It is an object of this invention to address one or more of these drawbacks.

SUMMARY ~~OF THE INVENTION~~

The problem underlying the ~~invention~~ disclosure is seen most generally in the provision of an improved, cost- and resource-saving process to produce saposin lipoprotein particles.

The problem is solved by the process defined in claim 1. Advantageous ~~embodiments~~ examples are described in the dependent claims and further herein below.

The ~~invention~~ disclosure provides a process for producing a saposin lipoprotein particle, wherein the produced saposin lipoprotein particle comprises

- a saposin-like protein,
- lipids, and
- optionally, a hydrophobic agent, wherein the hydrophobic agent is different from the lipids and

(I) wherein the process comprises the following steps:

a) providing the lipids, and optionally the hydrophobic agent;

b.1) contacting the saposin-like protein with a support that is capable of selectively binding the saposin-like protein to the support in a liquid environment;

c.1) contacting the support-bound saposin-like protein with the lipids and, optionally, the hydrophobic agent, to allow for the self-assembly of the saposin lipoprotein particle on the support;

d) optionally eluting the support-bound saposin lipoprotein particle; or

(II) wherein alternatively the process comprises the following steps:

a) providing the hydrophobic agent and the lipids;

b.2) contacting the hydrophobic agent with a support that is capable of selectively binding the hydrophobic agent to the support;

c.2) contacting the support-bound hydrophobic agent with the saposin-like protein to allow for the self-assembly of the saposin lipoprotein particle on the support;

d) optionally eluting the support-bound saposin lipoprotein particle.

In one aspect, the present disclosure refers to a process for producing a saposin lipoprotein particle, wherein the produced saposin lipoprotein particle comprises

- a saposin-like protein (SAPLIP) belonging to the SAPLIP family of lipid interacting proteins, characterized by the saposin fold, a conserved alpha-helical three-dimensional structure that is stabilized by highly conserved intramolecular disulphide bonds, and
- lipids, and

(I) wherein the saposin lipoprotein particle optionally further comprises a hydrophobic agent, wherein the hydrophobic agent is different from the lipids, and wherein the process comprises the following steps:

a) providing the lipids, and optionally the hydrophobic agent;

b.1) contacting the saposin-like protein with a support that is capable of selectively binding the saposin-like protein to the support in a liquid environment, wherein the support is a solid support;

c.1) contacting the support-bound saposin-like protein with the lipids and, optionally, the hydrophobic agent, to allow for the self-assembly of the saposin lipoprotein particle on the support;

d) optionally eluting the support-bound saposin lipoprotein particle; or

(II) wherein the saposin lipoprotein particle further comprises a hydrophobic agent, wherein the hydrophobic agent is different from the lipids, and wherein the process comprises the following steps:

a) providing the hydrophobic agent and the lipids;

b.2) contacting the hydrophobic agent with a support that is capable of selectively binding the hydrophobic agent to the support, wherein the support is a solid support;

c.2) contacting the support-bound hydrophobic agent with the saposin-like protein to allow for the self-assembly of the saposin lipoprotein particle on the support;

d) optionally eluting the support-bound saposin lipoprotein particle.

In the process according to the ~~invention~~disclosure, either the hydrophobic agent or the saposin-like protein are selectively bound to a support and particle assembly occurs in this support-bound state by contacting the support-bound hydrophobic agent or the saposin-like protein with the remaining components of

the saposin lipoprotein particle, which allows for the self-assembly of the saposin lipoprotein particles on the support. This is different from the processes of the prior art, which relied on assembly processes, in which the individual components are all present in free (i.e. non-support-bound) form and their self-assembly into saposin lipoprotein particles is based on the free, random motion of all involved particle components in the liquid environment.

The process according to the ~~invention~~disclosure has the advantage that the saposin-like protein or the hydrophobic agent is selectively bound to a support, which directly yields support-bound saposin lipoprotein particles or from which the later assembled particles can be eluted. This limits the amount of formed Salipro particles to the amount of pre-bound particle components, i.e. either the amount of bound saposin-like protein or bound hydrophobic agent. Consequently, any material that remains unbound can be recycled. The recycling can concern the saposin-like protein or the hydrophobic agent which is not bound to the support in step b.2) and b.1), respectively. The recycling can, however, also concern surplus particle components, which were contacted with the support-bound material to allow for self-assembly of the Salipro particles in step c.1)/c.2), but did not incorporate into particles. Such recycling of the "unused" components allows for cost- and resource-saving particle assembly.

