Fig. 1

**Abstract:** Herein is reported a lipid particle comprising an apolipoprotein, a phosphatidylcholine and a lipid, such as a phospholipid, fatty acid or steroid lipid. In one embodiment the lipid particle comprises only one apolipoprotein. In one embodiment the lipid particle consists of one apolipoprotein, a phospholipid, a lipid, and a detergent. In one embodiment the lipid is a second phosphatidylcholine, wherein the first phosphatidylcholine and the second phosphatidylcholine differ in one or two fatty acid residues or fatty acid residue derivatives which are esterified to the glycerol backbone of the phosphatidylcholine. In one embodiment the apolipoprotein is selected from an apolipoprotein that has the amino acid sequence selected from SEQ ID NO: 01, 02, 06, 66, and 67, or is a variant thereof that has at least 70% sequence identity with the selected sequence.

Declarations under Rule 4.17:
— of inventorship (Rule 4.17(iv))

Published:
— without international search report and to be republished upon receipt of that report (Rule 48.2(g))
— with sequence listing part of description (Rule 5.2(a))
Tetranectin-apolipoprotein A-I, lipid particles containing it and its use

The current invention is in the field of lipoproteins and lipid particles. It is reported herein a lipid particle comprising an apolipoprotein, a phosphatidylcholine and a lipid, as well as a tetranectin-apolipoprotein A-I, and the use thereof.

Background of the Invention

Plasma lipoproteins are soluble protein-lipid complexes that carry out lipid transport and metabolism in blood. Several major classes of lipoproteins are distinguished on the basis of their density, size, chemical compositions, and functions. Among them high-density-lipoprotein (HDL) particles alternatively denoted as high-density-lipid particles, are made up of several subclasses that vary in their average molecular weight of from 180 kDa to 360 kDa. Their average lipid and protein content is 50% by weight of each. Phosphatidylcholine (PC) accounts for 38% of the total lipid followed by cholesteryl esters and small amounts of other polar and non-polar lipids, including free cholesterol. The main protein component is apolipoprotein A-I (Apo A-I), representing about 60% of total protein weight in human HDL.

HDL particles and its major polypeptide apolipoprotein A-I participate in the reverse cholesterol transport (RCT). Therein the apolipoprotein A-I increases the efflux of cholesterol from cells, e.g. from cells of the wall of blood vessels, the binding of the lipid and the activation of the lecithin-cholesterol-acetyl-transferase and thereby the elimination of cholesterol via plasmatic flow by the liver. This is an active transport process involving the cell membrane protein ATP-binding-cassette-transporter-A-I (ABCA-I).

Apolipoprotein A-I and apolipoprotein-based therapeutics, e.g. reconstituted HDL particles, were already identified in the late 70ties and early 80ties of the last century. For apolipoprotein A-I-Milano containing lipid particles the clinical proof (meaning significant plaque reduction in arteriosclerotic patients) could be shown. Apolipoprotein A-I-Milano, a dimeric form of wild-type apolipoprotein A-I, was designed according to a naturally occurring mutant of the apolipoprotein A-I molecule. The dimer formation is enabled by the exchange of amino acid residue 173 (arginine) by cysteine allowing the formation of a disulfide bond.

In WO 2009/131704 nanostructures are reported, which are suitable for sequestering cholesterol and other molecules, comprising a core comprising an
inorganic material. In WO 2006/125304 pharmaceutical compositions for treating or preventing coronary artery disease are reported. Compositions encoding apolipoproteins that are related to lipid metabolism and cardiovascular disease are reported in US 2002/0142953. In WO 2005/084642 an apoprotein-cochelate composition is reported. In WO 2009/036460 modified human apolipoprotein A-I polypeptides and their uses are reported. Plant production of dimeric and/or oligomeric forms of human apolipoprotein A-I protein muteins is reported in WO 2008/017906. In WO 2007/137400 a method and compound for the treatment of valvular stenosis is reported. In WO 2006/100567 charged lipoprotein complexes and their uses are reported.


