Therapeutic delivery vesicles

Technical Field

The present invention pertains, inter alia, to therapeutic delivery vesicles, for instance exosomes or microvesicles, comprising polypeptide constructs, methods for producing said therapeutic delivery vesicles, pharmaceutical compositions and medical uses thereof.

Background Art

Exosomes and microvesicles are membrane-bound vesicles that differ based on their process of biogenesis and biophysical properties, including size and surface protein markers. Exosomes are homogenous small particles ranging from 40 to 150 nm in size and they are normally derived from the endocytic recycling pathway. In endocytosis, endocytic vesicles form at the plasma membrane and fuse to form early endosomes. These mature and become late endosomes where intraluminal vesicles bud off into an intra-vesicular lumen. Instead of fusing with the lysosome, these multivesicular bodies directly fuse with the plasma membrane and release exosomes into the extracellular space. Exosome biogenesis, protein cargo sorting, and release involve the endosomal sorting complex required for transport (ESCRT complex) and other associated proteins such as Alix and Tsg101.

In contrast, another type of extracellular vesicles, namely microvesicles, are produced directly through the outward budding and fission of membrane vesicles from the plasma membrane, and hence, their surface markers are largely dependent on the composition of the membrane of origin. Further, they tend to constitute a larger and more heterogeneous population of extracellular vesicles, ranging from 150 to 1000 nm in diameter. However, both types of

vesicles have been shown to deliver functional mRNA, miRNA and proteins to recipient cells.

To maintain a physiological balance in receptor signalling and response several receptors exists both in a membrane bound form and in a soluble form. The membrane form is normally capable of signalling, whereas the soluble form is signalling-incompetent. The soluble form often occurs as the extracellular part of the membrane-bound form. The soluble part is generated in two different ways: (1) alternative splicing of the pre-mRNA, and (2) by cleavage of extracellular proteases (often metalloproteases). The soluble form binds its ligand and thereby sequesters the ligand, inhibiting its binding with the membrane-bound form, meaning that the overall signalling from that pathway will decrease. The soluble form often increases when the signalling pathway is very active. For instance, the soluble forms of the two tumour necrosis factor receptor alpha (TNFRαs) increase in pathological conditions such as sepsis and inflammation in order to reduce the inflammatory process.

Decoy receptors have received substantial interest from a therapeutic point of view, since they provide a highly specific and tailored approach to decrease the physiological concentration of a protein of interest. The therapeutic modality is reliant on administration of decoy receptors in order to decrease the activity of a particular signaling pathway. Decoy receptors are often fused with the Fc-part of an antibody to increase their half-life and to increase the avidity of the receptors when two come in close range from each other. One example of this strategy is Etanercept, which is the sTNFR2 fused with an Fc-fragment. Etanercept is clinically approved for treating rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and moderately to severely active polyarticular juvenile idiopathic arthritis, and it has been shown to be safe and effective over the last 19 years since its initial approval. Several other decoy receptor fusion proteins are in clinical trials, targeting for example VEGF, EGF, FGF and angiopoietin.

Although successfully applied in various therapeutic contexts and for a large number of ailments, decoy receptors and other biologics (biopharmaceuticals) suffer from a number of drawbacks relating to for instance pharmacokinetics, toxicity, pharmacodynamics, and therapeutic efficacy.

Summary of the Invention

It is hence an object of the present invention to overcome the above-identified problems associated with the use of biologics, and specifically decoy receptors, and to satisfy the existing needs within the art, namely to provide for optimized therapeutic efficacy, significantly improved pharmacokinetics, as well as reduced side-effects of biopharmaceutical polypeptides, such as decoy receptors and biologics (biopharmaceuticals) in general.

Thus, the present invention pertains to, in a first aspect, a therapeutic delivery vesicle having attached to its membrane a polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor present at least partially on the outside of the delivery vesicle, and wherein the at least one therapeutic polypeptide decoy receptor is signalling-incompetent, i.e not capable of transmitting signals it transmits under normal circumstances. Normally the decoy receptor binds to and sequesters a circulating ligand or a ligand that may in fact also be present inside a target cell and/or on the surface of a target cell. Without wishing to be bound by any theory, it is surmised that therapeutic polypeptide decoy receptor present on the surface of therapeutic delivery vesicle sequesters its interaction partner(s), i.e. the target and/or ligand, in virtually the same manner as the free therapeutic polypeptide decoy receptor, albeit with a significantly improved half-life, reduced clearance, decreased side-effects, and generally significantly enhanced pharmacokinetics, and therapeutic efficacy, by virtue of its attachment on a

suitable delivery vesicle, for instance an exosome, a microvesicle, an apoptotic body, a liposome, or any other type of naturally derived or artificially produced vesicle. The at least one therapeutic polypeptide decoy receptor present on the therapeutic delivery vesicle may be partially or completely devoid of its signalling domain, so as to make it a signalling-incompetent therapeutic polypeptide receptor, and the signalling domain may be partially or completely replaced by the carrier polypeptide, but the incapacity to contribute to signalling may also derive from alterations in the polypeptide sequence. For instance, in various embodiments of the present invention it may be sufficient to replace certain amino acids to render the therapeutic polypeptide receptor signalling-incompetent, and/or the therapeutic polypeptide receptor may be rendered signalling-incompetent merely by attaching the decoy receptor to the carrier polypeptide (using recombinant technology) which is transporting the entire polypeptide construct to the surface of the extracellular vesicle.

Importantly, exosomes and other types of cell-derived vesicles (which constitute a conceivable source of delivery vesicles in accordance with the present invention) may have therapeutic activities per se. For example, vesicles derived from e.g. mesenchymal stem cells but also from other cells are known to be innately immunosuppressive as they carry several miRNAs, proteins and bioactive lipids that for instance suppress cytotoxic T-cells and trigger expansion of regulatory T-cells. The repressive effect on the immune system is a prerequisite also for subsequent tissue regeneration following tissue injury. Hence, choosing for instance an appropriate source of cells for derivation of exosomes and/or other types of extracellular vesicles will provide an additional therapeutic advantage as compared to using receptor decoys/monoclonal antibodies (such as etanercept and infliximab) only.

In further aspects, the instant invention relates to therapeutic delivery vesicles and pharmaceutical compositions comprising the vesicles in accordance with the present invention for use in medicine, and more specifically for use in the

treatment, alleviation, and/or prophylaxis of various diseases and disorders that may be treated using biopharmaceutical therapeutics (biologics).

Thus, the present invention essentially pertains to the use of exosomes and other vesicles (notably derivable from cellular and/or biological sources, but alternatively also artificially produced vesicles such as liposomes) as delivery or administration vehicles for biopharmaceuticals, specifically polypeptide-based biologics, and more specifically decoy receptors (also known as sink receptors). The present invention hence relates to the use of exosomes (and other types of vesicles) comprising various polypeptides, as defined herein, in the treatment of a large number of diseases and disorders, as herein disclosed.

In additional aspects, the instant invention pertains to methods of producing the therapeutic delivery vesicles of the present invention, generally comprising the steps of (i) providing at least one polynucleotide construct encoding at least one therapeutic polypeptide decoy receptor (which is preferably signalling-incompetent, for instance via being partially or completely devoid of its signalling domain) that binds to a suitable target and/or ligand, (ii) introducing said at least one polynucleotide construct into a cell capable of producing exosomes, and, (iii) collecting (harvesting) at least one delivery vesicle produced by the cell of step (ii). The present invention also relates to delivery vesicles produced by said methods, as well as various aspects and embodiments related to kits, compositions, and cell culture media in accordance with the present invention.

In further aspects, the present invention relates to methods for increasing the yield of extracellular vesicles (which may be in their native form, i.e. completely free from therapeutic polypeptide constructs) comprising exposing the cells (which are the source of the extracellular vesicles) to inhibitors of autophagy. In yet a further aspect, the present invention pertains to methods for increasing the regenerative capacity of extracellular vesicles, by exposing

the cell source to stress-inducing conditions (for instance oxygen deprivation and/or serum starvation). Exposure of vesicle-producing cells to stress-inducing conditions results in enrichment of metabolically active proteins and/or anti-apoptotic proteins, which leads to enhanced regenerative effects.

The present invention thus provides delivery vesicles, methods, compositions, and uses, as well as various other aspects and embodiments, for improving the delivery, administration, and characteristics of e.g. biopharmaceutical polypeptide agents. The present invention results in optimized therapeutic efficacy (for instance due to therapeutic polypeptide decoy receptor multivalency and the inherent regenerative therapeutic effects of exosomes *per se*), significantly improved pharmacokinetics (via e.g. reduced renal clearance), improved biodistribution to certain organs, such as the brain, as well as reduced side-effects of biopharmaceutical polypeptides (via e.g. fusion with recipient cells to confer direct cellular protection), such as decoy receptors and other types of biologics.

Brief Description of the Drawings

Figure 1 shows treatment efficacy, in a mouse colitis model, of exosomes comprising a signalling-incompetent therapeutic polypeptide decoy receptor, which is comprised of a signalling-incompetent soluble tumour necrosis factor receptor 1 (sTNFR1) (which may be either one of sTNFR 1A (SEQ ID No 30) or sTNFR 1B (SEQ ID No 31)), fused to the carrier polypeptide CD63 (SEQ ID No 14). The exosomes displaying signalling-incompetent sTNFR1-CD63 successfully treat the induced colitis (solid black line) with only a minor initial loss in body weight. Signalling-competent exosomes (short-dashed grey line) show clear anti-therapeutic efficacy since they deliver more signalling receptors to the cells, whereas mock-treated mice (dotted line) did not display

any clinically relevant efficacy. Unmodified exosomes (i.e. exosomes devoid of any artificially introduced polypeptide constructs) (long-dashed grey line) also induced moderate colitis remission, probably due to their inherent anti-inflammatory effects.

Figure 2 shows Western blot confirmation of the presence, on the exosomal surface, of the CD63-sTNFR1 polypeptide used for the colitis treatment of Figure 1.

Figure 3 shows the neutralizing activity of the sTNFR1 decoy exosomes of Figure 1 on TNFα-mediated cytotoxicity. The exosomes displaying signalling-incompetent sTNFR1-CD63 exhibit good TNFα neutralization in a dose-dependent manner (solid black line), and the signalling-competent sTNFR1-CD63-containing exosomes are, as in Figure 1, aggravating the response (short-dashed grey line). Unmodified exosomes (long-dashed grey line) also show a moderate effect due to their anti-inflammatory properties.

Figure 4 shows a graph over the tumour inhibitory effects of exosomes displaying either signalling-incompetent soluble vascular endothelial growth factor receptor (sVEGFR) (SEQ ID No 17) fused to CD63 (solid black line) or signalling-incompetent sVEGFR fused to syndecan (SEQ ID NO 23-26) (dashed black line). Mice treated with exosomes comprising the above polypeptide constructs had a considerably reduced tumour burden post treatment, whereas treatment with exosomes comprising signalling-competent sVEGFR polypeptide did again display a worsened outcome compared to control (short-dashed grey line).

Figure 5 shows treatment efficacy in MDX mice of exosomes comprising the therapeutic decoy polypeptide receptor activin (SEQ ID No 40-43) fused to either the carrier proteins syndecan (solid black line) or synaptotagmin (SEQ ID No 27) (long-dashed grey line). Bi-weekly treatment with the above

therapeutic delivery exosomes resulted in a considerably greater body weight increase than treatment with unmodified exosomes and mock treatment.

Figure 6 shows the amino acid sequences of various polypeptide constructs as per the present invention. Some of the constructs comprise His tags (for facilitated purification) whereas others comprise EGFP (to facilitate detection) but the constructs may naturally be applied both with and without said additional moieties/sequences.

Figure 7 illustrates the increased therapeutic efficacy seen when using exosomes (comprising a therapeutic polypeptide comprising soluble tumour necrosis factor receptor 1 (sTNFR1) fused to the carrier polypeptide CD63) obtained via ultrafiltration-liquid chromatography (UF-LC) purification (solid black line), compared to the same exosomes obtained via a conventional ultracentrifugation (UC) process (crossed solid black line). The increased therapeutic efficacy is most likely due to the improved biophysical stability of the exosomes obtained via UF-LC, which in turn results in less accumulation in lung tissue.

