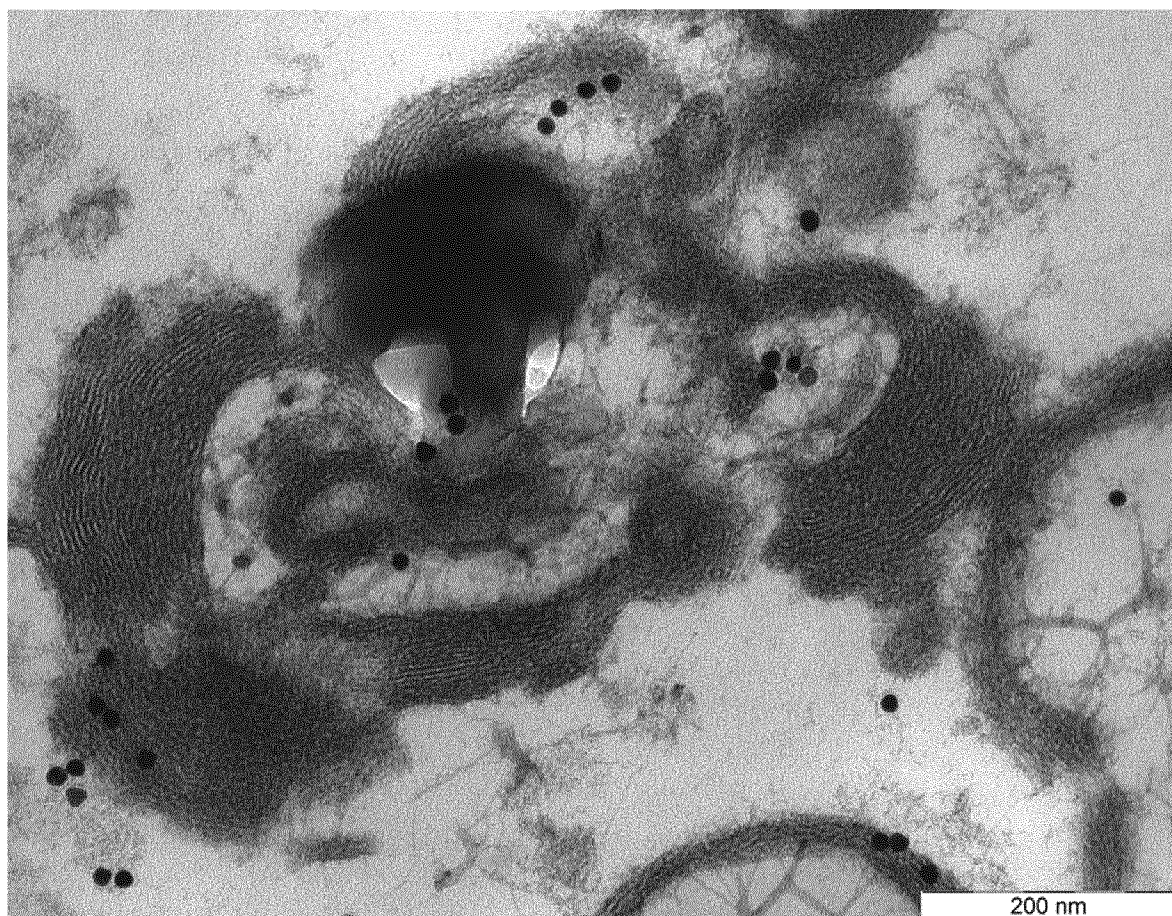


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/078431

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N15/88 A61K9/50 A61K48/00 A61K38/00 ADD.		
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) C12N A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARINA G. YEFIMOVA ET AL: "Myelinosomes act as natural secretory organelles in Sertoli cells to prevent accumulation of aggregate-prone mutant Huntingtin and CFTR", HUMAN MOLECULAR GENETICS, vol. 25, no. 19, 4 August 2016 (2016-08-04), pages 4170-4185, XP055569043, gb ISSN: 0964-6906, DOI: 10.1093/hmg/ddw251 figure 3 page 4171, right-hand column, paragraph 2 page 4182, left-hand column, paragraph 2 page 4182, right-hand column, paragraph 3 ----- -/-	6-15
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Brouns, Gaby

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