In WO 03/097696 methods and compositions for the treatment of ischemic reperfusion are reported. Nanoscale bound bilayers, methods of use and production are reported in WO 2009/097587. In WO 2007/098122 methods for the treatment of macular degeneration and related eye conditions are reported. Apolipoprotein Analogues are reported in WO 02/38609. In WO 2005/041866 pharmaceutical formulations are reported. Methods and dosing regimens for the treatment and prevention of coronary syndromes are reported. Gene therapy, approaches to supply apolipoprotein A-I agonists and their use to treat dislipidemic disorders are reported in WO 99/16409. In WO 2008/106660 isolated phospholipid-protein particles are reported. Method for the prevention and treatment of diastolic dysfunction employing an apolipoprotein (APO A-I) mimetic peptide/phospholipid complex are reported in WO 2010/083611. In WO 2008/156873 APO A-I peptide mimetics are reported. Encapsulated HDL mimetic peptides are reported in WO 2008/094905. In WO 98/56906 a trimerising module is reported.

**Summary of the Invention**

Herein is reported as one aspect a tetranectin-apolipoprotein A-I with improved production properties, especially less side-product formation during cultivation and improved downstream processing properties.
Also herein is reported as one aspect a lipid particle comprising an apolipoprotein, a phosphatidylcholine and a further lipid, such as a phospholipid, lysosphospholipid, galactocerebrosides, gangliosides, cerebrosides, glycerides, fatty acid, triglyceride, or steroid lipid, cholesterol, cholesterol esters or an analog or derivative thereof.

In one embodiment the lipid particle comprises only one type of apolipoprotein.

In one embodiment the lipid particle is consisting of one apolipoprotein, a phosphatidylcholine, a further lipid, and a detergent.

In one embodiment the further lipid is a phosphatidylcholine, wherein both of the phosphatidylcholines differ in one or two carboxylic acid moieties or carboxylic acid moiety derivatives which are esterified to the phosphoglycerol backbone of the phosphatidylcholine.

In a further embodiment the apolipoprotein is a human apolipoprotein A, in another embodiment a human apolipoprotein conjugated to a multimerization domain, and in still a further embodiment a tetranectin-apolipoprotein A-I. In one embodiment the apolipoprotein is selected from an apolipoprotein that has the amino acid sequence selected from SEQ ID NO: 01, 02, 06, 66, and 67, or is a variant thereof that has at least 70 % sequence identity with the selected sequence.

In one embodiment the lipid particle comprises

a) an apolipoprotein,

b) a phosphatidylcholine, and

c) a further lipid,

wherein the apolipoprotein is a tetranectin-apolipoprotein A-I when the lipid is phosphatidylethanolamine, phosphatidylinositol, 1-palmitoyl-2-oleoyl-phosphatidyl serine, sphingosine 1-phosphate, cholate, or dimyristoyl phosphatidylglycerol, and/or

wherein the apolipoprotein is not apolipoprotein A-I Milano when the lipid is a small alkyl chain phospholipid, phosphatidylglycerol, phosphatidylserine, phosphatidic acid, sphingomyelin, sphingolipid, ganglioside, cerebroside, lysolecithin, cephaline, cardiolipine, dicetylphosphate, or cholesterol.
In another embodiment the lipid particle comprises
   a) an apolipoprotein,
   b) a phosphatidylcholine, and
   c) a further lipid,

wherein the apolipoprotein is a tetranectin-apolipoprotein A-I when the lipid is phosphatidylethanolamine, sphingosine I-phosphate, or cholate, and/or

wherein the apolipoprotein is not apolipoprotein A-I Milano when the lipid is a small alkyl chain phospholipid, sphingomyelin, sphingolipid, ganglioside, cerebroside, lysolecithin, cephaline, cardiolipine, dicetylphosphate, or cholesterol.

In one embodiment the further lipid is any lipid except phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and phosphatidic acid.

In one embodiment the further lipid is a second phosphatidylcholine. In one embodiment the phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC.

In one embodiment the molar ratio of the phosphatidylcholine to the lipid is of from 99:1 to 1:99. In another embodiment the molar ratio of the phosphatidylcholine to the lipid is of from 99:1 to 10:90. In a further embodiment the molar ratio of the phosphatidylcholine to the lipid is of from 99:1 to 25:75. In another embodiment the apolipoprotein is non-covalently associated with the phosphatidylcholine and the lipid.

