

Description

Title of Invention: ENGINEERED EXOSOMES WITH INCREASED LOADING OF PROTEIN CARGO AND METHODS THEREOF

Field of the Invention

[0001] The present disclosure relates to engineered exosomes, in particular to an exosome with increased accumulation of protein cargo anchored external to the membrane of the exosome. The present disclosure also relates to a method for increasing the accumulation of protein cargo, e.g., anchored external to the membrane of the exosome. Also disclosed are composition comprising the engineered exosome and uses of the engineered exosome for drug delivery.

Background

[0002] Exosomes possess unique characteristics such as stability, low immunogenicity, and high biocompatibility which make them ideal drug delivery platforms. These features enable exosomes to effectively enter target cells, avoiding recognition and elimination by the immune system, and therefore, deliver foreign protein and nucleic acid drugs to target cells. Exosomes have shown promise as drug delivery vehicles in therapeutic research. For instance, loading patient-specific neoantigens into dendritic cell-derived exosomes for cancer immunotherapy has shown promising results, with exosome-based nano vaccines inhibiting tumor growth and promoting tumor cell uptake more effectively than liposomes. Additionally, modification of bone marrow mesenchymal stem cell-derived exosomes with a specific short peptide for heme oxygenase-1 (HSSP) and loading temozolomide or siRNA into these exosomes have demonstrated excellent tumor cell targeting capability, due to the overexpression of heme oxygenase-1 in glioblastoma. Thus, delivering protein drugs safely and effectively has emerged as a major challenge in current research. Exosomes can be a promising option in this regard, as protein drugs can be encapsulated into them by either genetic engineering or exogenous loading methods for efficient drug delivery.

[0003] Various attempts were reported to load protein drugs into exosomes, including incubation, electroporation, sonication, freeze-thaw cycling, transfection and others. One of the major challenges reside with protein loading into exosomes is its limited loading efficiency. Exosomes are intrinsically packed with natural proteins and nucleic acids, which, significantly increases the difficulties in desired cargo loading. Although there are approaches to engineer exosomes in order to enhance loading capacity, the cargo

loading efficiency of exosomes remains much lower than that of unpacked synthetic liposomes.

Summary of the Invention

- [0004] In a first aspect, provided is a method of improving a loading amount of a polypeptide of interest in an engineered exosome, the method comprising (a) constructing a vector comprising a polynucleotide encoding the polypeptide of interest and a Fc domain, (b) transfecting a donor cell with the vector, (c) culturing the donor cell in a condition to allow an expression of the polynucleotide, so that a fusion protein comprising the polypeptide of interest and the Fc domain is formed, and (d) isolating an exosome from the culture of the donor cell to obtain the engineered exosome loaded with the fusion protein, wherein the loading of the polypeptide of interest, when fused to the Fc domain, is increased compared to that without the Fc domain.
- [0005] In a second aspect, provided is an engineered exosome loaded with a fusion protein comprising a polypeptide of interest and a Fc domain, wherein the polypeptide is loaded at a higher amount compared to that when the polypeptide of interest is not fused to the Fc domain.
- [0006] In a third aspect, provided is composition comprising the engineered exosome provided herein and a carrier.
- [0007] These and other aspects and advantages of the disclosure will be apparent from the detailed description provided in the following.

Brief Description of the Figures

- [0008] Figure 1. Immunoblotting analysis of Fc-fusion protein of cell pellets or exosomes derived from pcDNA-CD63-L-FC5 & pcDNA-CD63-L-FC5-hFC (A) , pcDNA-CD63-L-TfR & pcDNA-CD63-L-TfR-hFC (B) transfected HEK293, with blank HEK293 used as mock.
- [0009] Figure 2. A. Binding assay of ACE2 with spike protein was performed with COVID-19 Spike-ACE2 Binding Assay Kit (RayBiotech, CoV-SACE2-1) . Exosomes were derived from HEK 293 cells transfected with plasmids CD63-L-ACE2, CD63-L-ACE2-Fc and mock treated. ACE2 protein was used as a positive control. B. SARS-CoV-2 pseudovirus neutralization assay with different viral strains, including wild type (WT) , and mutants (XBB. 1.5, BA. 4&BA. 5) was performed.

Detailed Description of the Invention

- [0010] Definition
- [0011] The words “a” and “an” when used in the present specification in concert with the word comprising, including the claims, denote “one or more.”
- [0012] As used herein, the terms “or” and “and/or” are utilized to describe multiple components in combination or exclusive of one another. For example, “x, y, and/or

z” can refer to “x” alone, “y” alone, “z” alone, “x, y, and z,” “(x and y) or z,” “x or (y and z),” or “x or y or z.” It is specifically contemplated that x, y, or z may be specifically excluded from an embodiment.

- [0013] Throughout this application, the term “about” is used according to its plain and ordinary meaning in the area of cell and molecular biology to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.
- [0014] “Homology” or “identity” or “similarity” refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An “unrelated” or “non-homologous” sequence shares less than 40%identity, though preferably less than 25%identity, with one of the sequences of the present disclosure.
- [0015] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%) of “sequence identity” to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art.
- [0016] The term “linker” as used herein refers to a short fragment of amino acid (AA) or nucleotide sequence containing two or more amino acids or nucleotides which may be same or different.
- [0017] As used herein, “cell line” refers to a population of cells formed by one or more sub-cultivations of a primary cell culture. Each round of subculturing is referred to as a passage. When cells are subcultured, they are referred to as having been passaged. A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a P10 culture. The primary culture, i.e., the first culture following the isolation of cells from tissue, is designated P0. Following the first subculture, the cells are described as a secondary culture (P1 or passage 1) . After the second subculture, the cells become a tertiary culture (P2 or passage 2) , and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore, the number of population doublings of a culture is greater than the passage number. The expansion of cells (e.g., the number of population doublings) during the period between passaging

depends on many factors, including but not limited to seeding density, substrate, medium, growth conditions, and time between passaging.