Figure 8 shows inhibition of autophagy resulting in increased exosomal yields. The graph displays, from left to right, cells treated with culture media only (as a baseline control), growth factor-supplemented media (for instance supplemented with IGF-1), media comprising autophagy inhibitors (3-methyladenine and bafilomycin) or media comprising autophagy activators (rapamycin). Treatment with autophagy inhibitors results in a strong increase in the exosomal yield, which can be exploited in the production of exosomes as per the present invention.

Figure 9 shows how EAE-mice treated with exosomes comprising displaying signalling-incompetent therapeutic decoy receptors for IL6 (SEQ ID No 1-2), IL-1β (SEQ ID No 3-5) and TNFα (solid black line and solid black lines with circles, squares, and diamonds) display a very moderate disease

manifestation compared to mock treated control (dotted line), whereas signalling-competent (TNFR1 signalling-competent) exosomes treated mice in fact display a worsened disease (dashed grey line).

Figure 10 illustrates the effects of stress-inducing conditions on enrichment of anti-apoptotic and metabolically active proteins, according to GO-terms (GO-terms: translation, metabolic processes, glycolysis and ribosomal proteins). As compared to cells cultured under normal conditions, exosomes obtainable from cells cultured under stress-inducing conditions (e.g. serum-starvation and/or oxygen deprivation) are enriched in proteins for survival and metabolic processes, which is beneficial for regenerative medicine applications.

Detailed Description of the Invention

The present invention pertains inter alia to, in a first aspect, a therapeutic delivery vesicle having attached to its membrane a polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor present at least partially on the outside of the delivery vesicle, and wherein the at least one therapeutic polypeptide decoy receptor binds to a circulating ligand.. In further aspects, the instant invention relates to delivery vesicles and pharmaceutical compositions in accordance with the present invention for use in medicine, as well as to methods of producing delivery vesicles, kits, compositions, and cell culture media.

Where features, embodiments, or aspects of the present invention are described in terms of Markush groups, a person skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. The person skilled in the art will further recognize that the invention is also thereby described in terms of any combination of individual members or subgroups of members of Markush

groups. Additionally, it should be noted that embodiments and features described in connection with one of the aspects and/or embodiments of the present invention also apply mutatis mutandis to all the other aspects and/or embodiments of the invention. For example, the at least one therapeutic polypeptide decoy receptor described in connection with therapeutic delivery vesicles is to be understood to be potentially relevant/applicable/present also in the context of the methods of producing delivery vesicles or in the context of the pharmaceutical compositions, or in connection with the polypeptide and/or polynucleotide constructs as per the present invention. Furthermore, certain embodiments described in connection with certain aspects, for instance the administration routes of the therapeutic delivery vesicles, as described in relation to aspects pertaining to treating certain medical indications, may naturally also be relevant in connection with other aspects and/or embodiment such as aspects/embodiments pertaining to the pharmaceutical compositions of the present invention. As a general remark, the therapeutic polypeptide decoy receptors and the carrier polypeptides in accordance with the present invention may be freely combined in any and all possible combinations without deviating from the scope and the gist of the invention, and the sequences may deviate strongly from the original sequences as long as any given carrier polypeptide retains its ability to carry the therapeutic polypeptide decoy receptor to the surface of an extracellular vesicle, and as long as any given therapeutic polypeptide decoy receptor retains its ability to bind to its target in a therapeutically efficacious manner. As long as their biological properties are retained the polypeptide sequences may deviate with as much as 50% (calculated using for instance BLAST or ClustalW) as compared to the native polypeptide, although a sequence identity that is as high as possible is preferable. The combination (fusion) of the carrier and the decoy receptor polypeptides implies that certain segments of the respective polypeptides may be replaced and/or modified, meaning that the deviation from the native sequence may be large as long as the key properties are conserved.

For convenience and clarity, certain terms employed herein are collected and described below. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "therapeutic delivery vesicle", interchangeably termed "delivery vesicle", shall be understood to relate to any type of vesicle that is, for instance, obtainable from a cell, for instance a microvesicle (any vesicle shedded from the plasma membrane of a cell), an exosome (any vesicle derived from the endo-lysosomal pathway), an apoptotic body (from apoptotic cells), a microparticle (which may be derived from e.g. platelets), an ectosome (derivable from e.g. neutrophiles and monocytes in serum), prostatosome (obtainable from prostate cancer cells), cardiosomes (derivable from cardiac cells) etc. Furthermore, the terms "therapeutic delivery vesicle" and "delivery vesicle" shall also be understood to potentially also relate to lipoprotein particles, such as LDL, VLDL, HDL and chylomicrons, as well as liposomes, lipid-like particles, lipidoids, etc. Essentially, the present invention may relate to any type of lipid-based structure (vesicular or with any other type of suitable morphology) that can act as a delivery or transport vehicle for the therapeutics polypeptide construct(s).

The term "attached to its membrane" shall be understood as attached in the sense of how a biological polypeptide is normally attached to a vesicular membrane, i.e. predominantly via non-covalent interactions but possibly also via covalent bonds. The attachment to the membrane may comprise attachment inside the vesicle, in the vesicle membrane, on the outside of the vesicle, or any combination thereof. A typical example of a polypeptide being "attached" to the membrane of a vesicle is a transmembrane polypeptide that spans the bi-layer vesicular membrane of an exosome from the intra-exosomal space out through the exosomal membrane to the extra-exosomal milieu.

The term "polypeptide construct" (or interchangeably "therapeutic polypeptide construct") shall be understood to relate to any polypeptide that comprises a "carrier polypeptide", as defined herein, and a "therapeutic polypeptide decoy receptor", as defined herein. The polypeptide construct may be attached to the membrane of the delivery vesicle in accordance with the present invention, preferably in a transmembrane manner (i.e. with the polypeptide construct extending from the inside of the delivery vesicle, through the delivery vesicle membrane, to the outside of the delivery vesicle). When the therapeutic polypeptide decoy receptor is having a transmembrane configuration it may be preferable if the carrier polypeptide is present substantially on the inside of the delivery vesicle whereas the therapeutic polypeptide decoy receptor is present at least partially on the outside of the delivery vesicle, to be able to exert its therapeutic effect.

The term "signalling-incompetent" shall be understood as not capable of transmitting biochemical signals, i.e. a polypeptide that is signalling-incompetent is merely binding its extracellular interaction partner but upon binding no signals (i.e. no intracellular, no intravesicular, or no extracellular) are being generated or transmitted. For example, in the case of the soluble TNFR (sTNFR), even if transferred to immune cells a signalling-incompetent TNFR cannot exert a biological response as it lacks crucial signalling components. Similarly, mimics of the EGFR (SEQ ID No 18) may lack the transmembrane domain which prohibits insertion into the membrane of recipient cells, thereby not being able to transmit biological signals, e.g. in the event of a transfer to a recipient cell. Importantly in a clinical context, in the case of TNFr, transfer a of signalling incompetent receptor to recipient cells offer another layer of protection as they then directly protect the cells from excessive TNF signalling by competing for ligand binding. For clarity, both the therapeutic polypeptide decoy receptor and/or the entire polypeptide construct may be signalling-incompetent. For instance, the therapeutic polypeptide decoy receptor may be rendered signalling-incompetent through e.g. a site-

specific mutation, but a signalling-competent therapeutic polypeptide receptor may be made signalling-incompetent through replacement of and/or attachment to its signalling-domain by a carrier polypeptide.

The term "carrier polypeptide" shall be understood to relate to any polypeptide that can be utilized to transport a polypeptide construct to a suitable vesicular location. More specifically, the term "carrier polypeptide" shall be understood as comprising any polypeptide that enables transporting or shuttling of a polypeptide construct (which said "carrier polypeptide" forms part of) to, at least partially, the vesicular membrane and, at least partially, the extra-vesicular side (for instance the surface) of a delivery vesicle in accordance with the present invention. Examples of carrier polypeptides are for instance Lamp2b (SEQ ID No 22), CD9 (SEQ ID No 12), CD81 (SEQ ID No 13), CD63, syndecan, ALIX (SEQ ID No 28), syntenin (SEQ ID No 29), and synaptotagmin, but numerous other polypeptides capable of transporting a polypeptide construct at least partially to the extra-vesicular side of delivery vesicle are comprised within the scope of the present invention.

The terms "therapeutic polypeptide decoy receptor", "polypeptide decoy receptor", and "decoy receptor" are used interchangeably herein and shall be understood to relate to any polypeptide (often called a decoy or a sink receptor) that can be utilized for therapeutic purposes through sequestering (binding) a suitable target and/or ligand (normally a circulating ligand or a ligand present on a cell that is itself in circulation but potentially any ligand on the surface of and/or inside a target cell), thereby exerting its therapeutic effect. The decoy receptor binds to and/or sequesters its target, which in essence inhibits the target from carrying out its function which may contribute to a disease and/or disorder to be treated. The signal transduction processes of the major receptor classes are described in some detail below, to exemplify certain therapeutic polypeptide decoy receptors in accordance with the present invention. However, as above-mentioned, the decoy receptors of the

present invention are preferably signalling-incompetent, to ensure therapeutic efficacy and safety.

The terms "ligand" and "target" in the context of the present invention shall be understood to comprise any molecule that is bound (normally with high affinity, e.g. a Kd of less than 100 μM but preferably below 1 μM or more preferably below 100 nM or even more preferably below 100 nM) by the therapeutic polypeptide decoy receptors according to the instant invention. The ligand (which may be any polypeptide or carbohydrate/polysaccharide or essentially any molecule, for instance present on the surface of a cell) is normally circulating freely in the blood or in any other bodily fluid (TNFα is an example of such a freely circulating polypeptide ligand for the TNFα receptor) but it may also be a polypeptide or any other type of molecule present on a cell and/or inside a cell which circulates in a bodily fluid, for instance blood. An example of a cell-bound target may be CD19, which is present on a circulating B cells, and which can be bound by a therapeutic polypeptide receptor such as the commercially available rituximab. The term "ligand" shall thus be understood to comprise both polypeptide and non-polypeptide molecules (for instance carbohydrates or any other type of molecule).

The phrase "binds to a ligand/target" shall be understood as the therapeutic polypeptide decoy receptor having the capacity to bind to a ligand and/or a target in the human and/or animal body, meaning that the decoy receptor may bind to and sequester its ligand, to exert a therapeutic effect by inhibiting the ligand/target from carrying out its normal physiological function. The ligand/target is normally circulating in the blood stream or is exposed to the extracellular environment through being present on the surface of a target cell.

Signal transduction normally occurs *in vivo* when an extracellular ligand binds to a cell surface receptor and activates it. In turn the receptor changes intracellular proteins/molecules, which starts a signalling pathway. There are

two main receptor groups; extracellular receptors and intracellular receptors. The extracellular receptors can be further divided into different classes:

1. G-protein coupled (7-TM) receptors
2. Tyrosine and histidine kinases
3. Integrins
4. Toll gate receptors
5. Ligand-gated ion channel

7-TM receptors have 7 transmembrane regions and are linked to a heterotrimeric G-protein. Upon ligand binding the receptor undergoes a conformational change and the G-protein becomes active. The activated G protein subunits detaches from the receptor and initiate signaling via many downstream effector proteins such as phospholipases and ion channels. Adrenergic- and chemokine receptors belong to this family. 7-TM receptors can be made signaling-incompetent by removing the binding site for the G-proteins. For instance, replacing the intracellular binding site of G-proteins with the syntenin binding site of Syndecans would direct a signaling-incompetent receptor to an exosome.

Tyrosine kinase receptors (RTKs) are transmembrane proteins with an intracellular kinase domain and an extracellular ligand-binding domain. Examples of ligands are growth factors, insulin, etc. To induce a signal the RTKs need to form dimers at the plasma membrane. When a dimer is formed the interaction between the intracellular domains initiates auto-phosphorylation of the tyrosine residues which causes a conformational change in the receptor. The kinase domains of the receptors are subsequently activated and phosphorylate downstream signaling molecules that create a signaling cascade.