In one embodiment the molar ratio of POPC to DPPC is of from 99:1 to 1:99. In another embodiment the molar ratio of POPC to DPPC is of from 99:1 to 10:90. In a further embodiment the molar ratio of POPC to DPPC is of from 99:1 to 25:75.

In another embodiment the apolipoprotein is non-covalently associated with the POPC and DPPC.

In one embodiment the apolipoprotein is a multimer comprising three apolipoprotein monomers. In another embodiment the multimer comprises three tetranectin-apolipoprotein A-I monomers.

In one embodiment the lipid particle comprises less than 0.75 % by weight detergent. In one embodiment the detergent is selected from sugar-based
detergents, polyoxyalkylene-based detergents, bile-salt based detergents, synthetic detergents, and a combination thereof. In another embodiment the detergent is cholic acid.

In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is of from 40 to 120, in one embodiment of from 50 to 110, in one embodiment of from 54 to 102, in one embodiment of from 60 to 90, in one embodiment of from 65 to 70.

In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90. In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the combined number of phosphatidylcholine molecules and lipid molecules per apolipoprotein monomer in the lipid particle is about 66.

In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is of from 40 to 115, in a further embodiment of from 50 to 110, and in another embodiment of from 54 to 102.

In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90. In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the combined number of POPC and DPPC molecules per apolipoprotein monomer in the lipid particle is about 66.

In one embodiment the lipid particle is capable of binding to a receptor selected from the group consisting of cubulin, Scavenger receptor class B, type 1 (SR-B1), ATP-binding cassette 1 (ABCA-1), Lecithin-cholesterol acyltransferase (LCAT), Cholesteryl-ester transfer protein (CETP), or Phospholipid transfer protein (PLTP).
A further aspect as reported herein is a pharmaceutical composition comprising a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein.

One aspect as reported herein is a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use as a medicament.

One aspect as reported herein is the use of a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for the manufacture of a medicament.

One aspect as reported herein is the use of a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for the manufacture of a medicament

- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacityification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacityification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.

One aspect as reported herein is the use of a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranection-apolipoprotein A-I as reported herein, or a fusion protein as reported herein in the manufacture of a medicament.

One aspect as reported herein is a method for the manufacture of a medicament
- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranection-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.
One aspect as reported herein is a method for
- secondary prevention in patients with an acute coronary syndrome, or
- the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetraneectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.

One aspect as reported herein is a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetraneectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use in treating
- acute coronary syndrome, or
- atherosclerosis, or
- atherosclerotic plaques in blood vessels of a subject, or
- valvular stenosis in a subject, or
- septic shock, or
- angina pectoris, or
- myocardial infarction, or
- unstable angina pectoris, or
- arterial stenoses, or
- peripheral artery diseases (PAD), or
- carotis stenosis, or
- cerebral arterial stenosis, or
- coronary arterial stenosis, or
- vascular demencia, or

- amaurosis fugax.

One aspect as reported herein is a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use in
- inducing reverse cholesterol transport, or
- inducing plaques pacification, or
- cleaning or dissolving or stabilizing atherosclerotic plaques, or
- redistributing cholesterol from the wall of arteries to the liver, or
- increasing the number of HDL particles, or
- removal of endotoxins.

One aspect as reported herein is a method of treating an individual having acute coronary syndrome, or atherosclerosis, or atherosclerotic plaques in blood vessels, or valvular stenosis, or septic shock, or angina pectoris, or myocardial infarction, or unstable angina pectoris, or arterial stenoses, or peripheral artery diseases (PAD), or carotis stenosis, or cerebral arterial stenosis, or coronary arterial stenosis, or vascular demencia, or amaurosis fugax comprising administering to the individual an effective amount of a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein.