Direct assembly of saposin lipoprotein particles on a support is particularly advantageous if the particles anyhow need to be used in a support-bound state. This is the case for many chip-based analytical applications such as biosensor applications, in particular for optical biosensor applications such as Surface Plasmon Resonance. These are described in more detail herein below. In the past, free saposin lipoprotein particles had first to be prepared by the prior art processes and then required a second coupling step to immobilize them on the support. With the method of the ~~invention~~disclosure, it is possible to perform the actual particle assembly on the support, which saves additional costs and process steps.

It was surprising that Salipro particles could be assembled also when one particle component, i.e. the saposin-like protein or the hydrophobic agent, is bound to a support. The binding of one of the particle components to the support limits its freedom to get into contact with other particle components in solution and also its freedom for spatial interactions with the particle components. Consequently, an impairment of particle assembly with regard to efficiency, ultimately also influencing the overall yield of obtained particles, was expected.

It has to be borne in mind that saposin lipoprotein particle assembly must be assumed to involve significant rearrangements in saposin protein structure and lipid organization (e.g., from random motion of free lipids to highly ordered nanomembrane bilayer structures). The saposin protein likely undergoes a conformational change when switching from the closed lipid-free form to the open lipid-

bound state. Particle self-assembly is thought to involve a step in which the saposin-like protein captures and embraces the lipids and the hydrophobic agent that will be incorporated into the particle. Whereas it was known that this self-assembly occurs in solution, it was entirely unexpected to see that it worked equally well and fast when one of the principal particle components (i.e. the saposin protein or the hydrophobic agent) is immobilized on a solid support.

It was unexpected to discover that high quality support-bound Salipro particles can be obtained with the process according to the ~~invention~~disclosure, which provides an easy, rapid and efficient way of producing support-bound Salipro particles that maintain a uniform quality and composition over time. The ~~invention~~disclosure in principle provides a more efficient and "straightforward process for obtaining support-bound Salipro particles by intertwining assembly and coupling of the Salipro particles. In this way, hydrophobic agents can – in one continuous process – be solubilized into stable particles mimicking their natural membrane environment and at the same time be immobilized on a support for direct use in applications such as lab-on-a-chip or biosensor applications such as SPR.

The saposin lipoprotein particles obtained by the process of the ~~invention~~disclosure are in principle the same as those known from the prior art, just that they are directly in support-bound form. The saposin lipoprotein particles are also referred to herein as "Salipro particle(s)". They are nanoparticles that comprise a saposin-like protein, a hydrophobic agent and lipids. These components and advantageous ~~embodiments~~examples thereof will be further defined below. The saposin-like protein belongs to the well-known and conserved SAPLIP family of lipid interacting proteins or is a derivative or truncated form thereof.

Practical experiments of the inventors have revealed that the size of the Salipro particles self-adjusts to the nature of the incorporated hydrophobic agent and lipids, e.g., to the size of the incorporated membrane protein. Salipro particles are surprisingly flexible in size. Also in the process according to the ~~invention~~disclosure, it has been observed that the Salipro particles adjust their size to the nature of the incorporated hydrophobic agents. This is advantageous over the size limitation of other non-saposin-derived prior art particles. This flexibility also enables the incorporation of very large hydrophobic agents, such as (multimeric) membrane proteins. For these in particular the process of the ~~invention~~disclosure has the advantage that they can be reconstituted in their natural environment, e.g., the membrane lipids or other cell components associated with the membrane protein and potentially required for maintaining the protein's structure and/or function.

Surprisingly, Salipro particles display a certain degree of thermostability, and also seem to be amenable to freeze-drying and rehydration when bound to supports without major quality deterioration observable. This enables Salipro particles that are obtained by the process of the ~~invention~~disclosure to be used in applications in which portability and storage stability is an issue. With the process of the ~~invention~~disclosure it is

possible to obtain support-bound Salipro particles that can be custom-made in advance, stored and shipped to end users later on. It is also possible to employ them in applications over a longer period of time, for example, lab-on-a-chip experiments that reuse the same chip multiple times.

The support-bound Salipro particles obtained with the method of the ~~invention~~disclosure have proven to be capable of the incorporation of a variety of lipids, membrane proteins and hydrophobic compounds at a physiological pH, giving rise to nanoscale complexes that are soluble, but support-bound and stable in an aqueous environment. For the hydrophobic agents tested thus far, in particular for many complex membrane proteins, the incorporation into the support-bound Salipro particles has had no negative effect on their biological function. By contrast, for many of these, incorporation into Salipro particles efficiently mimics the hydrophobic agent's natural membrane environment and therefore has a positive effect on their biological function.