The tyrosine receptors can be made signaling-incompetent by removing or mutating the kinase domain or the tyrosine domain. This can be done in a

similar manner as with the 7-TM receptors. Further, the extracellular domain of tyrosine receptors could be fused with an exosomal protein, such as CD63, Lamp2b, etc.

Integrins are transmembrane proteins that are important for cell attachment to other cells as well as to the extracellular matrix. The integrins also take part in the transduction of signals from the extracellular matrix proteins such as fibronectin and collagen. Integrins change their conformation upon ligand binding; integrins lack a kinase domain which means the integrins need adaptor molecules to relay the signal into the cell. There are several adaptor molecules and integrin-linked kinases. The integrins can exist in two different conformations: an inactive form and an active form. The inactive form is common on non-activated leucocytes; when the leucocytes are activated the cell changes its integrins to an active state. Integrins are signaling incompetent without its adaptor molecules, so the binding sites for the adaptor molecules and kinases can be removed to render the receptors signaling-incompetent.

Toll-like receptors have four known adaptor molecules which are activated upon ligand binding. This four adaptor molecules, Myd88, TIRAP, TRIF, and TRAM, subsequently activate intracellular molecules, and the Toll-like receptors inhibit or activates thousands of genes when activated. The Toll-like receptors can be made signaling-incompetent through removal of either the binding site and/or the interaction sites for the adaptor molecules.

The various polypeptides mentioned in the present application (for instance carrier polypeptides such as Lamp2b or CD63, and therapeutic polypeptide decoy receptors such as sTNFR or VEGFR , etc.) shall be understood to relate also to homologous polypeptides having sequence identities to the polypeptide in question preferably above 50%, more preferably above 60%, more preferably above 70%, more preferably above 80%, and more preferably above 90%.

In a first aspect, the present invention relates to a therapeutic delivery vesicle having attached to its membrane a polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor present at least partially on the outside of the delivery vesicle and wherein the at least one therapeutic polypeptide decoy receptor is signalling-incompetent, to enable binding and sequestering of its target molecule without the generation and/or transmission of any signals. In a preferred embodiment, the at least one therapeutic polypeptide decoy receptor binds to a circulating ligand but it may naturally also bind to a target molecule present on a target cell. The therapeutic polypeptide construct may comprise at least one therapeutic polypeptide decoy receptor (interchangeably termed a "decoy receptor", but said therapeutic polypeptide decoy receptors may also relate to therapeutic polypeptides not necessarily classified as decoy receptors as such) that is signalling-incompetent (or alternatively in some embodiments signalling-competent), fused to a carrier polypeptide. Naturally, one single delivery vesicle may comprise more than one polypeptide construct (i.e. a plurality of constructs are present on a single exosome), and also more than one type of polypeptide construct (a single exosome could, for example, comprise a plurality of (1) constructs comprising the VEGF receptor, as the decoy receptor, and a carrier polypeptide, such as Lamp2b, and (2) constructs comprising the EGF receptor, as the decoy receptor, and the carrier polypeptide CD63). The inventors have unexpectedly realized that using extracellular vesicles (such as exosomes) as delivery vehicles for therapeutic polypeptide decoy receptors (e.g. biopharmaceuticals) results not only in enhanced pharmacokinetics but unexpectedly also increases the efficacy of the therapeutic polypeptide decoy receptors, possibly as a result of regenerative effects exerted by exosomes and other vesicles *per se*. Additionally, employing extracellular vesicles as delivery vectors for therapeutic polypeptides does not only facilitate production in comparison with

classical biologics, but the fact that each delivery vesicle potentially comprises a considerable plurality of therapeutic constructs (which in turn may comprise a plurality of therapeutic polypeptide decoy receptors) potentially leads to a receptor multivalency that enhances the therapeutic efficacy and improves treatment outcomes.

In preferred embodiments, the at least one therapeutic polypeptide decoy receptor may be partially or completely devoid of its signalling domain, to make it signalling-incompetent. This may be achieved either via truncating or mutating the polynucleotide encoding the signalling domain, or via completely removing said polynucleotide, in order to block any signalling from the therapeutic polypeptide decoy receptor. In a further embodiment, the signalling domain of therapeutic polypeptide decoy receptor may be partially or completely replaced by the carrier polypeptide, to possibly minimize the size of the polypeptide construct.

The inventors have realized that it is, surprisingly, in some instances preferable to utilize therapeutic polypeptide decoy receptors that are signalling-incompetent, in order to avoid generating signals that otherwise may negatively impact the therapeutic efficacy.

In one embodiment as per the present invention, the carrier polypeptide may be located partially inside the therapeutic delivery vesicle and/or partially in the therapeutic delivery vesicle membrane and/or partially outside the therapeutic delivery vesicle. In a preferable embodiment, the carrier polypeptide is present substantially on the inside of the delivery vesicle or in its membrane, whereas the therapeutic polypeptide decoy receptor is present at least partially on the outside of the delivery vesicle, to be able to exert its therapeutic effect. Thus, the polypeptide construct may preferably be present in transmembrane form (i.e. a transmembrane polypeptide construct), with the carrier polypeptide present substantially on the inside or in the vesicular membrane and the therapeutic polypeptide decoy receptor present

substantially on the outside of the delivery vesicle (and either the carrier polypeptide and/or the therapeutic polypeptide decoy receptor extending through the membrane of the delivery vesicle). In one embodiment, more than one carrier polypeptide may be used, in order to improve the expression of the therapeutic polypeptide decoy receptor on the surface (outside) of the therapeutic delivery vesicle.

The location of the carrier polypeptide in the membrane may vary depending on the application and the therapeutic polypeptide in question; with the primary consideration being that the therapeutic polypeptide decoy receptor is capable of interacting with its interaction partner, normally a circulating ligand, which is normally present extracellularly for instance in the blood or in any other bodily fluid or on a circulating target cell. In further embodiments, the at least one therapeutic polypeptide decoy receptor may be fused to the carrier polypeptide via a chemical bond selected from the group comprising a peptide (amide) bond, a thio-ether bond, a di-sulfide bridge, and a biotin-streptavidin interaction. The formation of a peptide (amide) bond may naturally be achieved via recombinant technology (i.e. via expression of a suitable polynucleotide in a cell capable of producing suitable delivery vesicles) but such a bond may also be generated using various conjugation strategies commonly employed within the art, for instance EDC/NHS-mediated conjugation, sulfo-NHS conjugation, or any other type of amide (peptide) conjugation approach. However, other methodologies for coupling the at least one therapeutic polypeptide decoy receptor and the at least one carrier polypeptide may comprise forming a disulfide bridge between e.g. two cysteine residues, or utilizing the natural interaction between biotin and streptavidin to connect the therapeutic polypeptide decoy receptor and the carrier polypeptide. An alternative to using fusion constructs and chemical bond formation is to place a lipid tag on the therapeutic polypeptide decoy receptor and non-covalently coat the surface of the delivery vesicle with the therapeutic polypeptide decoy receptor by means of simple lipid intercalation.

Such lipid tags may include cholestoryl, stearyl, di-stearyl, myristoyl, palmitoyl, decanoyl, and other suitable lipids know to a person skilled in the art.

The carrier polypeptide may be selected from the group comprising Lamp2b, CD63, syndecan, synaptotagmin, ALIX (CHAMP 4) domain, ALIX-syntenin binding domain, ESCRT-proteins, PDGF, syntenin-PDZ, P6- and P9-domain, CD81, CD9, and any combination thereof. Again, the primary consideration behind selecting an appropriate carrier polypeptide pertains to its ability to efficiently carry the therapeutic polypeptide decoy receptor to an appropriate vesicular location (normally its surface, or at least to a part of the therapeutic delivery vesicle membrane that enables the therapeutic polypeptide decoy receptors to interact with and bind to the ligand, i.e. its interaction partner, to exert its therapeutic effect). Further in accordance with the present invention, the carrier polypeptide may comprise the cytoplasmic part of syndecan. The cytoplasmic part of syndecan has a PDZ-binding domain which binds the syntenin-ALIX complex, and the Syntenin-ALIX complex subsequently forms an extracellular vesicle and/or an exosome (the PDZ domain would therefore essentially guide the receptor to the extracellular vesicle).

The at least one therapeutic polypeptide decoy receptor as per the present invention may be selected from the group comprising receptors, for example, from the following receptor families: insulin, PDGF (SEQ ID No 15-16), FGF (SEQ ID No 36-39), EGF, NGF (SEQ ID No 32), VEGF, HGF, TRK, EPH, AXL, LTK, TIE, ROR, DDR, RET, KLG, RYK, MuSK, Type I and Type II TGF (SEQ ID No 33-35), activin, and TNF, PTCH1 (SEQ ID No 44), interleukins (IL) 1, 6, 12, 17, 23, and others (SEQ ID No 1-11), angiopoetin, HER phagedisplay peptides binding ligands for the receptors above (and possibly also phage display towards receptors in order to occupy binding space via allosteric hindrance), 7-TM receptors, integrins, selectins (e.g. selectins E, P, and L (SEQ ID No 19-21), ligands of integrins/selectins membrane-bound antibodies, T-cell receptors, NK-cell receptors, Toll-like recptors, PAMP, etc.

Also, engineered receptors that bind several ligands such as both VEGF and angiopoetin (DAAP) are in accordance with the present invention, and further all of the above-mentioned receptor families and specific receptors are in line with the present invention. Further in accordance with the present invention, the polypeptide construct attached to therapeutic delivery vesicles may comprise more than one therapeutic polypeptide decoy receptor, and also more than one carrier polypeptide, in order to optimize e.g. the therapeutic effects or the transport of the therapeutic polypeptide decoy receptor to delivery vesicle membrane (or its surface).

The polypeptide construct as per the present invention may consequently be formed from any combination of the at least one therapeutic polypeptide decoy receptor and the at least one carrier polypeptide. Exemplary embodiments comprise, for instance, (i) at least one therapeutic polypeptide decoy receptor from the TNF family (e.g. TNFR1) combined with a carrier polypeptide selected from CD63 (SEQ ID No 14), Lamp2b, syndecan, synaptotagmin, or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (ii) at least one therapeutic polypeptide decoy receptor from the VEGF family (for instance VEGFR) combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin, or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (iii) at least one therapeutic polypeptide decoy receptor from the FGF family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin, or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (iv) at least one therapeutic polypeptide decoy receptor from the EGF family combined with a carrier polypeptide selected

from CD63, Lamp2b, syndecan, synaptotagmin, or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (v) at least one therapeutic polypeptide decoy receptor from the activin family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin, (vi) at least one therapeutic polypeptide decoy receptor from the interleukin receptor family (e.g. IL6R (SEQ ID No 1) or IL12R beta 1 (SEQ ID No 4) or IL1R Type 1 (SEQ ID No 3)) combined with a carrier polypeptide selected from e.g. CD63, Lamp2b, syndecan, synaptotagmin, or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle).

In yet another advantageous embodiment of the present invention, a suitable carrier polypeptide (such as CD63, Lamp2b, CD9, CD81, synaptotagmin, or syndecan, etc.) is fused to PTCH1, creating a polypeptide construct capable of sequestering sonic hedgehog (SHH), which is an important signalling molecule implicated in various cancers. The inventors have experimentally seen decreased tumour burden in mice treated with exosomes comprising various carrier polypeptides coupled to PTCH1 and to a specifically selected SHH-binding domain of PTCH1. For instance, in the experimental setup reported in Figure 4, exosomes comprising polypeptide constructs comprising CD63 or Lamp2b as the carrier polypeptide and PTCH1 as the therapeutic polypeptide decoy receptor showed similar anti-tumour efficacy as the syndecan-VEGFR constructs shown in the graph in question.