One aspect as reported herein is a method of inducing reverse cholesterol transport, or inducing plaques pacification, or cleaning or dissolving or stabilizing atherosclerotic plaques, or redistributing cholesterol from the wall of arteries to the liver, or increasing the number of HDL particles, or removing endotoxins in an individual comprising administering to the individual an effective amount of a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein to induce reverse cholesterol transport, or to induce plaques pacification, or to clean or dissolve or stabilize atherosclerotic plaques, or to redistribute cholesterol from the wall of arteries to the liver, or to increase the number of HDL particles, or to remove endotoxins.
In one embodiment the non-normal lipid level is in a body fluid. In another embodiment the body fluid is whole blood or blood serum.

In one embodiment the non-normal lipid level is an increased cholesterol level.

In one embodiment the lipid containing deposition is a plaque in a blood vessel.

In one embodiment the disease is a cardiovascular disease.

One aspect as reported herein is a method of treating a disease or condition characterized by non-normal lipid levels or a lipid containing deposition within body components comprising

i) administering a therapeutically effective amount of a lipid particle as reported herein to a subject in need of a treatment or an artificial system, and

ii) optionally monitoring the lipid level or the lipid containing deposition of a subject for a change.

One aspect as reported herein is a method for secondary prevention in patients with an acute coronary syndrome comprising administering to a subject in need thereof a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein.

One aspect as reported herein is a diagnostic composition comprising a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein wherein the apolipoprotein is labeled allowing for the detection of the labeled apolipoprotein or lipid particle within a sample or subject.

One aspect as reported herein is the use of a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for diagnosis.

One aspect as reported herein is the use of a lipid particle as reported herein for the prevention or treatment of a subject suffering from a disease or condition characterized by the presence of a non-normal lipid level or a lipid containing deposition.
One aspect as reported herein is a nucleic acid encoding a tetranection-apolipoprotein A-I as reported herein, or a fusion protein as reported herein as well as a cell comprising a nucleic acid as reported herein.

One aspect as reported herein is a tetranection-apolipoprotein A-I that has the amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67, or a pharmaceutically acceptable salt thereof, or a prodrug thereof. In one embodiment the tetranection-apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67 with one or more conservative amino acid modifications. In one embodiment the tetranection-apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67 wherein one or more amino acids are substituted, added or deleted.

One aspect as reported herein is a tetranection-apolipoprotein A-I that has an amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67. In one embodiment the amino acid sequence has at least 70% sequence identity with an amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67.

In one embodiment the tetranection-apolipoprotein A-I monomer, or the tetranection-apolipoprotein A-I trimer is capable of binding to a receptor selected from the group consisting of cubilin, Scavenger receptor class B, type 1 (SR-B1), ATP-binding cassette 1 (ABCA-1), Lecithin-cholesterol acyltransferase (LCAT), Cholesteryl-ester transfer protein (CETP), or Phospholipid transfer protein (PLTP).

One aspect as reported herein is a multimer comprising three tetranection-apolipoprotein A-I monomers, wherein the tetranection-apolipoprotein A-I monomers are not covalently bound to each other.

One aspect as reported herein is a fusion protein comprising the amino acid sequence of SEQ ID NO: 01, SEQ ID NO: 02, or SEQ ID NO: 66, a nucleic acid encoding the fusion protein, and a plasmid comprising the nucleic acid encoding the fusion protein.

In one embodiment the fusion protein comprises in N- to C-terminal direction

- the amino acid methionine (M),
- a fragment of an interferon sequence that has the amino acid sequence of CDLPQTHSL (SEQ ID NO: 55),
- a GS linker,
- a hexa-histidine tag that has the amino acid sequence of HHHHHH (SEQ ID NO: 56),
- a GS linker,
- an IgA protease cleavage site that has the amino acid sequence of VVAPPAP (SEQ ID NO: 60), and
- a tetranectin-apolipoprotein A-I that has the amino acid sequence of SEQ ID NO: 02.

In one embodiment the fusion protein has the amino acid sequence of SEQ ID NO: 57.