The ~~invention~~disclosure also provides the advantage that hydrophobic agents, for which direct immobilization on supports without impairment or loss of their biological function is not possible, can be indirectly immobilized by incorporation into a Salipro particle prepared according to process (I) of the ~~invention~~disclosure, i.e. in which the Salipro particle is bound to the support not via the hydrophobic agent but via a binding moiety in the saposin-like protein. This allows the hydrophobic agent to remain unmodified and/or not in direct contact with the support, while still being effectively bound to a support.

As described above, the provision of support-bound Salipro particles, which can be optionally also eluted from the support, is a very useful tool in chip-based applications such as lab-on-a-chip or biosensor (e.g., SPR) applications.

It appears that by selectively binding the hydrophobic agent or the saposin-like protein to a support and by contacting them with the respective other mandatory particle components, the process of the ~~invention~~disclosure provides a robust technique to assemble Salipro particles, which are stable in aqueous solutions over a wide pH range, in particular at physiological pH, and allows larger particles than the 3.2 nm saposin A-derived lipoprotein particles obtained from synthetically prepared liposomes according to the prior art teaching of Popovic et al.

As described in the introduction, the importance of membrane proteins in therapeutic developments necessitates the discovery of innovative methods for interrogating membrane proteins in cell free mediums, preferably in a detergent-free environment.

The Salipro particles obtainable by the process of the ~~invention~~disclosure fulfill this requirement. The Salipro particles, once obtained, are stable in cell free media and detergent-free environments, and are directly bound to supports that they may later on be used on.

In a particular advantageous ~~embodiment~~ example of the ~~invention~~disclosure, the hydrophobic agent and the lipids that are to be incorporated into the saposin lipoprotein particles are provided in form of biological membranes comprising the hydrophobic agent and the lipids that are to be incorporated into the saposin lipoprotein particles. In particular, viral, archaeal, eukaryotic or prokaryotic membranes can be directly employed in the process of the ~~invention~~disclosure as source materials for the hydrophobic agent and the lipids. The biological membranes can be provided in the form of cells, viruses or organelles, all of which can be intact, treated with an amphiphilic agent, or lysed. The amphiphilic agent can be selected from the group consisting of detergents, amphiphilic peptides, amphiphilic polymers, other amphiphilic compounds and mixtures thereof. Examples of amphiphilic polymers that can be used according to the ~~invention~~disclosure are maleic acid copolymers, in particular styrene-maleic acid copolymer (SMA) or diisobutylene-maleic acid copolymer (DIBMA). Amphiphilic peptides and amphiphilic polymers can be considered specialty detergents. Suitable SMAs are, e.g., described in Postis, Vincent, et al. "The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy." Biochimica et Biophysica Acta (BBA)-Biomembranes 1848.2 (2015): 496-501. DIBMAs are commercially available, e.g. from anatrace as BMA101. Preferably, the amphiphilic agent is a detergent as defined herein. Preferably, the cells are eukaryotic cells, in particular non-human animal or human cells. Surprisingly the research leading up to this ~~invention~~disclosure has shown that no purification of the biological membranes is required. Rather, when using the method of the ~~invention~~disclosure, saposin lipoprotein particles can be prepared directly from intact, amphiphilic agent-treated, or lysed cells, viruses or organelles.

By using biological membranes as starting material in the process of the ~~invention~~disclosure, hydrophobic agents can be incorporated into Salipro particles together with their native membrane environment. Compared to the process of WO 2018/033647 A1, process alternative (II) of the ~~invention~~disclosure has the advantage that only the Salipro particles comprising the hydrophobic agent of interest are prepared, and not a full library of Salipro particles representing the entire membrane proteome/lipidome, from which the particles comprising the hydrophobic agent of interest again need to be isolated. In addition, the process of the ~~invention~~disclosure has the advantage over WO 2018/033647 A1 that support-bound particles can be obtained in a straightforward, simplified and continuous “one-step process”, which is cost-effective and desirable especially if the Salipro particles are to be used in support-bound form.

By using biological membranes as starting material in process alternative (I) of the ~~invention~~disclosure, a library of Salipro particles can be obtained wherein the library comprises a heterogenic mixture of saposin

lipoprotein particles with different membrane lipid and membrane protein compositions. Compared to the process of WO 2018/033647 A1, this ~~embodiment~~ example of the process of the ~~invention~~disclosure has the advantage that support-bound particles can be obtained in a continuous "one-step process", which is cost-effective and desirable especially if the library of Salipro particles is to be used in support-bound form.

