Figure 8: Flow Cytometry analysis of ABD EVs' capacity to bind albumin compared to control EVs.

Figure 9: Further ABD-EV albumin *in vitro* binding assays testing a range of additional constructs,

Figure 10: Further *in vivo* half-life extension experiments assays testing a range of additional constructs.

Figure 11: *In vitro* albumin binding data combined with *in vivo* plasma half-life extension data for EVs expressing ABD fused to a single pass transmembrane EV protein.

Figure 12: *In vivo* biodistribution of ABD EVs showing lymph node and tumor accumulation of ABD EVs compared to control EVs.

Figure 13: Graphs showing ABD EV half-life in plasma after delivery by different routes of administration.

Figure 14: Graphs showing improved plasma half-life of ABD EVs derived from an alternative cell source.

Figure 15: Gradient separation (Dot-Blot quantification) & wide-field microscopy images showing co-location of albumin and ABD expressing EVs.

Figure 16: Graph showing *in vivo* ABD binding assay expressed as quantified Dot-Blot confirming that *in vivo* EVs expressing ABD bind albumin.

Figure 17: Further gradient separation analysis by Dot-Blot showing ABD EVs bind albumin.

Figure 18: Schematic outlining construct design for fusion proteins.

Detailed Description of the Invention

linkers, spacers and/or scaffold sequences allows flexibility and enables ABD to be positioned optimally for display on the surface of the EV.

Linkers according to the invention are useful in providing increased flexibility, improving pharmacokinetics (PK), increasing expression and improving biological activity of the fusion polypeptide constructs, and also to the corresponding polynucleotide constructs, and may also be used to ensure avoidance of steric hindrance and maintained functionality of the fusion polypeptides. Exemplary linkers according to the present invention include glycine or serine linkers, which increase stability or flexibility, such as $(GGGGS)_n$ (n=1, 2, 4) or $(Gly)_6$, $(Gly)_8$, rigid linkers such as (EAAAK)n (n=1-3, and A(EAAAK)4ALEA(EAAAK)4A, bending linkers $(XP)_n$ or cleavable linkers such as disulphide, protease sensitive sequences.

In a specific embodiment, an EV of the invention comprises more than one ABD, i.e. a plurality of ABDs. The plurality of ABDs may be the same or different from one another. As described above this may be as a result of more than one ABD being present in a single fusion protein, alternatively it may be as a result of multiple fusion proteins being loaded into a single EV. Said multiple fusion proteins may comprise the same or different EV protein. Without wishing to be bound by theory, the presence of more than one ABD on a single EV can be advantageous as it increases the amount of albumin coating the EV and thus increases the shielding effect of the ABD, which in turn can increase the half-life of the ABD EV. In some embodiments, the EVs of the invention comprises at least one ABD EV fusion protein, wherein the at least one ABD EV fusion protein comprises at least one, or at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten ABDs.

EVs comprising ABDs may be produced using any of the methods disclosed herein.

In one embodiment of the present invention, the EVs of the invention are further loaded with a therapeutic cargo, optionally wherein the therapeutic cargo is a protein, nucleic acid, virus, viral genome, antigen or small molecule or combination thereof.

cytosine arabinoside, proteasome inhibitors such as bortezomib, or kinase inhibitors such as imatinib or seliciclib, or non-steroidal anti-inflammatory drugs (NSAIDs) such as naproxen, aspirin, or celecoxib, antibiotics such as heracillin, or antihypertensives such as angiotensin-converting enzyme (ACE) inhibitors such as enalapril, ARBs such as candesartan, etc. The present invention is naturally applicable also to other small molecules without departing from the gist of the invention, as would be clear to a person skilled in the art.

In certain embodiments, the therapeutic cargo carried by the ABD EVs may be present on the inside of the EV, on the outside of the EV or in the membrane of the EV. The desired location of the therapeutic cargo will depend on the nature of the cargo and its mechanism of action, for instance, a membrane protein will preferably be located in the membrane of the EV, a decoy receptor will preferably be present on the surface of the EV, but a cargo designed to be delivered into the cytosol or nucleus of the recipient cell, such as a silencing RNA, will preferably be located inside the lumen of the EV.

The therapeutic cargo may be loaded passively into the EVs by the therapeutic cargo being present in the cytosol of the EV producing cells. Such passive loading applies, for instance, to nucleic acids, small molecules, viruses, soluble proteins or membrane proteins that are naturally loaded into the EVs.