- [0018] The terms “reduce,” “inhibit,” “diminish,” “suppress,” “decrease,” “prevent” and grammatical equivalents (including “lower,” “smaller,” etc.) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity and/or magnitude of the symptoms in the untreated subject.
- [0019] As used herein, the term “therapeutically effective amount” is synonymous with “effective amount”, “therapeutically effective dose”, and/or “effective dose” and refers to the amount of compound that will elicit the biological, cosmetic, or clinical response being sought by the practitioner in an individual in need thereof. As one example, an effective amount is the amount sufficient to reduce hair loss. The appropriate effective amount to be administered for a particular application of the disclosed methods can be determined by those skilled in the art, using the guidance provided herein. For example, an effective amount can be extrapolated from in vitro and in vivo assays as described in the present specification. One skilled in the art will recognize that the condition of the individual can be monitored throughout the course of therapy and that the effective amount of an exosome or composition disclosed herein that is administered can be adjusted accordingly.
- [0020] As used herein, the terms “treatment,” “treat,” or “treating” refers to intervention in an attempt to alter the natural course of the individual or cell being treated and may be performed either for prophylaxis or during the course of pathology of a disease or condition. Treatment may serve to accomplish one or more of various desired outcomes, including, for example, preventing occurrence or recurrence of disease, alleviation of symptoms, and diminishment of any direct or indirect pathological consequences of the disease, lowering the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.
- [0021] The term “subject,” as used herein, may be used interchangeably with the term “individual” or “patient” and generally refers to an individual in need of a therapy. The subject can be a mammal, such as a human, dog, cat, horse, pig, or rodent.
- [0022] “Carrier”, as used herein, refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic agent is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil,

sesame oil and the like. A saline solution is a preferred carrier when the composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations, and the like. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

- [0023] The term “N-terminal amino acid residue” or “N-terminus” refers to the first amino acid residue (amino acid number 1) of a polypeptide or peptide. The term “C-terminal amino acid residue” or “C-terminus” refers to the last amino acid residue (amino acid number n, wherein n = the total number of residues in the peptide or polypeptide) of a polypeptide or peptide.
- [0024] The tetraspanin protein family members, such as CD63, CD81, and CD9, ubiquitously expressed on exosomes and extensively used as exosome biomarkers, are involved in physiological processes, for instance cell adhesion, cell motility, and signal transduction. CD63, the first characterized tetraspanin, has two extracellular loops of unequal sizes and two short cytoplasmic domains, is involved in the signal transduction processes of various types of immune cells. Sequential domain deletion has identified the transmembrane helix 3 (TM3) being necessary and sufficient for membrane anchoring and exosome targeting. The amino acid sequence of human CD63 is available from e.g., GenBank: AH151903.1, the amino acid sequence of TM3 of which corresponds to aa. 70 to 133, which is also shown as SEQ ID NO: 9 in the present disclosure.
- [0025] The term “anchoring polypeptide” is a polypeptide that is anchored on the exosome membrane when the exosome is generated by a cell. A transmembrane protein is a typical anchoring polypeptide in the context of the present disclosure. By “anchoring” or its grammatical variants, it means that at least a fragment of the polypeptide is embedded in the exosome membrane. The anchoring polypeptide may be fully or partly embedded in the exosome membrane. In some embodiments, the anchoring polypeptide is fused with a polypeptide heterologous to the exosome naturally produced by the same cell, such as the ACE2 polypeptide. Exemplary anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or

an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins. A detailed review regarding GPI anchors in exosome is available from, e.g., Michel Vidal, Exosomes and GPI-anchored proteins: Judicious pairs for investigating biomarkers from body fluids, Advanced Drug Delivery Reviews, Volumes 161–162, 2020 (incorporated herein by reference in its entirety). In a preferable embodiment of the present disclosure, the anchoring polypeptide comprises or consists of the transmembrane helix 3 (TM3) of CD63 protein.

[0026] As used herein, the term "antibody" generally refers to a polypeptide of the immunoglobulin family that is capable of binding a corresponding antigen non-covalently, reversibly, and in a specific manner. For example, a naturally occurring IgG antibody is a tetramer comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain (HC) is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain (LC) is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

[0027] The term "antibody" includes, but is not limited to, monoclonal antibodies, human antibodies, humanized antibodies, camelid antibodies, and chimeric antibodies. The antibodies can be of any isotype/class (e.g., IgG, IgE, IgM, IgD, IgA and IgY) or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). The term "antibody" as used herein also includes an antigen binding fragment of the antibody referred to.

[0028] As used herein, the term "antigen binding fragment" generally refers to a polypeptide including one or more portions of an antibody that retain the ability to specifically

interact with (e.g., by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Examples of binding fragments include, but are not limited to, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the VH and CH1 domains; a Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a dAb fragment (Ward et al., Nature 341: 544-546, 1989), which consists of a VH domain; and an isolated complementarity determining region (CDR) or other epitope-binding fragments of an antibody.

[0029] Antigen binding fragments also comprises single domain antibodies, maxibodies, minibodies, nanobodies, intrabodies, diabodies, triabodies, tetrabodies and bis-scFv (see, e.g., Hollinger and Hudson, Nature Biotechnology 23: 1126-1136, 2005). Antigen binding fragments can also comprise single chain molecules comprising a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al, Protein Eng. 8: 1057-1062, 1995). A fragment of a conventional antibody may also be a single domain antibody, such as a heavy chain antibody or VHH.

[0030] As used herein, the term "monoclonal antibody" as used herein generally refers to polypeptides, including antibodies and antigen binding fragments that have substantially identical amino acid sequence or are derived from the same genetic source. This term also includes preparations of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

[0031] As used herein, the term "polypeptide" is used herein interchangeably with the term "protein" and refers to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. Unless otherwise indicated, a particular polypeptide sequence also implicitly encompasses conservatively modified variants thereof.

[0032] Methods

[0033] One aspect of the present disclosure relates to a method of improving a loading amount of a polypeptide of interest in an engineered exosome, the method comprising (a) constructing a vector comprising a polynucleotide encoding the polypeptide of interest and a Fc domain, (b) transfecting a donor cell with the vector, (c) culturing the donor cell in a condition to allow an expression of the polynucleotide, so that a fusion protein comprising the polypeptide of interest and the Fc domain is formed, and

(d) isolating an exosome from the culture of the donor cell to obtain the engineered exosome loaded with the fusion protein, wherein the loading of the polypeptide of interest, when fused to the Fc domain, is increased compared to that without the Fc domain.

- [0034] In some embodiments of the present disclosure, the Fc domain may be located at either N terminus or C terminus of the polypeptide of interest. In some embodiments, the Fc domain is located at C terminus of the polypeptide of interest. In some embodiments, the Fc domain is located at N terminus of the polypeptide of interest.
- [0035] In some embodiments of the present disclosure, the Fc domain may be located at either N terminus or C terminus of the fusion protein. In some embodiments, the Fc domain is located at C terminus of the fusion protein. In some embodiments, the Fc domain is located at N terminus of the fusion protein.
- [0036] In the present disclosure, the Fc domain may be fused to the polypeptide of interest directly or via a linker. The linker may preferably be a peptide linker. The peptide linker could be any peptide linker that is available in the art useful for linking different domains or functional regions in a fusion protein. In some embodiments, the peptide linker consists of glycine and serine, e.g., (G4S)_n, in which n is an integer from 1 to 3. In preferable embodiments, the Fc domain is fused to the polypeptide of interest directly, for example, at the C terminus of the polypeptide of interest.
- [0037] Therefore, the fusion protein may have the structures of:
- [0038] [NH₂] - [POI] - [linker] - [Fc] - [COOH] ,
- [0039] [NH₂] - [POI] - [Fc] - [COOH] ,
- [0040] [NH₂] - [Fc] - [linker] - [POI] - [COOH] , or
- [0041] [NH₂] - [Fc] - [POI] - [COOH] ,
- [0042] wherein [NH₂] and [COOH] represent N and C termini of the fusion protein, [POI] represents the polypeptide of interest, [Fc] represents Fc domain, [linker] represents a peptide linker, and "-" represents a covalent bond.
- [0043] In some embodiments, the fusion protein may comprise more than one polypeptide of interest and one or more Fc domain. In these embodiments, the fusion protein may comprise two different polypeptides of interest and one or more Fc domain, for example, located at the C terminus of the second polypeptides of interest in the N to C termini direction. A peptide linker may be present between different polypeptides of interest. An exemplary peptide linker has an amino acid sequence shown in SEQ ID NO: 11, and a nucleotide sequence shown in SEQ ID NO: 12.
- [0044] For example, the fusion protein may have the structures of:
- [0045] [NH₂] - [POI 1] - [linker] - [POI 2] - [Fc] - [COOH] , or
- [0046] [NH₂] - [Fc] - [POI 1] - [linker] - [POI 2] - [COOH] ,