One aspect as reported herein is a cell comprising a nucleic acid encoding the fusion protein as reported herein. In one embodiment the cell is selected from the E.coli strains such as CSPZ-2, K12 strain 294 (ATCC 31446), B, X 1776 (ATCC 31537), W3110 (ATCC 273325), BL21, RM_82, SCS_110, G, XL-1_F_, SE_13009, LA_5709, C 600, CSH_1, TG_1, UT400, and UT5600.

One aspect as reported herein is a lipid particle comprising

a) a tetranectin-apolipoprotein A-I that has an amino acid sequence selected from the amino acid sequences of SEQ ID NO: 01, SEQ ID NO: 02, SEQ ID NO: 66, and SEQ ID NO: 67,

b) a phosphatidylcholine, and

c) a lipid.

In one embodiment the lipid is selected from phosphatidylethanolamine, phosphatidylinositol, 1-palmitoyl-2-oleoyl-phosphatidyl serine, sphingosine I-phosphate, cholate, or dimyristoyl phosphatidylglycerol.

In one embodiment the lipid is any lipid except phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and phosphatidic acid.

In one embodiment the lipid particle comprises

a) a tetranectin-apolipoprotein A-I that has an amino acid sequence selected from the amino acid sequences of SEQ ID NO: 01, SEQ ID NO: 02, SEQ ID NO: 66, and SEQ ID NO: 67,

b) a first phosphatidylcholine, and

c) a second phosphatidylcholine.
In one embodiment the first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine for producing the lipid particle is of from 99:1 to 1:99. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine for producing the lipid particle is of from 99:1 to 10:90. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine for producing the lipid particle is of from 99:1 to 25:75. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine for producing the lipid particle is of from 99:1 to 50:50. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine for producing the lipid particle is about 75:25.

In one embodiment the first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine in the lipid particle is of from 99:1 to 1:99. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine in the lipid particle is of from 99:1 to 10:90. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine in the lipid particle is of from 99:1 to 25:75. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine in the lipid particle is of from 99:1 to 50:50. In one embodiment the molar ratio of the first phosphatidylcholine to the second phosphatidylcholine in the lipid particle is about 75:25.

In one embodiment the apolipoprotein is non-covalently associated with the first phosphatidylcholine and the lipid. In one embodiment the tetranection-apolipoprotein A-I is non-covalently associated with the first phosphatidylcholine and the second phosphatidylcholine.

In one embodiment the tetranection-apolipoprotein A-I is a multimer comprising three tetranection-apolipoprotein A-I monomers.

In one embodiment the lipid particle comprises less than 0.75 % by weight detergent. In one embodiment the detergent is selected from sugar-based detergents, polyoxyalkylene-based detergents, bile-salt based detergents, synthetic detergents and a combination thereof. In one embodiment the detergent is cholic acid or a Zwittergent.
In one embodiment the combined number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 40 to 120, in a further embodiment of from 50 to 110, and in another embodiment of from 54 to 102. In one embodiment the phospholipid is a phosphatidylcholine.

One aspect as reported herein is a pharmaceutical composition comprising a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranecktin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein.

One aspect as reported herein is a lipid particle as reported herein, or an apolipoprotein A-I multimer as reported herein, or a tetranecktin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use as a medicament.

One aspect as reported herein is the use of a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranecktin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for the manufacture of a medicament

- for prevention of secondary Major Adverse CV Events (MACE),
- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranecktin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
for the treatment of vascular demencia, or
for the treatment of amaurosis fugax.

One aspect as reported herein is the use of a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein in the manufacture of a medicament.

One aspect as reported herein is a method for the manufacture of a medicament
for secondary prevention in patients with an acute coronary syndrome, or
for prevention of secondary Major Adverse CV Events (MACE), or
for the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
for inducing reverse cholesterol transport and/or plaques pacification, or
for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
for preventing or treating a valvular stenosis in a subject, or
for increasing the number of HDL particles in a subject, or
for initiation of reverse cholesterol transport in a subject, or
for the removal of endotoxins, or
for the prevention of septic shock
for the treatment of angina pectoris, or
for the treatment of myocardial infarction, or
for the treatment of unstable angina pectoris, or
for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
for the treatment of vascular demencia, or
for the treatment of amaurosis fugax.
One aspect as reported herein is a method for

- secondary prevention in patients with an acute coronary syndrome, or
- for prevention of secondary Major Adverse CV Events (MACE), or
- the prevention or treatment of atherosclerosis wherein a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranection-apolipoprotein A-I as reported herein, or a fusion protein as reported herein is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.