In certain embodiments, the therapeutic cargo is actively loaded into the ABD EVs of the present invention. One form of active loading of cargos involves exogenous active loading, which involves cargo being loaded using any known exogenous loading method including: electroporation, transfection with transfection reagents such as cationic transfection agents, lipofectamine (RTM), conjugation of the cargo to a membrane-anchoring moiety, such as a lipid or cholesterol tail, or loading by means of a CPP, either in the form of a CPP-cargo conjugate or in the form of a CPP-cargo non-covalent complex. Again, this type of active loading may result in the therapeutic cargo protein being located on the inside of the EV, on the outside of the EV or located within the membrane of the EV.

In certain embodiments, the therapeutic cargo is actively loaded into the ABD EVs of the present invention by the use of fusion proteins. In this case, the therapeutic cargo

fragment/domain thereof present on the matrix, and then running a solution with a suitable pH through the chromatography column. The pH of the solution that is intended to trigger release of the EVs from the column may be below pH 8, below pH 7, or below pH 6. Both the process of capturing the EVs and the process of releasing the EVs may be repeated multiple times, e.g. anywhere from once to up to e.g. 500 times.

In further embodiments, the albumin or fragment/domain thereof may be attached to the chromatography matrix via different types of chemical and biochemical linkages and bonds. Covalent bonds between the matrix and the protein comprising the albumin or fragment/domain thereof, e.g. HSA, may be conventional amide bonds, disulfide bonds, ether bonds, esther bonds, thio-ether bonds, thio-esther bonds, glutathione-GST interactions, streptavidin-biotin interaction, etc. The matrix may be chemically activated to facilitate binding to the albumin protein or fragment/domain thereof using chemical conjugation moieties such as N-hydroxysuccinimide (NHS; for NHC-EDC/EDAC coupling), thiols, cyanogen bromide (CNBr), epoxy, thiopropyl, primary amines, sulfhydryls, carboxylic acids, aldehydes, iodoacetyl, azlactones, carbonyldiimidazole (CDI), maleimide, etc., as is well known to a person skilled in the art.

In preferred embodiments, the processes of the present invention are carried out in chromatography columns, which comprise the chromatography matrix comprising the albumin or fragment/domain thereof.

The chromatography matrix for use in capturing ABD EVs may consist of essentially any type of material suitable as a stationary chromatography phase. Non-limiting examples include one or more of agarose, dextran, lectin, heparin, cellulose, starch, dextran, agar, agarose, poly(meth)acrylate, polyacrylamide, polysulfone, a polyvinyl polymer, polystyrene, silica, alumina, zirconium oxide, titanium oxide, polysaccharide-mineral structure, polysaccharide-synthetic polymer, synthetic polymer-mineral structure, or any combination thereof. The matrix may be in the form of beads, fibers, irregularly shaped particles, membranes, flat structure, porous mineral materials or essentially any type of suitable stationary phase. Naturally, the albumin or fragment/domain thereof attached to the matrix may also be directly attached to

percentage of double positive EVs is likely to be significantly higher than that presented in Figure 8. Nevertheless, the data provided in Figure 8 clearly show that albumin binds to engineered ABD-EVs. The data correlate well with the fluorescent signals detected by SpectraMax and nanoluc signals detected by luminometer.

Example 9: Additional ABD-EV albumin *in vitro* binding assays

Following the success of the *in vitro* binding assay described in Example 7 and shown in Figures 7A and 7B. The same protocol as outlined in Example 7 was used but a wider range of constructs were tested. These constructs include a range of different EV proteins (human CD81, CD9 and CD63) and a range of different locations for the ABD (1st loop, second loop or both loops(x2)).

Figures 9 A-F show similar results to Figure 7 indicating that FITC-albumin is bound on the surface of EVs when ABD is engineered into a fusion construct with a range of different EV proteins and at a range of different locations within the EV protein (1st loop, second loop or both loops). This shows that the binding of albumin to the ABD is not dependent on the type of EV protein present in the fusion construct, nor the location of the ABD within the fusion protein.

Importantly, experiments where ABD is fused into both loops show that it is possible to engineer functional proteins into more than one loop of a multi-pass transmembrane EV protein without the EV protein being disrupted or affecting membrane insertion.

Example 10: Additional *in vivo* half-life extension experiments

Following the success of the experiments shown in Example 3 additional constructs were tested *in vivo* to assess the effect of ABD in combination with a range of different EV proteins and ABD located in different loops. The constructed tested were:

- **mouseCD81-ABD2nd loop**

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