- [0047] wherein [NH₂] and [COOH] represent N and C termini of the fusion protein, [POI 1] represents a first polypeptide of interest, [POI 2] represents a second polypeptide of interest, [FC] represents Fc domain, [linker] represents a peptide linker, and “-” represents a covalent bond.
- [0048] In the present disclosure, the Fc domain may be derived from any species, preferably from a mammalian, for example a non-human primate or human. In preferable embodiments, the Fc domain is a human Fc domain. Preferably, the Fc domain may be derived from human IgG, IgA, IgE, or IgM. In particularly preferable embodiments, the Fc domain may be derived from human IgG1, IgG2, IgG3 or IgG4. Illustratively, the Fc domain may have an amino acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO: 3. Other exemplary Fc domains are available from National Center for Biotechnology Information (NCBI) through accession numbers such as 6P6D_A, 4L4J_B, 5W38_B, or 4C55_B.
- [0049] The polypeptide of interest (also referred to herein as cargo or protein cargo) can be any polypeptide desirable to be delivered by the engineered exosome. In some embodiments, the polypeptide of interest is selected from a group consisting of a cytokine, a hormone, an antibody, a transporter, an enzyme, a receptor, a ligand, a membrane protein, an antigen, a neoantigen, a ribonuclear protein, a nucleic acid binding protein, and a reporter protein.
- [0050] It will be appreciated that size may be a factor in choosing the most suitable protein cargos. In certain embodiments the cargo is no more than about 160 kDa, preferably no more than about 120 kDa, and most preferably no more than about 80 kDa. It will be appreciated that, in general, the smaller the protein the easier the delivery of the protein.
- [0051] Exemplary polypeptides of interest include, but are not limited to, antibodies, intrabodies, nanobodies, scFvs, affibodies, bi- and multispecific antibodies or binders including bispecific T-cell engagers (BiTEs), receptors, ligands, transporters, enzymes for e.g. enzyme replacement therapy (ERT) or gene editing, tumour suppressors (e.g., PTEN), viral or bacterial inhibitors, cell component proteins, DNA and/or RNA binding proteins, DNA repair inhibitors, nucleases, proteinases, integrases, transcription factors, growth factors, apoptosis inhibitors and inducers, toxins (for instance pseudomonas exotoxins), structural proteins, neurotrophic factors such as NT3/4, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and its individual subunits such as the 2.5S beta subunit, ion channels, membrane transporters, proteostasis factors, proteins involved in cellular signaling, translation- and transcription related proteins, nucleotide binding proteins, protein binding proteins, lipid binding proteins, glycosaminoglycans (GAGs) and GAG-binding

proteins, metabolic proteins, cellular stress regulating proteins, inflammation and immune system regulating proteins such as cytokines and inhibitors of such cytokines (cytokines may include: CXCL8, GMCSF, interleukins including: IL-1 family, IL-2, IL-4, IL-6, IL-6-like, IL-9, IL-10, IL12, IL-13, IL-17, Interferons including INF-alpha/beta/gamma, TNF family members, CD40 and CD40L, TRAIL, and TGF-beta family) mitochondrial proteins, and heat shock proteins, etc. The cargo may be a reporter protein such as GFP or nanoLuc.

- [0052] In one preferred embodiment, the encoded protein is a CRISPR-associated (Cas) polypeptide (such as Cas6, Cas9, Cas12, Cas12a/cpf1, Cas13) with intact nuclease activity which is associated with (i.e., carries with it) an RNA strand that enables the Cas polypeptide to carry out its nuclease activity in a target cell once delivered by the peptide. Alternatively, in another preferred embodiment, the Cas polypeptide may be catalytically inactive, to enable targeted genetic engineering. Yet another alternative may be any other type of CRISPR effector such as the single RNA guided endonuclease Cpf1. The inclusion of Cpf1 is a particularly preferred embodiment as herein disclosed, as it cleaves target DNA via a staggered double-stranded break. In yet another exemplary embodiment, the Cas polypeptide may also be fused to a transcriptional activator (such as the P3330 core protein), to specifically induce gene expression.
- [0053] Additional preferred embodiments include therapeutic protein cargos selected from the group comprising enzymes or transporters for lysosomal storage disorders, such as: glucocerebrosidases such as imiglucerase, alpha-galactosidase, alpha-L-iduronidase, iduronate-2-sulfatase and idursulfase, arylsulfatase, galsulfase, acid-alpha glucosidase (GAA), sphingomyelinase, galactocerebrosidase, galactosylceramidase, ceramidase, alpha-N-acetylgalactosaminidase, beta-galactosidase, lysosomal acid lipase, acid sphingomyelinase, NPC1, NPC2, heparan sulfamidase, N-acetylglucosaminidase, heparan- α -glucosaminide-N-acetyltransferase, N-acetylglucosamine 6-sulfatase, galactose-6-sulfate sulfatase, galactose-6-sulfate sulfatase, hyaluronidase, alpha N-acetyl neuraminidase, GlcNAc phosphotransferase, mucolipin 1, palmitoylprotein thioesterase, tripeptidyl peptidase I, palmitoyl-protein thioesterase 1, tripeptidyl peptidase 1, battenin, linclin, alpha-D-mannosidase, beta-mannosidase, aspartylglucosaminidase, alpha-L-fucosidase, cystinosis, cathepsin K, sialin, LAMP2, and hexoaminidase.
- [0054] Additional preferred embodiments include therapeutic protein cargos selected from the group comprising enzymes associated with Urea cycle disorders including N-Acetylglutamate synthase, carbamoyl phosphate synthetase, ornithine transcarbamoylase, argininosuccinic acid synthase, argininosuccinic acid lyase, arginase,

mitochondrial ornithine transporter, citrin, y+L amino acid transporter 1, uridine monophosphate synthase UMPS.