One aspect as reported herein is a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetranection-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use in treating or preventing

- acute coronary syndrome, or
- secondary Major Adverse CV Events (MACE), or
- atherosclerosis, or
- atherosclerotic plaques in blood vessels of a subject, or
- valvular stenosis in a subject, or
- septic shock, or
- angina pectoris, or
myocardial infarction, or
unstable angina pectoris, or
arterial stenoses, or
peripheral artery diseases (PAD), or
carotis stenosis, or
cerebral arterial stenosis, or
coronary arterial stenosis, or
vascular demencia, or
amaurosis fugax.

One aspect as reported herein is a lipid particle as reported herein, or a apolipoprotein A-I multimer as reported herein, or a tetraneectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein for use in
- inducing reverse cholesterol transport, or
- inducing plaques pacification, or
cleaning or dissolving or stabilizing atherosclerotic plaques, or
- redistributing cholesterol from the wall of arteries to the liver, or
- increasing the number of HDL particles, or
- removal of endotoxins.

One aspect as reported herein is a method for prevention of secondary Major Adverse CV Events (MACE) when applied post-intervention in patients presenting at the ER with an acute CV event, or a method for treating an individual having acute coronary syndrome, or having atherosclerosis, or having atherosclerotic plaques in blood vessels, or having valvular stenosis, or having septic shock, or having angina pectoris, or having myocardial infarction, or having unstable angina pectoris, or having arterial stenoses, or having peripheral artery diseases (PAD), or having carotis stenosis, or having cerebral arterial stenosis, or having coronary arterial stenosis, or having vascular demencia, or having amaurosis fugax comprising administering to the individual an effective amount of a lipid particle as reported herein, or a multimer as reported herein, or a fusion protein as reported herein, or a tetraneectin-apolipoprotein A-I as reported herein.

One aspect as reported herein is a method of inducing reverse cholesterol transport, or inducing plaques pacification, or cleaning or dissolving or stabilizing atherosclerotic plaques, or redistributing cholesterol from the wall of arteries to the liver, or increasing the number of HDL particles, or removing endotoxins in an individual comprising administering to the individual an effective amount of a lipid
particle as reported herein, or a multimer as reported herein, or a fusion protein as reported herein, or a tetranectin-apolipoprotein A-I as reported herein to induce reverse cholesterol transport, or to induce plaques pacification, or to clean or dissolve or stabilize atherosclerotic plaques, or to redistribute cholesterol from the wall of arteries to the liver, or to increase the number of HDL particles, or to remove endotoxins.

In one embodiment the non-normal lipid level is in a body fluid. In another embodiment the body fluid is whole blood or blood serum.

In one embodiment the non-normal lipid level is an increased cholesterol level.

In one embodiment the lipid containing deposition is a plaque in a blood vessel.

In one embodiment the disease is a cardiovascular disease.

One aspect as reported herein is a method of treating a disease or condition characterized by non-normal lipid levels or a lipid containing deposition within body components comprising

i) administering a therapeutically effective amount of a lipid particle as reported herein to a subject in need of a treatment or an artificial system, and

ii) optionally monitoring the lipid level or the lipid containing deposition of a subject for a change.

One aspect as reported herein is a method for secondary prevention in patients with an acute coronary syndrome comprising administering to a subject in need thereof a lipid particle as reported herein, or a multimer as reported herein, or a fusion protein as reported herein, or a tetranectin-apolipoprotein A-I as reported herein.

One aspect as reported herein is a method for prevention of secondary Major Adverse CV Events (MACE) comprising administering post-intervention to a subject in need thereof a lipid particle as reported herein, or a multimer as reported herein, or a fusion protein as reported herein, or a tetranectin-apolipoprotein A-I as reported herein wherein the subject presents at the ER with an acute CV event.