The Salipro particles or the library of Salipro particles obtainable by the process of the ~~invention~~disclosure can be for use in medicine, for use in a diagnostic method, a cosmetic treatment or for use as vaccination formulation. Further, the particles obtainable by the process of the ~~invention~~disclosure can be used as a tool for diagnostics, drug development, drug screening, drug discovery, antibody development, development of therapeutic biologics, for membrane or membrane protein purification, for membrane protein expression, for membrane and/or membrane protein research, for the isolation, identification and/or study of membranes and/or membrane proteins or creation of a lipidome or membrane proteome database.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a process for producing a saposin lipoprotein particle, wherein the saposin lipoprotein particle comprises

- a saposin-like protein,
- lipids, and
- optionally, a hydrophobic agent, wherein the hydrophobic agent is different from the lipids and

(I) wherein the process comprises the following steps:

a) providing the lipids, and optionally the hydrophobic agent;

b.1) contacting the saposin-like protein with a support that is capable of selectively binding the saposin-like protein to the support in a liquid environment;

c.1) contacting the support-bound saposin-like protein with the lipids and, optionally, the hydrophobic agent, to allow for the self-assembly of the saposin lipoprotein particle on the support;

d) optionally eluting the support-bound saposin lipoprotein particle; or

(II) wherein alternatively the process comprises the following steps:

a) providing the hydrophobic agent and the lipids;

b.2) contacting the hydrophobic agent with a support that is capable of selectively binding the hydrophobic agent to the support;

c.2) contacting the support-bound hydrophobic agent with the saposin-like protein to allow for the self-assembly of the saposin lipoprotein particle on the support;

Claims

1. Process for producing a saposin lipoprotein particle, wherein the produced saposin lipoprotein particle comprises

   – a saposin-like protein (SAPLIP) belonging to the SAPLIP family of lipid interacting proteins, characterized by the saposin fold, a conserved alpha-helical three-dimensional structure that is stabilized by highly conserved intramolecular disulphide bonds, and

   – lipids, and

   ~~– optionally, a hydrophobic agent, wherein the hydrophobic agent is different from the lipids and~~

   (I) wherein the saposin lipoprotein particle optionally further comprises a hydrophobic agent, wherein the hydrophobic agent is different from the lipids, and wherein the process comprises the following steps:

   a) providing the lipids, and optionally the hydrophobic agent;

   b.1) contacting the saposin-like protein with a support that is capable of selectively binding the saposin-like protein to the support in a liquid environment, wherein the support is a solid support;

   c.1) contacting the support-bound saposin-like protein with the lipids and, optionally, the hydrophobic agent, to allow for the self-assembly of the saposin lipoprotein particle on the support;

   d) optionally eluting the support-bound saposin lipoprotein particle; or

   (II) wherein the saposin lipoprotein particle further comprises a hydrophobic agent, wherein the hydrophobic agent is different from the lipids, and wherein the process ~~alternatively the process~~ comprises the following steps:

   a) providing the hydrophobic agent and the lipids;

   b.2) contacting the hydrophobic agent with a support that is capable of selectively binding the hydrophobic agent to the support, wherein the support is a solid support;

   c.2) contacting the support-bound hydrophobic agent with the saposin-like protein to allow for the self-assembly of the saposin lipoprotein particle on the support;

   d) optionally eluting the support-bound saposin lipoprotein particle.

2. Process according to claim 1, alternative (I), wherein the support comprises a capture moiety, and the saposin-like protein comprises a binding moiety, wherein the capture moiety is capable of selectively binding the binding moiety in the saposin-like protein.

3. Process according to claim 1, alternative (II), wherein the support comprises a capture moiety, and the hydrophobic agent comprises a binding moiety, wherein the capture moiety is capable of selectively binding the binding moiety in the hydrophobic agent.

4. Process according to any one of the preceding claims, wherein the support is in the form of

i. beads,

ii. a bed,

iii. a membrane, ~~and/~~or

iv. ~~a solid support, in particular~~ a solid support with a planar surface.

5. Process according to any one of the preceding claims, wherein the lipids are selected from the group consisting of viral, archaeal, eukaryotic and prokaryotic lipids, and mixtures thereof.

6. Process according to any one of the preceding claims, wherein in step a) the hydrophobic agent and the lipids are provided in form of a viral, archaeal, eukaryotic or prokaryotic membrane, which comprises the hydrophobic agent and the lipids that are to be incorporated into the saposin lipoprotein particles.