In additional embodiments, the therapeutic delivery vesicle in accordance with the present invention may further comprise at least one targeting entity

present. Said targeting entity is normally at least partially present on the outside of the delivery vesicle, in order to enable targeting of the vesicles to tissues, cell types, or organs of interest, e.g. to increase the concentration of delivery vesicles locally at the site where the therapeutic efficacy needs to be as high as possible. The targeting entity may be a peptide or a polypeptide (e.g. an antibody or an antibody fragment), but it may also be a small molecule (such as a vitamin), a carbohydrate, a nucleic acid (such as an aptamer), or any other type of molecule that may confer targeting properties to therapeutic delivery vesicles.

Further in accordance with the present invention, the therapeutic delivery vesicle may be selected from the group comprising exosomes, microvesicles, apoptotic bodies, microparticles, ectosomes, prostatosome cardiosomes, liposomes, lipid-like materials or structures, lipidoids, VLDL particles, LDL particles, HDL particles, chylomicrons, etc.

Generally, the present invention pertains to delivery vesicles as per the instant invention for use in medicine, and more specifically the present invention relates to the use in the prophylaxis and/or alleviation and/or treatment of diseases and disorders selected from the group comprising Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, kidney failure, graft-vs-host disease, Duschenne muscular dystrophy and other muscle diseases, neurodegenerative disease including Alzheimer´s disease, Parkinson´s diease, Huntingtons disease, ALS, cancer-induced cachexia, anorexia, diabetes mellitus type 2, and cancers (for instance cancers sensitive to EGF,

VEGF, FGF). Some of the cancer types of relevance for the present invention comprises, for instance, Acute lymphoblastic leukemia (ALL),Acute myeloid leukemia, Adrenocortical carcinoma, AIDS-related cancers, AIDS-related lymphoma, Anal cancer, Appendix cancer, Astrocytoma, cerebellar or cerebral, Basal-cell carcinoma, Bile duct cancer, Bladder cancer, Bone tumor, Brainstem glioma, Brain cancer, Brain tumor (cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic glioma), Breast cancer, Bronchial adenomas/carcinoids, Burkitt's lymphoma, Carcinoid tumor (childhood, gastrointestinal), Carcinoma of unknown primary, Central nervous system lymphoma, Cerebellar astrocytoma/Malignant glioma, Cervical cancer, Chronic lymphocytic leukemia, Chronic myelogenous leukemia, Chronic myeloproliferative disorders, Colon Cancer, Cutaneous T-cell lymphoma, Desmoplastic small round cell tumor, Endometrial cancer, Ependymoma, Esophageal cancer, Extracranial germ cell tumor, Extragonadal Germ cell tumor, Extrahepatic bile duct cancer, Eye Cancer (Intraocular melanoma, Retinoblastoma), Gallbladder cancer, Gastric (Stomach) cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal stromal tumor (GIST), Germ cell tumor (extracranial, extragonadal, or ovarian), Gestational trophoblastic tumor, Glioma (glioma of the brain stem, Cerebral Astrocytoma, Visual Pathway and Hypothalamic glioma), Gastric carcinoid, Hairy cell leukemia, Head and neck cancer, Heart cancer, Hepatocellular (liver) cancer, Hodgkin lymphoma, Hypopharyngeal cancer, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi sarcoma, Kidney cancer (renal cell cancer), Laryngeal Cancer, Leukemias ((acute lymphoblastic (also called acute lymphocytic leukemia), acute myeloid (also called acute myelogenous leukemia), chronic lymphocytic (also called chronic lymphocytic leukemia), chronic myelogenous (also called chronic myeloid leukemia), hairy cell leukemia)), Lip and Oral, Cavity Cancer, Liposarcoma, Liver Cancer (Primary), Lung Cancer (Non-Small Cell, Small Cell),

Lymphomas ((AIDS-related lymphoma, Burkitt lymphoma, cutaneous T-Cell lymphoma, Hodgkin lymphoma, Non-Hodgkin (an old classification of all lymphomas except Hodgkin's) lymphoma, Primary Central Nervous System lymphoma)), Medulloblastoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Chronic Myeloid Leukemia (Acute, Chronic), Myeloma, Nasal cavity and paranasal sinus cancer, Nasopharyngeal carcinoma, Neuroblastoma, Oral Cancer, Oropharyngeal cancer, Osteosarcoma/malignant fibrous histiocytoma of bone, Ovarian cancer, Ovarian epithelial cancer (Surface epithelial-stromal tumor), Ovarian germ cell tumor, Ovarian low malignant potential tumor, Pancreatic cancer, Pancreatic islet cell cancer, Parathyroid cancer, Penile cancer, Pharyngeal cancer, Pheochromocytoma, Pineal astrocytoma, Pineal germinoma, Pineoblastoma and supratentorial primitive neuroectodermal tumors, Pituitary adenoma, Pleuropulmonary blastoma, Prostate cancer, Rectal cancer, Renal cell carcinoma (kidney cancer), Retinoblastoma, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma (Ewing family of tumors sarcoma, Kaposi sarcoma, soft tissue sarcoma, uterine sarcoma), Sézary syndrome, Skin cancer (nonmelanoma, melanoma), Small intestine cancer, Squamous cell, Squamous neck cancer, Stomach cancer, Supratentorial primitive neuroectodermal tumor, Testicular cancer, Throat cancer, Thymoma and Thymic carcinoma, Thyroid cancer, Transitional cell cancer of the renal pelvis and ureter, Urethral cancer, Uterine cancer, Uterine sarcoma, Vaginal cancer, Vulvar cancer, Waldenström macroglobulinemia, and/or Wilm's tumor (kidney cancer).

Furthermore, the present invention pertains to pharmaceutical compositions comprising therapeutic delivery vesicles as per the present invention, normally formulated with at least one pharmaceutically acceptable excipient. The at

least one pharmaceutically acceptable excipient may be selected from the group comprising any pharmaceutically acceptable material, composition or vehicle, for instance a solid or liquid filler, a diluent, an excipient, a carrier, a solvent or an encapsulating material, which may be involved in e.g. suspending, maintaining the activity of or carrying or transporting the therapeutic delivery vesicles from one organ, or portion of the body, to another organ, or portion of the body (e.g. from the blood to any tissue and/or organ and/or body part of interest).

The present invention also relates to cosmetic applications of the delivery vesicles, either with or without the polypeptide construct(s). Thus, embodiments of the present invention may pertain to skin care products such as creams, lotions, gels, emulsions, ointments, pastes, powders, liniments, sunscreens, shampoos, etc., comprising the delivery vesicles, in order to improve and/or alleviate symptoms and problems such as dry skin, wrinkles, folds, ridges, and/or skin creases. The delivery vesicles may exhibit beneficial effects without the polypeptide construct being present but the presence of a suitable polypeptide construct may further enhance the cosmetic efficacy. In one embodiment, the delivery vesicles as per the present invention may comprise a botulinum toxin (e.g. botox, for instance botulinum toxin types A-G) as the therapeutic polypeptide decoy receptor (botulinum toxins may not necessarily be used only for cosmetic applications but could also be applied for e.g. treatment of migraine headaches and dystonia). In a preferred embodiment, exosomes from a suitable exosome-producing cell are comprised in a cosmetic cream, lotion, or gel for use in the treatment (which is normally for cosmetic purposes) of wrinkles, lines, folds, ridges and/or skin creases.

In a further embodiment, the exosomes in accordance with the present invention may comprise a therapeutic polypeptide construct but may also be devoid of any artificially introduced therapeutic polypeptide construct or may

contain polypeptide constructs having merely e.g. cosmetic capacity. Both extracellular vesicles devoid of therapeutic polypeptide constructs and extracellular vesicles comprising therapeutic polypeptide construct may mediate anti-inflammatory, anti-apoptotic and cell proliferative effects that may enhance wound healing and skin regeneration. Experiments carried out using (i) exosomes without any therapeutic polypeptide construct and (ii) exosomes comprising a VEGFR1 therapeutic polypeptide construct (with either CD63 or Lamp2b as the carrier polypeptide) show that both strategies display strong cosmetic potency in alleviating e.g. telangiectasias (small dilated blood vessels located near the skin). Exosomes devoid of therapeutic polypeptides were also shown to alleviate cosmetic problems such as dry skin, wrinkles, rashes, etc., and additionally exosomes comprising e.g. TNFR-containing therapeutic polypeptides are highly potent in treating rashes, scaling, and potentially psoriasis and psoriasis-related problems.

In further embodiments, the delivery vesicles in accordance with the present invention may comprise therapeutic polypeptide decoy receptors such as collagen, laminins (for instance laminins 111, 211, 511, and/or 521), and/or cell-penetrating peptides (CPPs).

Optionally, glycosaminoglycans (GAGs) and/or other types of carbohydrates may be included in the delivery vesicles, to further augment effects related to maintaining the structural integrity of the skin.

In one aspect as per the present invention, the polypeptide construct may comprise a polypeptide construct which comprises virtually any therapeutic polypeptide decoy receptor that can bind to a circulating ligand fused to virtually any carrier polypeptide.

The pharmaceutical compositions as per the present invention are naturally suitable for use in medicine, and specifically in the prophylaxis and/or alleviation and/or treatment of diseases and disorders selected from the group

comprising Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, kidney failure, graft-vs-host disease, Duschenne muscular dystrophy and other muscle diseases, cancer-induced cachexia, anorexia, diabetes mellitus type 2, and cancer (for instance cancers sensitive to EGF, VEGF, FGF).

The therapeutic delivery vesicles as per the present invention may be administered to a human or animal subject via various different routes, for instance auricular (otic), buccal , conjunctival, cutaneous ,dental, electro-osmosis, endocervical, endosinusial, endotracheal, enteral, epidural, extra-amniotic, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronal (dental), intracoronary, intracorporus cavernosum, intradermal, intradiscal, intraductal, intraduodenal, intradural, intraepidermal, intraesophageal, intragastric, intragingival, intraileal, intralesional, intraluminal, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous, intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratympanic, intrauterine, intravascular, intravenous, intravenous bolus, intravenous drip, intraventricular, intravesical, intravitreal, iontophoresis, irrigation, laryngeal, nasal, nasogastric, occlusive dressing technique, ophthalmic, oral, oropharyngeal, other, parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (inhalation), retrobulbar, soft tissue, subarachnoid, subconjunctival,

subcutaneous, sublingual, submucosal, topical, transdermal, transmucosal, transplacental, transtracheal, transtympanic, ureteral, urethral, and/or vaginal administration, and/or any combination of the above administration routes.

In a further aspect, the present invention relates to a method of producing a therapeutic delivery vesicle, comprising the steps of (i) providing at least one polynucleotide construct encoding at least one polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor that binds a circulating ligand, (ii) introducing said at least one polynucleotide construct into a cell capable of producing suitable delivery vesicles (through translating the polynucleotide construct into the corresponding polypeptide construct), and, (iii) collecting at least one delivery vesicle produced by the cell of step (ii). The method may further comprise a purification step, wherein the therapeutic delivery vesicle is purified through a procedure selected from the group comprising liquid chromatography (LC), high-performance liquid chromatography (HPLC), spin filtration, tangential flow filtration, centrifugation, immunoprecipitation, etc, or any combination thereof.

The inventors have surprisingly realized that applying a sequential combination of filtration (preferably ultrafiltration (UF)) and size exclusion liquid chromatography (LC) results in optimized purification, which in turn leads to superior therapeutic efficacy. Further, as compared to ultracentrifugation (UC), which is routinely employed for purifying exosomes, sequential ultrafiltration-liquid chromatography (UF-LC) is considerable faster and possible to scale to higher manufacturing volumes, which is a significant drawback of current UC methodology. However, as will be described in greater details below, the most advantageous implication of the use of UF-LC instead of UC is the fact that exosomes (and other extracellular vesicles to which UF-LC is applied) retain their biophysical and biological properties,

which results in less accumulation in lung tissue upon *in vivo* administration and therefore improves the therapeutic efficacy.