- [0055] In other preferred embodiments, the cargo may be e.g. an intracellular protein that modifies inflammatory responses, for instance epigenetic proteins such as methylases and bromodomains, or an intracellular protein that modifies muscle function, e.g. transcription factors such as MyoD or Myf5, proteins regulating muscle contractility e.g. myosin, actin, calcium/binding proteins such as troponin, or structural proteins such as Dystrophin, mini-dystrophin, micro-dystrophin, utrophin, titin, nebulin, dystrophin-associated proteins such as dystrobrevin, syntrophin, syncoilin, desmin, sarcoglycan, dystroglycan, sarcospan, agrin, and/or fukutin. The cargos are typically proteins or peptides of human origin unless indicated otherwise by their name, any other nomenclature, or as known to a person skilled in the art, and they can be found in various publicly available databases such as Uniprot, RCSB, etc.
- [0056] In another preferred embodiment the cargo is an antigen/neoantigen, optionally wherein the antigen/neoantigen is suitable for use in cancer immunotherapy.
- [0057] Any antigen/neoantigen may be incorporated into the exosomes of the present disclosure. The antigens may be suitable for raising immune responses against pathogens such as bacteria, viruses, fungi or the antigen may be a tumor antigen useful in eliciting an immune response against a tumor for cancer immunotherapy. There may be one or more antigens/neoantigens present in any exosome according to the disclosure. The one or more antigens/neoantigens may be endogenous/autologous (coming from the subject itself) or exogenous/allogenic (coming from another subject) or in the case of more antigens/neoantigens being incorporated into/onto the exosomes the antigens/neoantigens may be any mix of autologous /allogenic antigens. Preferably the antigens are autologous. Moreover, the one or more antigens/neoantigens may have any origin such as e.g., viral or bacterial or may be a tumor antigen and furthermore may be immunostimulatory or immunosuppressive or a combination thereof. The antigen/neoantigen may be useful in the treatment of any disease by immunotherapy. The treatment of cancer by immunotherapy is a particularly preferred embodiment. Where the antigen is a neo-antigen, it may be identified by sequencing of a tumor to identify the neo-antigen.
- [0058] Exemplary tumor antigens are: Alphafetoprotein (AFP) , Carcinoembryonic antigen (CEA) , CA-125, MUC-1 , Epithelial tumor antigen (ETA) , Melanoma-associated antigen (MAGE) , WT-1 , NY-ESO-1 , LY6K, IMP3, DEPDC1 , CDCA-1 , abnormal products of ras, p53, KRAS, or NRAS, CTAG1 B, peptides derived from chromosomal translocations such as BCR-ABL or ETV6-AML1 , viral antigens such as peptides from HPV-related cancers, peptides derived from proteins such as tyrosinase, gp100/

pmel17, Melan-A/MART-1 , gp75/TRP1 , or TRP2, and overexpressed antigens such as MOK (RAGE-1) , ERBB2 (HER2/NEU) .

[0059] Where the cargo is an antigen or neoantigen the exosome or pharmaceutical composition comprising the exosome may optionally further comprise at least one adjuvant. Where the antigen is administered with an adjuvant to stimulate the immune response the adjuvant may be: an inorganic compound: such as aluminium hydroxide, aluminium phosphate, calcium phosphate hydroxide, a mineral oil such as paraffin oil, bacterial products such as killed bacteria *Bordetella pertussis*, *Mycobacterium bovis*, toxoids, a nonbacterial organic such as squalene, a detergent such as Quil A, a plant saponin, a cytokine such as IL-1 , IL-2, IL-12, or RIBI (muramyl dipeptides) or immunostimulating complexes (ISCOM) such as stimulator of interferon genes (STING) agonists which can include cyclic dinucleotides. Such adjuvants may protect the therapeutic exosome from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Adjuvants that can be incorporated to a vaccine are well-known by the person skilled in the art and will be selected, in such a way that they do not negatively affect the immunological activity of the exosome.

[0060] In the present disclosure, the fusion protein may be packaged within the lumen of the engineered exosome or displayed on the membrane of the engineered exosome. In some embodiments, the fusion protein is anchored on a membrane of the engineered exosome via an anchoring polypeptide. In these embodiments, the polynucleotide further encodes an anchoring polypeptide, so that the fusion protein further comprises the anchoring polypeptide.

[0061] In some embodiments, the anchoring polypeptide is a membrane protein of exosome, a membrane-targeting sequence, or an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs) , lactadherin (C1C2 domain) , vesicular stomatitis virus glycoprotein (VSVG) , prostaglandin F2 receptor negative regulator (PTGFRN) , an integrin, and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins. In preferable embodiments of the present disclosure, the anchoring polypeptide comprises a TM3 domain of CD63. In preferable embodiments, the anchoring polypeptide comprises a TM3 domain of CD63. In some embodiments, the anchoring polypeptide is full-length CD63 proteins, e.g., full-length human CD63 (see e.g., UniProtKB/Swiss-Prot: F8VZE2, P08962, Q5TZP3, Q8N6Z9, or Q9UCG6) . In some embodiments, the anchoring polypeptide is a truncated CD63 proteins that

comprises a TM3 domain and at least one of TM1, TM2, and TM4 domains. For example, the anchoring polypeptide may consist of TM2 and TM3 of CD63; TM3 and TM4 of CD63; or TM1, TM2, and TM3 of CD63. In some embodiments, the anchoring polypeptide consists of TM3 domain of CD63.

- [0062] In some embodiments, the fusion protein has one of the following structures:
- [0063] [NH₂] - [AP] - [linker] - [POI] - [Fc] - [COOH] ,
- [0064] [NH₂] - [Fc] - [AP] - [linker] - [POI] - [COOH] ,
- [0065] [NH₂] - [POI] - [Fc] - [linker] - [AP] - [COOH] , or
- [0066] [NH₂] - [Fc] - [POI] - [linker] - [AP] - [COOH] ,
- [0067] wherein [NH₂] and [COOH] represent N and C termini of the fusion protein, [POI] represents the polypeptide of interest, [AP] represents anchoring polypeptide, [Fc] represents Fc domain, [linker] represents a peptide linker, and “-” represents a covalent bond.
- [0068] In preferable embodiments, the fusion protein has the Fc domain right at the N or C terminus of the fusion protein, depending on the specific AP used. Therefore, in one preferable embodiment, the fusion protein has the structure of [NH₂] - [AP] - [linker] - [POI] - [Fc] - [COOH] , in which the Fc domain is at the C-terminus of the fusion protein, when the N-terminus of the fusion protein is located in the lumen of an exosome. In another preferable embodiment, the fusion protein has the structure of [NH₂] - [Fc] - [POI] - [linker] - [AP] - [COOH] , in which the Fc domain is at the N-terminus of the fusion protein, when the C-terminus of the fusion protein is located in the lumen of an exosome.
- [0069] In preferable embodiments, the TM3 domain of CD63 comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO: 1. In preferable embodiments, the TM3 domain of CD63 comprises an amino acid sequence as shown in SEQ ID NO: 1. In preferable embodiments, the TM3 domain of CD63 consists essentially of an amino acid sequence as shown in SEQ ID NO: 1.
- [0070] In preferable embodiments, the fusion protein has the structure of [NH₂] - [TM3] - [linker] - [POI] - [Fc] - [COOH] . In the preferable embodiments, the fusion protein, when anchored on an exosome, has the Fc domain located at the C terminus of the fusion protein and external to the membrane of the exosome.
- [0071] In the present disclosure, the donor cell is any cell line suitable for exosome production. In some embodiments, the donor cell is a stem cell, for example, a mesenchymal stem cell. In some embodiment, the donor cell is not a stem cell. In preferable embodiments, the donor cell is a mammalian cell, such as a CHO or HEK cell.