7. Process according to claim 6 in so far as it relates to claim 1, alternative (I), wherein in step c.1) the support-bound saposin-like protein is contacted with the viral, archaeal, eukaryotic or prokaryotic membrane provided in step a) to allow formation of a library of saposin like particles wherein the library comprises a heterogenic mixture of saposin lipoprotein particles with different membrane lipid and optionally membrane protein compositions.

8. Process according to any one of the preceding claims, wherein the saposin-like protein is saposin A, saposin B, saposin C, saposin D or a derivative ~~or truncated form~~ thereof,

wherein the derivative form of the SAPLIP is selected from

i. a protein having at least 80 % sequence identity to the full length sequence of SEQ ID NO. 1, 2, 3, 4, 5 or 6;

ii. a protein having at least 40% sequence identity to the full length sequence of SEQ ID NO. 1, 2, 3, 4, 5 or 6, wherein said protein is amphipathic and forms at least one alpha helix; and

iii. a protein comprising the sequence of SEQ ID NO. 1, 2, 3, 4, 5 or 6 in which 1 to 40 amino acids have been deleted, added, inserted and/or substituted

and ~~which~~ is capable of forming saposin lipoprotein particles in the process of claim 1.

~~9. Process according to claim 8, wherein the derivative or truncated form is selected from~~

~~i. a protein having at least 20 % sequence identity to the full length sequence of SEQ ID NO. 1, 2, 3, 4, 5 or 6; in particular wherein said protein is amphipathic, forms at least one alpha helix, and is capable of self-assembling together with lipids into lipoprotein particles when employed in the process of claim 1; and~~

~~ii. a protein comprising the sequence of SEQ ID NO. 1, 2, 3, 4, 5 or 6 in which 1 to 40 amino acids have been deleted, added, inserted and/or substituted.~~

9~~10~~. Process according to any one of the preceding claims, wherein the hydrophobic agent is selected from the group consisting of a hydrophobic organic compound and a hydrophobic biomolecule.

10~~1~~. Process according to claim ~~10~~9, wherein the hydrophobic organic compound and/or the hydrophobic biomolecule is selected from the group consisting of a biologically active agent, a drug, an active ingredient of a drug, an active ingredient of a cosmetic product, an active ingredient of a plant protective product, a dietary and/or nutritional supplement, a diagnostic probe, a contrast agent, a label and an indicator.

11~~2~~. Process according to any one of claims ~~10~~ 9 or ~~11~~10, wherein the hydrophobic biomolecule is a protein comprising a hydrophobic moiety, in particular a protein selected from the group consisting of a membrane protein, an integral transmembrane protein, an integral monotopic membrane protein, a peripheral membrane protein, an amphitropic protein in a lipid-bound state, a lipid-anchored protein and a chimeric protein with a fused hydrophobic and/or transmembrane domain.

12~~3~~. Process according to any one of the preceding claims, wherein the hydrophobic agent, the lipids and/or the saposin-like protein is in a detergent-solubilized state and wherein optionally the detergent is selected from the group consisting of alkylbenzenesulfonates or bile acids, cationic detergents and non-ionic or zwitterionic detergents such as lauryl-dimethyl amine-oxides (LDAO), Fos-Cholines, CHAPS/CHAPSO, saponins such as Digitonin and structurally related synthetic detergents such as glycol-diosgenin, alkyl glycosides such as short, medium or longer chain alkyl maltosides, in particular n-Dodecyl β-D-maltoside, glucosides, maltose-neopentyl glycol (MNG) amphiphiles, amphiphilic polymers (amphipols), styrene maleic acid co-polymer (SMA), ~~macrocycle or cyclic oligomers based on a hydroxyalkylation product of a phenol and an aldehyde (~~Calixarene~~)~~, and mixtures thereof.

134. Process according to any one of the preceding claims, wherein

i. the particles obtained in step c.2) and/or c.1) are disc-shaped, in particular wherein they are disc-shaped and do not comprise a hydrophilic or aqueous core;

ii. the particles of step c.2) and/or c.1) have an average maximum diameter of from 2 nm to 200 nm, in particular from 3 nm to 150, preferably from 3 nm to 100 nm;

iii. the self-assembly of the particle in step c.2) and/or c.1) is carried out at a pH from 2.0 to 10.0, in particular 6.0 to 10.0, preferably from 6.0 to 9.0, particularly preferred from 7.0 to 9.0, and most preferred from 7.0 to 8.0.