According to electron microscopy (EM) analysis, vesicles from both UF and UC preparations displayed a rounded or cup-shaped morphology. In most cases the vesicle diameter was measured to be around 100 nm, and this was in accordance with results obtained via nanoparticle tracking analysis (NTA). Of note however, a fraction of vesicles in the UC sample was clearly distorted (either disrupted or fused to form slightly larger vesicles) and such vesicles were not observed in UF samples. Hence, these results indicate that the UC methodology had a negative impact on vesicular integrity.

To verify the presence of vesicle aggregates in UC samples, the inventors next examined HEK293T CD63-EGFP labeled exosomes directly by fluorescence microscopy. Similar to the FCS results, only UC samples displayed visible large aggregates in the fluorescence channel, which were not seen with UF samples. This was corroborated using the fluorescent dye DiOC6 labeled exosomes. Thus the UF isolation method results in preservation of the inherent biophysical properties of vesicles compared to UC, which results in fusion/aggregation and disruption of vesicles.

The UF experiments in accordance with the present invention were performed primarily using filters with a molecular weight cut-off of 100-kDa, 250-kDa, and/or 500-kDa, or any sequential combination thereof. In preferred embodiments, 100-kDa cut-off is preferable but given the number of biomolecules with higher molecular weight, it is possible that media/cell-secreted components other than exosomes are entrapped in the filters and the UF protocol was therefore refined further using highly unconventional size exclusion liquid chromatography. UF samples were loaded on *inter alia* a Sephacryl S-300 or a Sephacryl S-500 size exclusion LC column where two distinct fractions were collected based on the UV flow cell absorbance at 280 nm. NTA revealed that 98% of the particles were recovered in fraction 1 where

the mode particle size was consistent in all three UF-LC replicates. Subsequent total protein staining on SDS-PAGE confirmed that many of the contaminating proteins originally seen in the UF sample were eluted in fraction 2 while fraction 1 did not have any detectable levels of protein. By using Western Blot (WB), exosomal markers such as Alix and CD9 were only detected in fraction 1 and not in fraction 2, indicating that fraction 1 contained pure exosomes. Moreover, the vesicle recovery rate following LC fractionation was 70 +/- 19%, hence LC did not hamper the gain in particle yield achieved by UF alone. WB corroborated this data, as the exosomal markers were more strongly expressed in LC fraction 1 compared to UC-purified samples. Furthermore, the protein per vesicle ratio was much lower for UF-LC compared to UC samples. EM was also performed on fraction 1 and 2, where intact cup-shaped vesicles were detected only in fraction 1, while protein aggregates were seen in fraction 2. Furthermore, when HEK293T CD63-EGFP labeled exosomes purified with UF-LC were visualized by fluorescent microscopy, EGFP positive vesicles appeared similar to the UF purified vesicles indicating that LC did not affect the biophysical properties of the vesicles. Thus, the inventors have discovered that using a two-step method combining UF with subsequent LC surprisingly allows for highly efficient isolation of high yields of biophysically intact exosomes free of protein contamination. The exosomes field currently relies completely on the perceived effectiveness of the UC method, which the present inventors have proved to be highly unreliable.

The UF-LC purification method of the present invention and the conventional UC method isolated vesicles with similar protein contents, as evidenced by the good proteomic overlap between the two methods. However, importantly, the UF-LC method results in high yield purification of vesicles devoid of non-exosomal contamination.

Despite similar proteomic profiles of exosomes isolated by the UF-LC method of the present invention and conventional UC methods, the inventors hypothesized that the distinct differences in exosome integrity between the purification methods (vesicle aggregation and fusion following UC purification) might influence their biological properties *in vivo*. Given that it is well-established that aggregated particles typically show lung accumulation following intravenous (IV) injection the inventors speculated that UC purified exosomes might preferentially distribute to lung tissues compared to UF-LC purified vesicles. To investigate this, the same number of near-infrared fluorescent dye (DiR) labeled exosomes (based on NTA calculations) were injected via the tail vein in adult Balb/c mice and the biodistribution was analysed using IVIS imaging 24 h post injection. As postulated, UC purified exosomes showed a 4.6 times ($p<0.0001$) stronger signal in the lungs compared to UF-LC purified vesicles. The signal from the liver was as expected higher in the UF-LC group ($p<0.0001$), since the total fluorescence injected only differed by 6%. Thus vesicles isolated using the highly advantageous UF-LC methods of the present invention are biophysically intact, do not preferentially accumulate in lung and therefore are better suited for *in vivo* therapeutic applications.

As can be realized from the above description, the UF-LC method is generally applicable to purification of any type of vesicles (such as exosomes, liposomes, etc.) and may be in a broad aspect comprise exposing any type of suitable vesicle preparation which needs to be purified to UF-LC as described herein. In one exemplary embodiment, the present invention thus pertains to obtaining extracellular vesicles from a suitable source (for instance vesicle produced by the methods of the present invention, which may optionally comprise polypeptide constructs which in turn comprise carrier polypeptides and therapeutic decoy receptors), exposing the vesicles (which are normally present in a medium also comprising various other components such as

proteins and peptides) to an ultrafiltration step followed by a size exclusion liquid chromatography (LC) step.

Further, in a preferred embodiment the present invention relates to a method of producing a therapeutic delivery vesicle, comprising the steps of (i) providing at least one polynucleotide construct encoding at least one polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor that binds a circulating ligand, (ii) introducing said at least one polynucleotide construct into a cell capable of producing suitable delivery vesicles (through translating the polynucleotide construct into the corresponding polypeptide construct), and, (iii) purifying the delivery vesicles of step (ii) using ultrafiltration (UF) followed by size exclusion liquid chromatography (LC). The delivery vesicles obtained using said methodology may therefore display significantly enhanced biophysical stability and biological properties than vesicles obtained via conventional UC methodology. In a further embodiment, at least one filter may be used for the UF step, and the filter(s) may have the same or different cut-offs, e.g. one could initially perform a first step with a cut-off of 100 kDa and in a second step a filter with a cut-off of 200 kDa. Naturally, the filter may be selected to have any appropriate cut-off, for instance 100 kDa, 200 kDa, 500 kDa, etc. Furthermore, in additional embodiments, the column used for the LC step may have essentially any suitable pore size. S-300, S-500 and S-1000 of a Sephacryl column worked equally well, consistently producing two well-defined peaks with one of said peaks being the vesicle-containing fraction.

In a further embodiment of the present invention, the production of extracellular vesicles (such as exosomes) may be increased by including autophagy inhibitors in the cell culture medium when growing the vesicle-producing cells. As portrayed in Figure 8, treating cells with autophagy inhibitors resulted in a surprising increase of the exosomal yield. Various

substances can be used to inhibit the different stages of the autophagy pathway, for instance including but not limited to Bafilomycin A and chloroquine (inhibiting the fusion of the autophagosome with the lysosome), and/or 3-methyladenine (which is a PI3K inhibitor that hinders the formation of pre-stages of the autophagosome). Thus, the present invention further relates to the use of autophagy inhibitors to increase the vesicle yield in cell culture. Autophagy-inhibiting substances as per the present invention may include beclin -1 inhibitors, PI3K inhibitors (e.g. 3-methyladenine), and inhibitors of the fusion between autophagosome and lysosome (e.g. Bafilomycin A and chloroquine). Thus, in one general aspect, the present invention relates to increasing the yield of extracellular vesicles by treating the vesicle-producing cell(s) with at least one inhibitor of autophagy.

In a preferred embodiment, the method of producing a therapeutic delivery vesicle as the present invention may hence comprise the steps of (i) providing at least one polynucleotide construct encoding at least one polypeptide construct, (ii) introducing said at least one polynucleotide construct into a cell capable of producing vesicles comprising the polypeptide construct translated from the polynucleotide construct, (iii) cultivating said cells in the presence of at least one autophagy inhibitor, (iv) purifying the delivery vesicles obtained from said cells using UF-LC purification.

The method of producing a therapeutic delivery vesicle may alternatively comprise the steps of (i) providing (a) at least one polynucleotide construct encoding at least one therapeutic polypeptide decoy receptor, and (b) at least one polynucleotide construct encoding at least one carrier polypeptide, (ii) introducing at least one of the polynucleotide construct (a) and at least one of the polynucleotide construct (b) into a cell capable of producing delivery vesicles, and (iii) collecting at least one delivery vesicle produced by the cell of step (ii). When employing this method, the at least one therapeutic polypeptide decoy receptor and the at least one carrier polypeptide may form

a single polypeptide construct through formation of e.g. a disulfide bridge between the carrier polypeptide and the therapeutic polypeptide decoy receptor, or through the formation of a biotin-streptavidin interaction, or through the formation of any other type of chemical bond, including the formation of a syndecan-syntenin-ALIX complex

In yet another aspect, the present invention pertains to a polypeptide construct comprising at least one therapeutic polypeptide decoy receptor that binds a target molecule, fused to at least one carrier polypeptide. Said polypeptide construct may in exemplary embodiments comprise, for instance, (i) at least one therapeutic polypeptide decoy receptor from the TNF family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin (or any derivatives or analogues thereof), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (ii) at least one therapeutic polypeptide decoy receptor from the VEGF family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin (or any derivatives or analogues thereof), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (iii) at least one therapeutic polypeptide decoy receptor from the FGF family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin (or any derivatives or analogues thereof), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle), (iv) at least one therapeutic polypeptide decoy receptor from the EGF family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin (or any derivatives or analogues thereof), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the

surface (or essentially any suitable location on a therapeutic delivery vesicle), (v) at least one therapeutic polypeptide decoy receptor from the activin family combined with a carrier polypeptide selected from CD63, Lamp2b, syndecan, synaptotagmin (or any derivatives or analogues thereof), or any other suitable carrier polypeptide capable of transporting the at least one therapeutic polypeptide decoy receptor to the surface (or essentially any suitable location on a therapeutic delivery vesicle).

Moreover, the present invention may in further aspects relate to a polynucleotide construct encoding at least one therapeutic polypeptide in accordance with the present invention, and, in an additional aspect, a cell comprising at least one polynucleotide construct and/or at least one polypeptide construct. In a further aspect, the present invention pertains to a therapeutic delivery vesicle obtainable by the methods as per the present invention. The cells that may be utilized for the purposes of the present invention comprise for instance mesenchymal cells, adult stem cells (eg. myoblasts), induced pluripotent stem (iPS) cells, cord blood stem cells, embryonic stem cells, and/or amniotic stem cells, blood-derived cells (eg. B-cells, macrophages, DC-cells, T-cells, NK-cells, platelets etc), immortalized eukaryotic cells or cell-lines (eg. neuroblastoma cells NSC34, N2a and SHSY5Y, HEK cells, C17.2 neuronal stem cells, bEND3 neuroendothelial cells, HeLa cells, U2OS cells etc), or any combination of these sources of cells.

The inventors have unexpectedly realized that the proteome of extracellular vesicles obtainable from cells exposed to stress-inducing culturing conditions (such as serum-starvation and oxygen deprivation) is enhanced in positive GO terms as compared to the proteome of extracellular vesicles from cells grown under normal control conditions. Extracellular vesicles (such as exosomes) from cells exposed to serum-starvation and/or oxygen deprivation (i.e. reduced oxygen supply) were analysed with state of the art LC/MS/MS

(proteomics) to examine the changes in proteome the different culturing conditions bring about. Surprisingly, stress-inducing conditions enriched anti-apoptotic and metabolically active proteins to a greater extent than normal culturing conditions according to GO-terms (Figure 10) (GO-terms: translation, metabolic processes, glycolysis and ribosomal proteins). Hence, serum-starvation and/or oxygen deprivation enrich proteins for survival and metabolic processes in the exosomes, which would be a benefit for regenerative purposes. The GO-terms were normalized to the proteome of exosomes from a reference control cell and the enrichment is expressed as fold increase over the expected value from the reference cell. Thus, in a further generally applicable aspect, the present invention relates to the use of stress-inducing conditions to enrich the proteome of an extracellular vesicle (e.g. an exosomes) for metabolically active proteins and/or anti-apoptotic proteins, i.e. positive GO terms such as translation, metabolic processes, glycolysis and/or ribosomal proteins. In a further embodiment, the method for obtaining extracellular delivery vesicles as according to the present invention may comprise a step where the cells from which the extracellular vesicles are obtained are exposed to stress-inducing conditions (for instance but not limited to oxygen deprivation and/or serum-starvation), in order to induce enrichment of metabolically active proteins and/or anti-apoptotic proteins to enhance the regenerative effects of the extracellular vesicles. When comparing control vesicles (without any therapeutic polypeptide) cultured under stress-inducing conditions with control vesicles (without any therapeutic polypeptide) cultured under normal conditions the stress-exposed extracellular vesicles generate a clearly enhanced therapeutic effect in various inflammatory models, indicating that the regenerative capabilities of the extracellular vesicles (notably exosomes) have increased due to the stress-inducing culturing conditions.