- [0072] In some embodiment, the loading amount of a polypeptide of interest in an engineered exosome is improved at least by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800%, 900%, or more, compared to the loading amount of the polypeptide of interest in the engineered exosome without fusion to a Fc domain. In some embodiment, the loading amount of a polypeptide of interest in an engineered exosome is 2 to 100 times (e.g., 2, 5, 10, 20, 50, 80 or 100 times) more than the loading amount of the polypeptide of interest in the engineered exosome without fusion to a Fc domain.
- [0073] Evaluation of protein cargo loading into exosomes includes, but are not limited to, Western blotting, Enzyme-Linked Immunosorbent Assay (ELISA), conventional Flow cytometry of EVs coupled to beads, and Mass Spectrometry, all of which are widely used in the art. More recently, several techniques have been described for single-vesicle analysis to allow for more accurate characterization of protein cargo loading, with some of them reaching single-molecule resolution. These comprise Transmission Electron Microscopy (TEM) coupled with immunogold labelling, Atomic Force Microscopy, Super-Resolution Fluorescence Microscopy, Fluorescence Correlation Spectroscopy, Nanoflow cytometry, Imaging Flow cytometry, Single-Particle Interferometric Reflectance Imaging Sensing (SP-IRIS), and Laser tweezers Raman spectroscopy, amongst others. For a detailed description of these methods see Silva, Andreia M et al. "Quantification of protein cargo loading into engineered extracellular vesicles at single-vesicle and single-molecule resolution." *Journal of extracellular vesicles* vol. 10, 10 (2021) : e12130.
- [0074] Engineered Exosome Loaded with Polypeptide of Interest
- [0075] Another aspect of the disclosure provides an engineered exosome loaded with a fusion protein comprising a polypeptide of interest and a Fc domain, wherein the polypeptide is loaded at a higher amount compared to that when the polypeptide of interest is not fused to the Fc domain.
- [0076] In some embodiments, the fusion protein further comprises an anchoring polypeptide, and the polypeptide of interest is anchored on a membrane of the engineered exosome via the anchoring polypeptide.
- [0077] The anchoring polypeptide, and the Fc domain has the same definition and preferable embodiments as described in the above in the Methods section.
- [0078] In some embodiments, the polypeptide of interest is not a polypeptide binding to CD206 or an antibody. In preferable embodiments, the polypeptide of interest is selected from a group consisting of a cytokine, a hormone, a transporter, an enzyme, a receptor, a ligand, a membrane protein, an antigen, a neoantigen, a ribonuclear protein, a nucleic acid binding protein, and a reporter protein. In more preferable

embodiments, the polypeptide of interest is selected from a group consisting of a cytokine, a hormone, an antigen, and a neoantigen.

[0079] In some embodiments, the engineered exosome may comprise more than one kind of fusion protein, with each kind of fusion protein comprising a different polypeptide of interest, and optionally a different anchoring polypeptide and/or a different Fc domain.

[0080] For example, the engineered exosome may comprise a first fusion protein comprising a first anchoring polypeptide, a first polypeptide of interest, and a Fc domain; a second fusion protein comprising a second anchoring polypeptide, a second polypeptide of interest, and a Fc domain; and a third fusion protein comprising a third anchoring polypeptide, a third polypeptide of interest, and a Fc domain, wherein the first, second, and third anchoring polypeptide can be different or same. In preferable embodiments, the first, second, and third anchoring polypeptides are same and consist of TM3 domain of CD63.

[0081] In alternative embodiments, the engineered exosome may comprise more or less kinds of fusion proteins, e.g., 2 or 4 kinds of fusion proteins.

[0082] In the embodiments where two or more kinds of fusion proteins are present, it is preferable that the fusion proteins are expressed from a single polynucleotide in which the fusion proteins are linked through a fragment of nucleotides encoding a self-cleavage peptide, such as a 2A peptide, e.g., T2A, E2A, P2A or any combination thereof. For example, the self-cleavage peptide is a T2A peptide. An exemplary T2A peptide has an amino acid sequence shown in SEQ ID NO: 13, and a nucleotide sequence shown in SEQ ID NO: 14.

[0083] In some embodiments, a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO: 2 or a degenerate sequence thereof.

[0084] In some embodiments, provided is a composition comprising the engineered exosome and a carrier. In preferable embodiment, the composition is formulated for topical or systemic administration. In preferable embodiment, the composition is a pharmaceutical composition comprising a pharmaceutically acceptable carrier. In preferable embodiment, the composition is a cosmetic composition comprising a cosmetically acceptable carrier.

[0085] Sequences Listings

SEQ ID NO:	Description	Sequences
1	CD63 TM3 AA	YCLMITFAIFLSLIMLVEVAAAIAGYVFRDKVMSEFNNNF RQQMENYPKNNHTA
2	CD63 TM3 DNA	TACTGTCTGATGATCACCTTCGCCATCTTTCTGTCCCTG ATCATGCTGGTGGAGGTGGCCGCCGCCATCGCCGGATA CGTGTTTCAGAGACAAGGTGATGAGCGAGTTCAACAAT AATTTTAGACAGCAGATGGAGAATTACCCTAAGAATAA CCACACCGCC
3	Human Fc (hFc) AA	AEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSAYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG K
4	Human Fc (hFc) DNA	GCCGAGCCCAAGTCCTGCGACAAGACCCACACATGTC CTCCCTGCCCTGCTCCTGAGCTGCTGGGCGGACCCAG CGTGTTTCTGTTCCCAACCAAGCCTAAGGACACACTGA TGATCTCCAGAACACCCGAGGTGACATGTGTGGTGGT GGACGTGAGCCACGAGGACCCAGAGGTGAAGTTTAAT TGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGA CCAAGCCCAGGGAGGAGCAGTACAATAGCGCCTACAG GGTGGTGTCCGTGCTGACAGTGCTGCACCAGGATTGG CTGAATGGCAAGGAGTACAAGTGCAAGGTGTCCAATA AGGCCCTGCCCCTCCTATCGAGAAGACCATCAGCAA GGCCAAGGGCCAGCCTAGGGAGCCTCAGGTGTACACC CTGCCTCCTAGCAGAGACGAGCTGACCAAGAACCAGG TGAGCCTGACATGCCTGGTGAAGGGCTTTTACCCTAGC GATATCGCCGTGGAGTGGGAGAGCAATGGCCAGCCTG AGAACAACACTACAAGACCACACCTCCCCTGCTGGATT CGATGGCAGCTTTTCTGTACAGCAAGCTGACAGTGG ACAAGAGCAGATGGCAGCAGGGCAACGTGTTTCAGCTG CAGCGTGATGCACGAGGCCCTGCACAACCACTACACC

		CAGAAAGAGCCTGTCCCTGAGCCCTGGCAAG
5	Anti-FC5 VHH AA	EVQLQASGGGLVQAGGSLRLSCAASGFKITHYTMGWFR QAPGKEREVFSRITWGGDNFYSNSVKGRFTISRDNAKN TVYLMNSLKPEDTADYYCAAGSTSTATPLRVDYWGKG TQVTVSS
6	Anti-FC5 VHH DNA	GAGGTGCAGCTGCAGGCTTCCGCGCGCGGACTGGTGC AGGCTGGAGGAAGCCTGAGACTGAGCTGCGCCGCCTC CGGCTTTAAGATCACCCACTACACAATGGGCTGGTTCA GACAGGCTCCTGGCAAGGAGAGAGAGTTTGTGAGCA GGATCACATGGGGCGGCGATAATACCTTCTACAGCAAC AGCGTGAAGGGCAGGTTTACCATCAGCAGAGACAACG CCAAGAACACAGTGTACCTGCAGATGAACTCCCTGAA GCCTGAGGATACCGCCGATTACTACTGTGCCGCCGGCA GCACCAGCACCGCCACACCTCTGAGAGTGGATTACTG GGGCAAGGGCACCCAGGTGACCGTGTCCAGC
7	Anti-TfR scFv AA	MASYELTQPPSVSVAPGQTARITCSGDALGNKYASWYQQ KPGQAPVLVIYEDSKRPSGIPERFSGSNSGNTATLTISGTQ AEDEADYYCSSGDSPCRAFGGGTKLTVLGS GGSTITSYN VYYTKLSSSGSEVQLVESGGGLVQPGGSLRLSCAASGFT FSSYAMSWVRQAPGKGLEWVSAISGGSGSTYYADSVKG RFTISRDN SKNTLYLQMNSLRAEDTAVYYCARHSIYRCFF AVWGQGT LTVSS
8	Anti-TfR scFv DNA	ATGGCCAGCTACGAGCTGACCCAGCCTCCTAGCGTGTG CGTGGCTCCTGGCCAGACCGCCAGGATCACCTGCAGC GGCGATGCCCTGGGCAACAAGTACGCCTCCTGGTACC AGCAGAAGCCTGGCCAGGCTCCTGTGCTGGTGATCTA CGAGGACAGCAAGAGACCCAGCGGCATCCAGAGAG GTTTCAGCGGCAGCAACAGCGGCAACACAGCCACACTG ACCATCTCCGGCACCCAGGCCGAGGACGAGGCCGATT ACTACTGCTCCAGCGGCGATAGCCCATGTAGAGCCTTC GGCGGCGGCACCAAGCTGACAGTGCTGGGCTCCGGCG GCTCCACCATCACCTCCTACAATGTGTACTACACCAAG CTGTCTCCTCAGCGGCTCCGAGGTGCAGCTGGTGGAGA GCGGCGGCGGACTGGTGCAGCCAGGAGGAAGCCTGA GACTGTCTGCGCCGCCAGCGGCTTCACATTCAGCAG CTACGCCATGAGCTGGGTGAGGCAGGCTCCTGGCAAG GGCCTGGAGTGGGTGAGCGCCATCAGCGGCAGCGGCG GAAGCACCTACTACGCCGATTCCGTGAAGGGCAGGTT CACAATCAGCAGGGACAATTCCAAGAATACCCTGTACC TGCAGATGAATTCCCTGAGGGCCGAGGATACAGCCGT GTACTACTGTGCCAGACACAGCATCTACAGATGCTTCT TCGCCGTGTGGGGCCAGGGCACACTGGTGACAGTGAG CAGC
9	ACE2 AA	QSTIEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITE

		ENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEIQNLTV KLQLQALQQNGSSVLSSEDKSKRLNTILNTMSTIYSTGKV CNPDPNPQECLLLEPGLNEIMANSLDYNERLWAWESWRS EVGKQLRPLYEEYVVLKNEMARANHYEDYGDYWRGD YEVNGVDGYDYSRGQLIEDVEHTFEEIKPLYEHLHAYVR AKLMNAYPSYISPIGCLPAHLLGDMWGRFWTNLYSLTVP FGQKPNIDVTDAMVDQAWDAQRFKEAEKFFVSVGLPN MTQGFWENSMLTDPGNVQKAVCHPTAWDLGKGDFRIL MCTKVTMDDFLTAHHEMGHIQYDMAYAAQPFLLRNGA NEGFHEAVGEIMSLSAATPKHLKSIGLLSPDFQEDNETEI NFLKQALTIVGTLPTFTYMLEKWRWMVFKGEIPKDQW MKKWWEMKREIVGVVEPVPHDETYCDPASLFHVSNDY SFIRYYTRTLYQFQFQEAALCQAAKHGEPHLKCDISNSTEA GQKLFNMLRLGKSEPWTALENVVGAKNMNVRPLLNY FEPLFTWLKDQNKNSFVGWSTDWSPYADQSIKVRISLKS ALGDKAYEWNNDNEMYLFRSSVAYAMRQYFLKVKNQMI LFGEDVRVANLKPRI SFNFVVTAPKNVSDIIPRTEVEKAI RMSRSRINDAFRLNDNSLEFLGIQPTLGPPNQPPVS
10	ACE2 DNA	GCTCCAATGGCCGAGGGAGGAGGCCAGAACCACCAC GAGGTGGTGAAGTTTATGGACGTGTACCAGAGATCCTA CTGTCACCCTATCGAGACCCTGGTGGATATCTTTCAGG AGTACCCAGACGAGATCGAGTACATCTTCAAGCCCTCC TGTGTGCCTCTGATGAGATGCGGCGGCTGCTGCAATGA CGAGGGCCTGGAGTGTGTGCCTACCGAGGAGTCCAAT ATCACAATGCAGATCATGAGAATCAAGCCTCATCAGGG CCAGCACATCGGCGAGATGAGCTTCTGCAGCACAAT AAGTGTGAGTGCAGGCCTAAGAAGGACAGGGCCAGG CAGGAGAAGAAGTCCGTGAGGGGCAAGGGCAAGGGC CAGAAGAGGAAGAGAAAGAAGAGCAGATACAAGTCC TGGTCCGTGTACGTGGGCGCCAGATGTTGTCTGATGCC CTGGTCCCTGCCTGGCCCACACCCTTGTGGCCCATGTT CCGAGAGGAGGAAGCACCTGTTCGTGCAGGACCCACA GACCTGCAAGTGCTCCTGTAAGAATACCGATAGCAGGT GCAAGGCCAGACAGCTGGAGCTGAATGAGAGGACCT GCAGATGTGACAAGCCCAGAAGG
11	Linker AA	GGGSGGGSGGGGS
12	Linker DNA	GGCGGCGGCGGCTCCGAGGAGGAGGAAGCGGAGGA GGCGGCAGC
13	T2A AA	GSGEGRGSLTCDVEENPGP
14	T2A DNA	GGCAGCGGCGAAGGCCGCGGCAGCCTGCTGACCTGCG GCGATGTGGAAGAAAACCCGGGCCCG