A particularly advantageous aspect of the present invention pertains to exposing cells from which extracellular vesicles are to be obtained to a

combination of both autophagy inhibitors and stress-inducing conditions, in order to (1) increase the yield of vesicles and (2) increase the regenerative capacity of the exosomes thus produced. Vesicles (such as exosomes) obtained via this combinatorial approach may be purified using the advantageous UF-LC protocol of the present invention, to truly ensure that the therapeutic efficacy of the extracellular vesicles is optimized.

Further, the present invention relates to a method of treatment comprising administering a therapeutically effective amount of therapeutic delivery vesicles to a subject in need thereof, wherein the method is aimed at improving, alleviating, and/or preventing diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, kidney failure, graft-vs-host disease, Duschenne muscular dystrophy and other muscle diseases, neurodegenerative disease including Alzheimer´s disease, Parkinson´s diease, Huntingtons disease, cancer-induced cachexia, anorexia, diabetes mellitus type 2, and cancers (for instance cancers sensitive to EGF, VEGF, FGF). Some of the cancer types of relevance for the present invention comprises, for instance, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia, Adrenocortical carcinoma, AIDS-related cancers, AIDS-related lymphoma, Anal cancer, Appendix cancer, Astrocytoma, cerebellar or cerebral, Basal-cell carcinoma, Bile duct cancer, Bladder cancer, Bone tumor, Brainstem glioma, Brain cancer, Brain tumor (cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic glioma), Breast cancer, Bronchial adenomas/carcinoids, Burkitt's lymphoma, Carcinoid

tumor (childhood, gastrointestinal), Carcinoma of unknown primary, Central nervous system lymphoma, Cerebellar astrocytoma/Malignant glioma, Cervical cancer, Chronic lymphocytic leukemia, Chronic myelogenous leukemia, Chronic myeloproliferative disorders, Colon Cancer, Cutaneous T-cell lymphoma, Desmoplastic small round cell tumor, Endometrial cancer, Ependymoma, Esophageal cancer, Extracranial germ cell tumor, Extragonadal Germ cell tumor, Extrahepatic bile duct cancer, Eye Cancer (Intraocular melanoma, Retinoblastoma), Gallbladder cancer, Gastric (Stomach) cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal stromal tumor (GIST), Germ cell tumor (extracranial, extragonadal, or ovarian), Gestational trophoblastic tumor, Glioma (glioma of the brain stem, Cerebral Astrocytoma, Visual Pathway and Hypothalamic glioma), Gastric carcinoid, Hairy cell leukemia, Head and neck cancer, Heart cancer, Hepatocellular (liver) cancer, Hodgkin lymphoma, Hypopharyngeal cancer, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi sarcoma, Kidney cancer (renal cell cancer), Laryngeal Cancer, Leukemias ((acute lymphoblastic (also called acute lymphocytic leukemia), acute myeloid (also called acute myelogenous leukemia), chronic lymphocytic (also called chronic lymphocytic leukemia), chronic myelogenous (also called chronic myeloid leukemia), hairy cell leukemia)), Lip and Oral, Cavity Cancer, Liposarcoma, Liver Cancer (Primary), Lung Cancer (Non-Small Cell, Small Cell), Lymphomas ((AIDS-related lymphoma, Burkitt lymphoma, cutaneous T-Cell lymphoma, Hodgkin lymphoma, Non-Hodgkin (an old classification of all lymphomas except Hodgkin's) lymphoma, Primary Central Nervous System lymphoma)), Medulloblastoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Chronic Myeloid Leukemia (Acute, Chronic), Myeloma, Nasal cavity and paranasal sinus cancer, Nasopharyngeal

carcinoma, Neuroblastoma, Oral Cancer, Oropharyngeal cancer, Osteosarcoma/malignant fibrous histiocytoma of bone, Ovarian cancer, Ovarian epithelial cancer (Surface epithelial-stromal tumor), Ovarian germ cell tumor, Ovarian low malignant potential tumor, Pancreatic cancer, Pancreatic islet cell cancer, Parathyroid cancer, Penile cancer, Pharyngeal cancer, Pheochromocytoma, Pineal astrocytoma, Pineal germinoma, Pineoblastoma and supratentorial primitive neuroectodermal tumors, Pituitary adenoma, Pleuropulmonary blastoma, Prostate cancer, Rectal cancer, Renal cell carcinoma (kidney cancer), Retinoblastoma, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma (Ewing family of tumors sarcoma, Kaposi sarcoma, soft tissue sarcoma, uterine sarcoma), Sézary syndrome, Skin cancer (nonmelanoma, melanoma), Small intestine cancer, Squamous cell, Squamous neck cancer, Stomach cancer, Supratentorial primitive neuroectodermal tumor, Testicular cancer, Throat cancer, Thymoma and Thymic carcinoma, Thyroid cancer, Transitional cell cancer of the renal pelvis and ureter, Urethral cancer, Uterine cancer, Uterine sarcoma, Vaginal cancer, Vulvar cancer, Waldenström macroglobulinemia, and/or Wilm's tumor (kidney cancer).

Unlike many other therapies, exosomes and other extracellular vesicles have the potential to cross the blood-brain barrier (BBB). Therapeutic delivery exosomes comprising signalling-incompetent decoy receptors for e.g. IL6, IL-1β and TNFα may thus modulate the various disorders of the central nervous system (CNS), and specifically various forms of neuro-inflammation. Neuro-inflammation is inflammation of the nervous system, including the central nervous system (CNS). The neuroinflammation may be acute, e.g. infection or traumatic events, or chronic, e.g. neurodegenerative diseases (including Alzheimer's disease, Parkinsons disease and demyelinating diseases, such as multiple sclerosis (MS)). In the CNS, glial cells, including microglia and astrocytes, have an important role in innate immunity. These cells, among others cell types in the brain, can produce cytokines and chemokines that act

as neuro-modulators. The most common cytokines in CNS neuro-inflammation include IL6, IL-1β and TNFα. Production of theses pro-inflammatory cytokines can cause neurotoxicity and may compromise the integrity of the blood-brain-barrier (BBB).

In an in vivo model of experimental autoimmune encephalomyelitis (EAE), mice treated with therapeutic delivery exosomes comprising various therapeutic polypeptide decoy receptor(s) (as described above) displayed a markedly improved disease phenotype, as illustrated by Figure 9. Thus, in a further aspect, the present invention pertains to delivery of any suitable receptor (for instance IL-6R, TNFR1, IL-1βR, and/or any combination thereof) to the CNS, to treat essentially any neuro-inflammation.

Furthermore, the instant invention additionally relates to reagents, kits, cell mediums, and cell culturing processes. For instance, cell culturing processes utilizing the methods for producing the therapeutic delivery vesicles of the present invention may be employed in a variety of suitable exosome-producing cell lines, such as mesenchymal cells, adult stem cells (e.g. myoblasts), induced pluripotent stem (iPS) cells, cord blood stem cells, embryonic stem cells, and/or amniotic stem cells, blood-derived cells (eg. B-cells, macrophages, DC cells, T-cells, NK-cells, platelets etc), immortalized eukaryotic cells or cell-lines (eg. neuroblastoma cells NSC34, N2a and SHSY5Y, HEK cells, C17.2 neuronal stem cells, bEND3 neuroendothelial cells, HeLa cells, U2OS cells etc), or any combination of these sources of cells. In further embodiments, the present invention pertains to cell culture medium, any suitable reagent for *in vitro* use, and/or a kit of parts comprising the therapeutic delivery vesicles. Particularly advantageous kits may comprise, optionally in separate contains, cell media for culturing vesicle-producing cells and autophagy inhibitors, to increase the vesicle (preferably exosome) production yield. The cell culture media as per the present invention may be tailored to contain very little or no serum in order to ensure that the

vesicles produced by the cells are expressing higher amounts of metabolically active and anti-apoptotic proteins, to increase their inherent regenerative capacity. For instance, in one preferred embodiment the present invention pertains to a kit comprising (i) culturing media for cell culture under serum-starvation conditions to enhance the regenerative effects of the extracellular vesicles (exosomes) produced, (ii) autophagy inhibitors such as chloroquine, bafilomycin A, and/or 3-methyladenine, or any combination thereof to increase the production yield of the extracellular vesicles (exosomes), and suitable cells for production of extracellular vesicles, for instance exosomes.

It shall be understood that the above described exemplifying aspects, embodiments, and alternatives, and variants can be modified without departing from the scope of the invention, inter alia with respect to the described constituents and components (e.g. the therapeutic delivery vesicles, the therapeutic polypeptide decoy receptors, and the carrier polypeptides, etc.), materials (e.g. therapeutic delivery vesicles, cell types, etc.), and method parameters (e.g. purification techniques, conjugation approaches, etc.) applied. The invention will now be further exemplified with the enclosed examples, which naturally also can be modified without departing from the scope of the invention.

Examples

Cell-based delivery vesicle production

A cell type producing a suitable therapeutic delivery vesicle, such as an exosome, a microvesicle, or any other type of cell-derived structure, is plated/seeded with an appropriate density in cell media. In the case of exosome production, an exosome-producing cell type is plated/seeded with an appropriate density in cell media. The cell media is removed after 24 hours and the plate is washed with PBS 3 times. New fresh exosome-depleted

media or serum free media is added. Exosomes are purified from the conditioned media. The time of incubation before the media is taken from the cells usually ranges from 48-72 hours depending on cell type, it may however be increased or decreased under certain circumstances.

The media that the cells are grown in is always depleted of foreign exosomes and microparticles by ultracentrifugation at 110 000 g overnight before incubation with the cells. Alternatively, a serum free media is applied in its place, such as OptiMEM or DMEM.

The conditioned media can be purified with different techniques; ultrafiltration with sequential LC or high performance liquid chromatography purification, ultra-centrifugation, or commercially available kits. Before ultra-filtration or ultracentrifugation, the conditioned media is cleared of cells and cell debris by spinning the media at 300 g for 5 minutes. The supernatant is subsequently spun again at 1500 g for 15 minutes and ran through a 0.2-micrometer filter. The conditioned media is thus cleared of vesicles and aggregates over 200 nanometer in size. The 0.2-micrometer filtration can be exchanged to a 15 000 g spin for 30 minutes.

By ultra-filtration or tangential flow the conditioned media is concentrated. The MWCO limit is in both methods used are 100 kDa. The concentrated media is further purified by LC or HPLC, using a suitable column, such as Sephacryl S-300. The first fraction from the LC/HPLC contains the exosomes.

By ultracentrifugation the conditioned media is spun at 110 000 g for 70 minutes, the supernatant is discarded and the pellet is re-suspended in PBS and once again centrifuged at 110 000 g for 70 minutes. The supernatant is discarded and the pellet re-suspended in PBS. To further purify the exosomes the second step of the purification process can be done with a 30% sucrose cushion. The cushion traps the exosomes. The exosomes are eluted from the

sucrose cushion by another centrifugation step in PBS at 110 000g for 70 minutes and then the pellet is re-suspended in PBS.

The exosome sample can be analyzed with western blot, ELISA, NTA and electron microscopy. The amount of decoy receptors in each sample may be determined by ELISA towards a polypeptide of interest. The dose given is then calculated as amount of polypeptide given from the concentration obtained from the ELISA.

Artificial vesicle production

Liposomes, lipid-like structures, lipidoids, and other types of artificially produced lipid-based delivery vesicles may also be utilized for the purposes of the present invention. These vesicles may be produced by techniques known in the art and the polypeptide construct comprising the carrier polypeptide and the therapeutic polypeptide decoy receptor may be loaded onto the vesicles using standard technology, for instance lipid-tagging, etc.

Validation of UF-LC purification protocol

Cell culture

NSC-34, a fusion of motor neuron enriched embryonic mouse spinal cord cells with mouse neuroblastoma, N2a, a mouse neuroblastoma cell line, B16F10, a mouse melanoma cell line and human embryonic kidney (HEK293T) cells were cultured at 37°C with 5% CO_2 in complete media comprised of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Cellgro), and penicillin/streptomycin (pen/strep, 5000 µg/ml, Cellgro). For exosome isolation, media were changed 24h after seeding to either pre-spun media or OptiMEM. Pre-spun media is DMEM supplemented with 10% FBS that had been pre-spun at 120,000*g* for 70 min

prior to making up the vesicle devoid media. Both OptiMEM and pre-spun media were supplemented with pen/strep. Conditioned media was then collected for exosome isolation 48h after incubation. For large-scale experiments, conditioned media collected from multiple flasks were pooled prior to isolation of exosomes.

Transfection of HEK293T cells

6 million cells were seeded one day prior to transfection in a 15 cm culture dish with DMEM complete media. Transfection of the CD63-EGFP plasmid was done using polyethyleneimine (PEI) at a 1:4 pDNA:PEI ratio. Briefly, 25 µg of plasmid and 100 µg of PEI were diluted in 500 µl of OptiMEM in separate tubes. After 5 min of incubation at room temperature (RT), the pDNA and PEI solutions were combined and incubated for a further 30 min at RT to form the DNA/PEI complexes. The complexes were then added dropwise to cells. After 4h, the cell growth media containing the complexes was removed; the cells were washed with phosphate buffer saline (PBS) and fresh OptiMEM, supplemented with P/S antibiotics was added on the cells. After 48h of incubation, the conditioned media was collected for exosome isolation.

Ultracentrifugation (UC) for isolation of exosomes

Isolation of exosomes by UC was performed as described in Supplementary Fig. S1a. Briefly, protocol 1 involves two low speed spins, 300*g* for 5 min followed by 1200*g* for 10 min to get rid of cell debris and larger particles. The supernatant was subsequently filtered through a 0.22 µm syringe filter before the final ultracentrifugation step at 120,000*g* for 70 min. Protocol 2 follows that of protocol 1 but includes an additional PBS wash at 120,000*g* for 70 min.Briefly, conditioned media was subjected to an initial low speed spin at 300*g* for 5 min, followed by a 10,000*g* spin for 30 min. The supernatant was

then ultracentrifuged at 120,000*g* for 70 min. Protocol 4 is similar to protocol 1 but lacks the 0.22 µm syringe filtration step.

Ultrafiltration (UF) for isolation of exosomes

The UF protocol involves the same initial low-speed spins as that of the UC protocol. Instead of a high-speed ultracentrifugation at the final step, the cell culture supernatants were spun in 100-kDa cut-off Amicon Ultra-15 spin filter (Millipore) while placental perfusates were spun in 300-kDa cut-off filter (Vivaspin, Sartorius Stedim) at 3500*g* for 15 min. PBS was then added to the filters and spun down to wash the samples.

Liquid chromatography fractionation of UF samples (UF-LC) from cell culture

UF samples, prepared as described above, were loaded onto a HiPrep 16/60 Sephacryl S-300 HR column for samples collected from OptiMEM conditioned media and a 26/60 S-500 HR column for samples collected from pre-spun conditioned media (GE Healthcare), connected to an ÄKTA prime (GE healthcare) equipped with a UV flow cell. Each individual fraction was collected according to the UV absorbance. The collected fractions were then concentrated using a 30-kDa cut-off Amicon Ultra-15 spin filter (Millipore) to 300-400 µl and stored in -80°C until further analyses.

Liquid chromatography fraction of UF samples (UF-LC) from placental perfusions

1 ml of the UF STBM sample was loaded onto an XK16/70 Sephacryl S-1000 column (GE Healthcare), connected to fraction collector (RediFrac, Pharmacia). A pump speed of 2 ml/min was used and 4 ml fractions were

collected. The collected fractions were then concentrated on a 30-kDa spin filter (Vivaspin, Sartorius Stedim), diluted to 300-400 µl and stored in -80°C until further analysis.

Western blotting

Western blotting was performed using either the Bio-Rad® Mini-PROTEAN® Tetra cell or the iBlot® system (Invitrogen, Life Technologies) according to the manufacturer's instructions. To cross-compare the yield of exosomes, we proceeded to load equal volumes of the re-suspended exosome pellet or filtrate on the gel.

For the Bio-Rad system, 15 µl of exosome samples with 15 µl of 2X Laemilli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol were mixed and heated at 100°C for 10 min. Samples were then loaded in a 1.5 mm, 10% Tris/Glycine SDS-polyacrylamide gel and ran at 170 V for 60-70 min in running buffer, until the dye front reached the bottom of the tank. Proteins on the gel were then transferred to a polyvinylidine fluoride (PVDF) membrane (Millipore) at 100 V for 60-70 min in transfer buffer containing 20% methanol. Membranes were then incubated in blocking buffer (5% fat-free milk in Tris buffer saline with 0.1% Tween-20 (TBS-T) for 60 min at room temperature (RT) with gentle shaking.

For the iBlot® system, 30 µl of sample was mixed with a sample buffer, containing 0.5 M ditiothreitol (DTT), 0.4 M sodium carbonate (Na_2CO_3), 8% SDS and 10% glycerol, and heated at 65 °C for 5 min. Samples were then loaded in a NuPAGE® Novex® 4-12% Bis-Tris Gel and ran at 120 V in running buffer until the dye front reached the bottom of the gel. The proteins

on the gel were transferred to an iBlot nitrocellulose membrane (Invitrogen) for 7 min with the iBlot system. Membranes were stained with Ponceau S dye that was later washed away with PBS before blocking with Odyssey blocking buffer for 60 min at RT with gentle shaking.

After the blocking step, the membrane was incubated with freshly prepared primary antibody solution (anti-CD9, anti-PDC6I (Alix), anti-Tsg101 and anti-calnexin; all at 1:1,000 dilution from Abcam, Cambridge UK) overnight at 4°C or 2 h at RT. Membranes were washed three times, 10 min each using washing buffer (TBS-T) with vigorous shaking before adding the secondary antibody solution (anti-mouse IgG DyLight-800 at 1:10,000 dilution if detecting Alix; anti-rabbit IgG DyLight-800 at 1:10,000 dilution for detecting CD9, Tsg101 and Calnexin) and incubated for 1 h at RT. After the secondary antibody incubation, membranes were washed three times, 10 min each and visualised by scanning both 700- and 800-nm channels on the LI-COR Odyssey CLx infrared imaging system. For subsequent probing of other proteins on the same membrane, the membrane was washed three times, 10 min each before re-incubation with the next primary antibody.

Nanoparticle tracking analysis

For particle size determination, nanoparticle tracking analysis (NTA) was performed with a NanoSight NS500 instrument equipped with the NTA 2.3 analytical software. For all our recordings, we used a camera level of 13 or 15 and automatic function for all post-acquisition settings: blur and minimum expected particle size, except in the detection threshold where we fixed it at 5. Samples were thawed on ice and diluted in PBS between 1:500 to 1:20,000 to achieve a particle count of between 2×10^8 and 2×10^9 per mL. Once the dilution of the sample was determined, sample was loaded in the sample

chamber and the camera focus was adjusted to make the particles appear as sharp dots of light. Using the *script control* function, we recorded five 30 or 60 s videos for each sample; incorporating a sample advance and 5 s delay between each recording. For GFP positive exosomes the same set up was used with one minor alteration, which was that the sample was under constant flow in the sample chamber not to bleach the GFP signal. These measurements were then analysed using the *batch process* function and results were exported to Microsoft Excel for further analysis.

Quantification of proteins and RNA in exosomes

Protein quantities in exosomes were quantified using the microBCA assay kit (Thermo Scientific) and levels of RNA were measured using the Quant-iT ™ RiboGreen® RNA assay kit (Life Technologies), according to the manufacturer's instructions.

Electron microscopy

5µl of exosome suspension was diluted 1:1 with PBS and added on formvar-carbon coated electron microscopy grids for 20 min. The grid was blotted with filter paper and 15 µl of 2% uranyl acetate (UA) was added on the grid for 1 min. Next, UA was removed and 15 µl of distilled water was added for 1 min. The water droplet was then removed and the grid was left to air dry for 15 min. The grids were then visualized in the electron microscope.

Fluorescence microscopy

CD63-EGFP positive exosomes were generated as described above. The particles were quantified by NTA and the UF-LC and UC samples were diluted to the same concentration of particles/ml. Before any measurements the

exosomes were re-suspended with a 27G needle. The samples were positioned on a microscope slide and covered with a coverslip and analysed Microscopy was performed using Olympus IX-81 inverted microscope (Olympus America, Center Valley PA, USA) equipped with 20X objective. The following fluorescence filter-set (Chroma Technology Corp., Bellows Falls, VT, USA) was used, with the central wavelength and bandwidth of the excitation and emission filters as indicated: GFP (Ex. 470/40 nm; Em. 525/50 nm)

Liquid chromatography tandem mass spectrometry (LC-MS/MS) of exosomes

Exosomes from UC and UF-LC were concentrated by speedvac and lysed with 1% SDS, 25 mM HEPES, 1 mM DTT. Lysates were heated to 95°C for 5 min followed by sonication for 1 min and centrifugation at 14,000*g* for 15 min. The supernatant was mixed with 1 mM DTT, 8 M urea, 25 mM HEPES, pH 7.6 and transferred to a 10-kDa cut-off centrifugation filtering unit (Pall, Nanosep®), and centrifuged at 14,000*g* for 15 min, followed by an addition of the 8 M urea buffer and centrifugation again. Proteins were alkylated by 50 mM iodoacetamide (IAA) in 8 M urea, 25 mM HEPES for 10 min, The proteins were then centrifuged at 14, 000*g* for 15 min followed by 2 more additions and centrifugations with 8 M urea, 25 mM HEPES. Trypsin (Promega) in 250 mM urea, 50 mM HEPES was added to the cell lysate at a ratio of 1:50 trypsin:protein and incubated overnight at 37°C. The filter units were centrifuged at 14,000*g* for 15 min followed by another centrifugation with MQ and the flow-through was collected. Peptides were cleaned by a strata-X-C-cartridge (Phenomenex).

Before analysis on the Q Exactive (Thermo Fischer Scientific, San Jose, CA, USA), peptides were separated using an Agilent 1200 nano-LC system. Samples were trapped on a Zorbax 300SB-C18, and separated on a NTCC-

360/100-5-153 (Nikkyo Technos., Ltd) column using a gradient of A (3% ACN, 0.1% FA) and B (95% ACN, 0.1% FA), ranging from 7% to 40% B in 240 min with a flow of 0.4 µl/min. The Q Exactive was operated in a data dependent manner, selecting top 5 precursors for fragmentation by HCD. The survey scan was performed at 70,000 resolution from 300-1700 *m/z,* using lock mass at *m/z* 445.120025, with a max injection time of 100 ms and target of 1×10^6 ions. For generation of HCD fragmentation spectra, a max ion injection time of 500 ms and AGC of 1×10^5 were used before fragmentation at 30% normalized collision energy, 17,500 resolution. Precursors were isolated with a width of 2 *m/z* and put on the exclusion list for 70 s. Single and unassigned charge states were rejected from precursor selection.

Proteome discoverer 1.3 with sequest-percolator was used for protein identification. Precursor mass tolerance was set to 10 ppm and for fragments to 0.02 Da. Oxidized methionine and was set as dynamic modification, and carbamidomethylation as static modification. Spectra were matched to a combined *mus musculus* and *bos taurus* ensembl 72 database, and results were filtered to 1% FDR. Identifications in bos taurus was considered to originate from FBS and removed. GO term enrichment analysis was done using Panther.