AA: Amino Acid Sequence

Examples

- [0086] Material and Methods
- [0087] Cell Lines
- [0088] The HEK-293 cell line (human embryonic kidney 293 cells) was purchased from the Cell Bank of the representative culture preservation committee of the Chinese Academy of Sciences (Shanghai, China) . HEK-293 cells were maintained in DMEM (high-glucose) containing 10% (vol/vol) FBS. The CHO-K1 cell line (Chinese Hamster Ovary Cell) was purchased from BeNa Culture Collection (Beijing, China) . CHO-K1 cells were maintained in F-12K (31765035, Thermo Fisher Scientific, United States) containing 10% (vol/vol) FBS, supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated in a humidified atmosphere containing 5%CO₂ at 37 °C.
- [0089] Antibodies
- [0090] Antibody used in this study was anti-CD63 antibodies (Cat. No. MA5-32085; Invitrogen) .
- [0091] Plasmid construction
- [0092] The amino acid sequences of CD63 (SEQ ID NO: 1) , Human Fc (SEQ ID NO: 3) and ACE2 (SEQ ID NO: 9) were derived from Uniprot. Amino acid sequences of anti-FC5 VHH (SEQ ID NO: 5) and Anti-TfR (SEQ ID NO: 7) were obtained from literatures. The corresponding DNA sequences were synthesized by General Biotechnology (Chuzhou, China) with plasmid pcDNA3.1 (+) (System Biosciences) . Recombinant Plasmids: CD63-L-FC5; CD63-L-FC-5-hFc; CD63-L-Anti-TfR; CD63-L-Anti-TfR-hFc; CD63-L-ACE2; CD63-L-ACE2-hFc; GFP was used as a target protein when scaffold protein was screening. CD63 designate TM3 of CD63, which has an amino acid sequence as shown in SEQ ID NO: 1; L represents linker (SEQ ID NO: 11) ; FC5 represents anti-FC5-VHH (SEQ ID NO: 5) ; hFc represent human IgG1 Fc (SEQ ID NO: 3) ; anti-TfR represents anti-TfR scFv (SEQ ID NO: 7) .
- [0093] Exosomes isolation
- [0094] The transfected HEK293 cell was seeded in T150 flasks for 24h, rinsed extensively with PBS and incubated in serum-free medium (SFM) for another 48h. The cultured cell-free extracellular medium containing exosomes was harvested by centrifugation at 300×g for 10 min to remove the cells. Then centrifuge at 10,000×g for 30 min to remove dead cells and cell debris. Finally, the clear supernatant was centrifuged for 70 min at 100,000×g to pellet the exosomes for twice. And the exosome pellet was re-suspended. All centrifugation steps were carried out at 4 °C.
- [0095] Characterization of Exosomes
- [0096] Analysis of the particle concentration and size distribution of exosomes

- [0097] The particle concentration and size distribution of exosomes from transfected HEK293 cells were analyzed by the nFCM (NanoFCM Inc., Xiamen, China). The nFCM analysis used two single photon counting avalanche photodiodes (APDs) to detect individual particle side scatter (SSC) and fluorescence simultaneously. Firstly, the exosomes pellet was prepared in PBS. Then, 200 nm PE and AF488 fluorophore-conjugated polystyrene beads were used for particle concentration and Silica Nanosphere Cocktail (NanoFCM Inc., Xiamen, China) for particle size distribution. The detector recorded particles passing by during a 1-min interval in each test. Each sample was diluted to reach a particle count within the optimal range of 3000-9,000 particles per minute. NanoFCM software (NanoFCM Profession V2.0) was used to convert flow rate and side scattering intensity to vesicle concentration and size. The percentage of GFP, the fused protein of the surface scaffold proteins was analyzed by NanoFCM.
- [0098] Identification of scaffold or target proteins on exosomes via Western blotting (WB)
- [0099] For the identification of target proteins expressed on exosomes, the purified exosomes were lysed with RIPA lysis buffer (Beyotime) supplemented with 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Beyotime) and phosphatase inhibitor (Beyotime), then heat denatured, separated by SDS-PAGE, and transferred onto PVDF membrane (Millipore, USA). The proteins were detected by incubation with an appropriate primary antibody, followed by incubation with a HRP-conjugated secondary antibody (Invitrogen). Enhanced chemiluminescence reagent (Millipore, MA, USA) was then used for the visualization of the membranes.
- [0100] Binding assay of ACE2 with spike protein
- [0101] HEK293 cells were prepared for transfection with CD63-L-ACE2, or CD63-L-ACE2-Fc. Twenty-four hours later, transfected cells were rinsed extensively with PBS and incubated in SFM for another 48h. Then the exosomes were collected and purified following the above exosomes isolation procedure. The binding assay of ACE2 from the purified exosomes with spike protein was performed with COVID-19 Spike-ACE2 Binding Assay Kit (RayBiotech, CoV-SACE2-1). The ACE2 protein was used as a positive control.
- [0102] SARS-CoV-2 pseudovirus neutralization assay
- [0103] The pseudovirus neutralization assay of SARS-CoV-2 with exosomes derived from CD63-L-ACE2-Fc transfected HEK293 cells was performed by GenScript ProBio (Nanjing, China). Three SARS-CoV-2 stains were used in this study, including wild type SARS-CoV-2 pseudovirus (WT) (SC2087A), mutants SARS-CoV-2 pseudovirus (XBB. 1.5) (SC2087-037) and SARS-CoV-2 pseudovirus (BA. 4&BA. 5) (SC2087-031). The cell line Opti-HEK293/ACE2 (RD00825) was used for pseudovirus infection and analysis. The inhibition rate was defined for evaluating the

neutralization activity. The calculation was as the following: $\text{Inhibition\%} = 100\% \times (\text{Luminescence control} - \text{Luminescence Compound}) / \text{Luminescence control}$.