Exosome biodistribution in mice

Conditioned cell supernatants were filtered through a 0.22 µm syringe filter and incubated with 1µM DiR (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide) (Invitrogen). The conditioned media with DiR was then ultracentrifuged at 110,000*g* for 70 min or concentrated with a 100 kDa Amicon Ultra spin filter (Millipore). The UC pellet was re-suspended and spun again in PBS to purify away-unbound DiR or LC fractionated as described above. The purified exosomes were quantified with

NTA and equal amounts of particles from both UC and LC preparations were injected in the tail vein of Balb/c mice (n=5). 24 h post injection, the organs were harvested and subjected to imaging in the In Vivo Imaging System (IVIS) Spectrum (Caliper). The IVIS was set to record the fluorescence for 2 seconds (excitation 710, emission 760) and the data obtained was then analysed with the IVIS software. All animal experiments were approved by The Swedish Local Board for Laboratory Animals. The experiments were performed in accordance with the ethical permission and designed to minimize the suffering and pain of the animals.

Colitis treatment using exosomes displaying signalling-incompetent CD63-sTNFR1

The well-studied TNBS-induced colitis model in mice was used, simulating the cytokine storm, the diarrhea, weight decrease, and gut inflammation seen in IBD patients. 24 mice were divided into four treatment groups, with 6 mice per group. The mice were pre-sensitized by applying 150 µl of a olive oil-acetate solution with 2% TNBS, on the skin, 1 week prior to colitis induction. Colitis was then induced by giving a rectal infusion of 100 µl solution containing 1.5 % TNBS in 40 % ethanol. Immediately post colitis induction, 30 µg exosomes in 200 µl were administrated intravenously in the tail vein. The mice were given either decoy signalling-incompetent TNFR1-CD63 exosomes, unmodified exosomes, signalling-competent TNFR1-CD63 exosomes, or PBS as mock treatment, depending on the assigned treatment group. The bodyweight was recorded daily.

As can be seen from Figure 2, administration of exosomes comprising decoy receptor as per the present invention leads to successful treatment of colitis in mice. The signalling-incompetent decoy exosomes successfully treat induced colitis within a few days with less loss in body weight, whereas unmodified

exosomes show a moderate effect. Signalling-competent decoy receptors in fact aggravate the condition.

Western blot of CD63-sTNFR1 on the exosomal surface

Western Blot was carried out towards the extracellular part of TNFR1, to verify the presence of the CD63-sTNFR1 polypeptide construct on the exosomal surface. Predicted molecular weight for the CD63-TNFR1 construct is 38.52 kDa, which can be seen in the vicinity of the 37 kDa reference band, both in the cell lysate sample and the exosome sample. The fusion protein is loaded onto the exosomes with great efficiency, since the band is so strong in the exosome fraction (the band around 15 kDa is an irrelevant unspecific band).

Neutralization of TNFα-mediated toxicity

The neutralizing activity of signalling-incompetent CD63-TNFR1 exosomes, signalling-competent CD63-TNFR1 exosomes and exosomes from N2a cells against human TNF-α was measured on the mouse WEHI 164 cell line treated with actinomycin D as previously described (Austgulen et al., 1986; Khabar et al., 1995), in order to verify the binding affinity for TNFα. Briefly, WHEI 164 cells were seeded in triplicate at 1×10^4 cells/well in a 96-well plate and cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS for 20 h. Subsequently, serially diluted exosomes (final concentration: 0.5-100 ug/ml) in the medium containing 2 µg/ml actinomycin D were added to the cell culture together with 0.1 ng/ml of human TNF-α. The cells were incubated for an additional 20 h at a temperature of 37 degrees Centigrade and cell viability was analyzed using a colorimetric MTT-based Cell Growth Determination kit (Sigma, St. Louis, MO). The ED_{50} value was calculated by complex sigmoid non-linear regression analysis using Sigma plot software (Systat software, Inc. Richmond, CA).

Anti-tumour efficacy of exosomes and liposomes displaying signalling-incompetent syndecan/CD63-sVEGFR1

30 mice were implanted with $1x10^6$ B16/F10 melanoma cells into the flank at day zero. The mice were then divided into five treatment groups, with 6 mice per group. After one week (day 7), the mice received intravenous injections of 30 µg exosomes in 200 µl which were repeated every second days for two weeks. The mice were given exosomes comprising signalling-incompetent syndecan-sVEGFR1, signalling-incompetent CD63-sVEGFR1 exosomes, unmodified exosomes, and exosomes comprising signalling-competent CD63-sVEGFR1, or PBS as mock treatment depending on assigned treatment group. The tumour volume was measured every second day.

The arrow in Figure 4 indicates start of treatment (day 7). The mice treated with signalling-incompetent decoy sVEGFR1 exosomes (syndecan-sVEGFR1 EXO and CD63-sVEGFR1 EXO) displayed the lowest tumour burden after treatment. Treatment with unmodified exosomes had a moderate effect on tumour size, whereas treatment with exosomes comprising signalling-competent CD63-sVEGFR1 resulted in an aggravated condition, compared to the mock-treated control group.

The above experiments were also repeated with liposomes comprising the same set of polypeptide decoy receptors and similar results were obtained.

Treatment of MDX mice

N2a cells were seeded at 3 million per 150 cm^2 flask and grown in DMEM with 10% FBS. After 24 hours the cells were PEI-transfected with plasmids encoding signalling-incompetent activin-syndecan or signalling-incompetent activin-synaptotagmin. 4 hours post transfection the media was changed to OptiMEM. 72 hours after the media change exosomes produced by the N2a cells were harvested by ultra-filtration and sequential LC purification. The exosomes were used immediately or stored at -20. MDX mice were obtained

from Charles River at a weight of around 18-19 grams. The mice were allocated into 4 groups with 6 mice in each group. The mice received injections of exosomes or PBS twice weekly for 12 weeks. The weight was recorded before each injection.

Figure 5 shows treatment efficacy in MDX mice of exosomes comprising the therapeutic decoy polypeptide receptor activin fused to either the carrier proteins syndecan (solid black line) or synaptotagmin (long-dashed grey line). Bi-weekly treatment with the above therapeutic delivery exosomes resulted in a considerably greater body weight increase than treatment with unmodified exosomes and mock treatment.

The above experiments were also repeated with chylomicrons and similar results were obtained.

Treatment of neuro-inflammation

In an in vivo model of experimental autoimmune encephalomyelitis (EAE), mice treated with decoy exosomes (as described above) displayed a markedly improved disease phenotype, fig X3. EAE was induced by immunization of mice with neuroantigen (myelin basic protein, MBP) and complete Freund's adjuvant (containing M. tuberculosis), followed by injection of pertussis toxin to produce severe and reliable EAE. Disease progression with clinical symptoms was recorded daily from disease onset (day 12-28). The symptoms are scored based on severity (0=normal mouse; no overt signs of disease; 1=limptail or hind limb weakness, but not both; 2=limptail and hind limb weakness; 3=partial hind limb paralysis; 4=complete hind limb paralysis; 5=moribund state; death by EAE: sacrifice for humane reasons), resulting in a mean clinical score used to assess disease state. Notable is that mice, with and without induced EAE, treated with exosomes derived from neural origin resulted in spasms and subsequent death, compared to treatment of

exosomes from other cell origins where this phenomenon was not present. Figure 9 illustrates the efficacy of the following delivery exosomes:

- Decoy exosomes comprising IL6R +TNFR1
- Decoy exosomes comprising IL6R
- Decoy exosomes comprising TNFR1
- Decoy exosomes comprising IL-1βR
- Unmodified exosomes
- Untreated control

As can be seen from Figure 9, EAE-mice treated with decoy exosomes displaying signalling-incompetent receptors for IL6, IL-1β and TNFα display a very moderate disease manifestation compared to mock treated control, whereas mice treated with signalling-competent (TNFR1 signalling-competent) therapeutic polypeptide receptor-containing exosome display a worsened disease.

Claims

1. A therapeutic delivery vesicle comprising attached to its membrane a polypeptide construct, wherein the polypeptide construct comprises at least one carrier polypeptide fused to at least one therapeutic polypeptide decoy receptor present at least partially on the outside of the delivery vesicle and wherein the at least one therapeutic polypeptide decoy receptor is signalling-incompetent.

2. The therapeutic delivery vesicle according to claim 1, wherein the polypeptide construct is a transmembrane polypeptide construct.

3. The therapeutic delivery vesicle according to any one of the preceding claims, wherein the at least one therapeutic polypeptide decoy receptor is fused to the carrier polypeptide via a chemical bond selected from the group comprising a peptide (amide) bond, a thio-ether bond, a di-sulfide bridge, a biotin-streptavidin interaction, and any combination thereof.

4. The therapeutic delivery vesicle according to any one of the preceding claims, wherein the at least one carrier polypeptide is selected from Lamp2b, CD9, CD81, CD63, syndecan, synaptotagmin, ALIX, syntenin, and any combination thereof.

5. The therapeutic delivery vesicle according to any one of the preceding claims, wherein the delivery vesicle is selected from the group comprising extracellular vesicles, exosomes, microvesicles, apoptotic bodies, microparticles, ectosomes, prostatosomes, cardiosomes, lipoprotein particles, LDL particles, VLDL particles, HDL particles, chylomicrons, liposomes, lipid-like particles, lipidoids and any combination thereof.

6. The therapeutic delivery vesicle according to any one of the preceding claims, wherein the at least one therapeutic polypeptide decoy receptor is selected from the group comprising receptors from the following

receptor families: insulin, PDGF, FGF, EGF, VEGF, HGF, TRK, EPH, AXL, LTK, TIE, ROR, DDR, RET, KLG, PTCH1, RYK, MuSK, activin, Type I TGF, Type II TGF, and TNF, interleukin (IL), and any combination thereof.

7. The therapeutic delivery vesicle according to any one of claims 1 to 6, for use in medicine.

8. The therapeutic delivery vesicle according to any one of claims 1 to 6, for use in the treatment and/or prophylaxis of Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, kidney failure, graft-vs-host disease, Duschenne muscular dystrophy and other muscle diseases, cancer-induced cachexia, anorexia, diabetes mellitus, neuro-inflammatory diseases and/or disorders, cancer (for instance cancers sensitive to EGF, VEGF, FGF), colon cancer, breast cancer, and glioma.

9. A pharmaceutical composition comprising a therapeutic delivery vesicle according to any one of claims 1 to 6 and at least one pharmaceutically acceptable excipient.

10. A signalling-incompetent polypeptide construct comprising at least one therapeutic polypeptide decoy receptor fused to at least one carrier polypeptide.

11. A polynucleotide construct encoding the signalling-incompetent polypeptide construct according to claim 10.

12. A cell comprising at least one polypeptide construct according to claim 10 and/or at least one polynucleotide construct according to claim 11.

13. A method for purifying extracellular vesicles, comprising the steps of:

    (i) exposing a sample comprising at least one extracellular vesicle to ultrafiltration;

    (ii) exposing the sample obtainable from step (i) to size exclusion liquid chromatography.

14. A method for increasing the production yield of extracellular vesicles comprising exposing vesicle-producing cells from which the extracellular vesicles are to be obtained to at least one autophagy inhibitor.

15. The method according to claim 14, wherein the at least one autophagy inhibitor is selected from the group comprising beclin -1 inhibitors, PI3K inhibitors (e.g. 3-methyladenine), inhibitors of the fusion between autophagosome and lysosome (e.g. Bafilomycin A and chloroquine), and any combination thereof.

16. A method for enriching metabolically active proteins and/or anti-apoptotic proteins in extracellular vesicles, comprising the step of exposing vesicle-producing cells from which the extracellular vesicles are to be obtained to stress-inducing conditions.

17. The method according to claim 16, wherein the stress-inducing conditions are oxygen deprivation and/or serum-starvation.