- [0104] Example 1. Fusion with a c-terminal human IgG Fc domain (hFC) increases the incorporation of fused cargo proteins onto exosomes.
- [0105] Two pairs of recombinant plasmids with or without hFC, CD63-L-FC5 and CD63-L-FC-5-hFc, CD63-L-Anti-TfR and CD63-L-Anti-TfR-hFc, were used for transfection and the exosomes were purified from the transfected cell supernatant. The immunoprecipitation analysis showed that more fused cargo proteins, FC5 and Anti-TfR mentioned, were sorting onto exosomes when similar expression level was shown on cells (see Figs. 1A and 1B) .
- [0106] Example 2. Accumulation of ACE2 fused with a C-terminal human IgG Fc domain on the surface of exosomes displayed remarkable neutralizing activity against SARS-CoV-2 pseudovirus.
- [0107] The binding assay (Fig. 2A) showed that exosomes derived from HEK293 cells transfected with plasmid ACE2-hFC displayed more than 2-fold binding activity when compared with exosomes from HEK293 cells transfected with ACE2 only, which suggested that more ACE2 protein was sorting onto the exosomes when fused with hFC. Moreover, the SARS-CoV-2 pseudovirus assay (Fig. 2B) confirmed that exosomes from HEK293 cells transfected with plasmid ACE2-hFC have high neutralizing activity against SARS-CoV-2 pseudovirus, not only the wild type, but also mutants XBB. 1.5 and BA.4 & BA.5.

Claims

- [Claim 1] A method of improving a loading amount of a polypeptide of interest in an engineered exosome, the method comprising
- (a) constructing a vector comprising a polynucleotide encoding the polypeptide of interest and a Fc domain,
 - (b) transfecting a donor cell with the vector,
 - (c) culturing the donor cell in a condition to allow an expression of the polynucleotide, so that a fusion protein comprising the polypeptide of interest and the Fc domain is formed, and
 - (d) isolating an exosome from the culture of the donor cell to obtain the engineered exosome loaded with the fusion protein,
- wherein the loading of the polypeptide of interest, when fused to the Fc domain, is increased compared to that without the Fc domain.
- [Claim 2] The method of claim 1, wherein the Fc domain has at least one feature selected from a group consisting of:
- (i) is located at N or C terminus of the polypeptide of interest;
 - (ii) is located at N or C terminus of the fusion protein;
 - (iii) is fused to the polypeptide of interest directly;
 - (iv) is a human Fc;
 - (v) is a Fc domain derived from human IgG, IgA, IgE, or IgM; and
 - (vi) has an amino acid sequence having at least 80% sequence identity to the amino acid sequence shown in SEQ ID NO: 3.
- [Claim 3] The method of claim 1 or 2, wherein the polynucleotide further encodes an anchoring polypeptide, so that the fusion protein further comprises the anchoring polypeptide, and the polypeptide of interest is anchored on a membrane of the engineered exosome via the anchoring polypeptide.
- [Claim 4] The method of claim 3, wherein the anchoring polypeptide is a membrane protein of exosome, a membrane-targeting sequence, or an anchoring functional fragment thereof; preferably, the membrane protein of exosome is selected from a group consisting of lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN), integrin, and any combination thereof; preferably the membrane-targeting sequence includes a glycosylphosphatidylinositol (GPI) anchor and a lipid-anchored protein; preferably,

- the anchoring polypeptide comprises a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, the anchoring polypeptide comprises a TM3 domain of CD63; more preferably, the anchoring polypeptide is a TM3 domain of CD63; preferably, the TM3 domain of CD63 comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 1.
- [Claim 5] The method of claim 3 or 4, wherein the fusion protein has a structure of [NH₂] - [AP] - [linker] - [POI] - [Fc] - [COOH] or [NH₂] - [Fc] - [POI] - [linker] - [AP] - [COOH], wherein [NH₂] and [COOH] represent N and C termini of the fusion protein, [POI] represents the polypeptide of interest, [AP] represents anchoring polypeptide, [Fc] represents Fc domain, [linker] represents a peptide linker, and “-” represents a covalent bond; more preferably, the fusion protein has the structure of [NH₂] - [TM3] - [linker] - [POI] - [Fc] - [COOH].
- [Claim 6] The method of any of claims 1 to 5, wherein the polypeptide of interest is selected from a group consisting of a cytokine, a hormone, an antibody, a transporter, an enzyme, a receptor, a ligand, a membrane protein, an antigen, a neoantigen, a ribonuclear protein, a nucleic acid binding protein, and a reporter protein.
- [Claim 7] The method of claim 1, wherein the donor cell is a mammalian cell; preferably, the donor cell is a HEK cell, CHO cell or a stem cell.
- [Claim 8] An engineered exosome loaded with a fusion protein comprising a polypeptide of interest and a Fc domain, wherein the polypeptide is loaded at a higher amount compared to that when the polypeptide of interest is not fused to the Fc domain.
- [Claim 9] The engineered exosome of claim 8, wherein the Fc domain has at least one feature selected from a group consisting of:
(i) is located at N or C terminus of the polypeptide of interest;
(ii) is located at N or C terminus of the fusion protein;
(iii) is fused to the polypeptide of interest directly;
(iv) is a human Fc;
(v) is a Fc domain derived from human IgG, IgA, IgE, or IgM; and
(vi) has an amino acid sequence having at least 80% sequence identity to the amino acid sequence shown in SEQ ID NO: 3.
- [Claim 10] The engineered exosome of claim 8 or 9, wherein the fusion protein further comprises an anchoring polypeptide, and the polypeptide of interest is anchored on a membrane of the engineered exosome via the anchoring polypeptide.

- [Claim 11] The engineered exosome of claim 10, wherein the anchoring polypeptide is a membrane protein of exosome, a membrane-targeting sequence, or an anchoring functional fragment thereof; preferably, the membrane protein of exosome is selected from a group consisting of lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN), integrin, and any combination thereof; preferably the membrane-targeting sequence includes a glycosylphosphatidylinositol (GPI) anchor and a lipid-anchored protein; preferably, the anchoring polypeptide comprises a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, the anchoring polypeptide comprises a TM3 domain of CD63; more preferably, the anchoring polypeptide is a TM3 domain of CD63; preferably, the TM3 domain of CD63 comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 1.
- [Claim 12] The engineered exosome of claim 10 or 11, wherein the fusion protein has a structure of [NH₂] - [AP] - [linker] - [POI] - [Fc] - [COOH] or [NH₂] - [Fc] - [POI] - [linker] - [AP] - [COOH], wherein [NH₂] and [COOH] represent N and C termini of the fusion protein, [POI] represents the polypeptide of interest, [AP] represents anchoring polypeptide, [Fc] represents Fc domain, [linker] represents a peptide linker, and "-" represents a covalent bond; more preferably, the fusion protein has the structure of [NH₂] - [TM3] - [linker] - [POI] - [Fc] - [COOH].
- [Claim 13] The engineered exosome of any of claims 8 to 12, wherein the polypeptide is not a polypeptide binding to CD206 or an antibody; preferably, the polypeptide of interest is selected from a group consisting of a cytokine, a hormone, a transporter, an enzyme, a receptor, a ligand, a membrane protein, an antigen, a neoantigen, a ribonuclear protein, a nucleic acid binding protein, and a reporter protein.
- [Claim 14] A composition comprising the engineered exosome of any of claims 8 to 13, and a carrier; preferably, the composition is a cosmetic or pharmaceutical composition; more preferably, the composition is formulated for topical or systemic administration.