One aspect as reported herein is a diagnostic composition comprising a lipid particle as reported herein, or a multimer as reported herein, or a fusion protein as reported herein, or a tetranectin-apolipoprotein A-I as reported herein, wherein the apolipoprotein is labeled allowing for the detection of the labeled apolipoprotein or lipid particle within a sample or subject.
One aspect as reported herein is the use of a lipid particle as reported herein for diagnosis.

One aspect as reported herein is the use of a lipid particle as reported herein for the prevention or treatment of a subject suffering from a disease or condition characterized by the presence of a non-normal lipid level or a lipid containing deposition.

One aspect as reported herein is a nucleic acid encoding a tetranectin-apolipoprotein A-I as reported herein, or a fusion protein as reported herein as well as a cell comprising a nucleic acid as reported herein.

One aspect as reported herein is a polypeptide that has the amino acid sequence of SEQ ID NO: 01 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 01.

One aspect as reported herein is a polypeptide that has the amino acid sequence of SEQ ID NO: 02 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 02.

One aspect as reported herein is a polypeptide that has the amino acid sequence of SEQ ID NO: 06 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 06.

One aspect as reported herein is a polypeptide that has the amino acid sequence of SEQ ID NO: 66 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 66.

One aspect as reported herein is a polypeptide that has the amino acid sequence of SEQ ID NO: 67 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 67.

One aspect as reported herein is a lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and

- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 90, and
wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 01.

In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

One aspect as reported herein is a lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and

- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90, and wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 02.

In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

One aspect as reported herein is a lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and

- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 06.
In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

One aspect as reported herein is a lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and

- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 66.

In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

One aspect as reported herein is a lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and

- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 67.
In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70. In one embodiment the number phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

**Detailed Description of the Invention**

**Definitions**

The term “apolipoprotein” denotes a protein that is comprised in a lipid or lipoprotein particle, respectively.

The term „apolipoprotein A-I“ denotes an amphiphilic, helical polypeptide with protein-lipid and protein-protein interaction properties. Apolipoprotein A-I is synthesized by the liver and small intestine as prepro-apolipoprotein of 267 amino acid residues which is secreted as a pro-apolipoprotein that is cleaved to the mature polypeptide having 243 amino acid residues. Apolipoprotein A-I is consisting of 6 to 8 different amino acid repeats consisting each of 22 amino acid residues separated by a linker moiety which is often proline, and in some cases consists of a stretch made up of several residues. An exemplary human apolipoprotein A-I amino acid sequence is reported in GenPept database entry NM-000039 or database entry X00566; GenBank NP-000030.1 (gi 4557321). Of human apolipoprotein A-I (SEQ ID NO: 06) naturally occurring variants exist, such as P27H, P27R, P28R, R34L, G50R, L84R, D113E, A-A119D, D127N, deletion of K131, K131M, W132R, E133K, R151C (amino acid residue 151 is changed from Arg to Cys, apolipoprotein A-I-Paris), E160K, E163G, P167R, L168R, E171V, P189R, R197C (amino acid residue 173 is change from Arg to Cys, apolipoprotein A-I-Milano) and E222K. Also included are variants that have conservative amino acid modifications.

In one embodiment the tetranectin-apolipoprotein A-I comprises a fragment of the cleavage site of Immunoglobulin A protease (IgA protease). The recognition sites known from IgA proteases comprise the following sequences with “▽” denoting the position of the cleaved bond:
Pro-Ala-Pro ↓ Ser-Pro (SEQ ID NO: 61)
Pro-Pro ↓ Ser-Pro (SEQ ID NO: 62)
Pro-Pro ↓ Ala-Pro (SEQ ID NO: 63)
Pro-Pro ↓ Thr-Pro (SEQ ID NO: 64)
Pro-Pro ↓ Gly-Pro (SEQ ID NO: 65),

wherein the first three are more frequently chosen and cleaved.

The term „apolipoprotein mimic“ denotes a synthetic polypeptide that mimics the function of the respective apolipoprotein. For example an „apolipoprotein A-I mimic“ is a synthetic polypeptide that shows comparable biological function with respect to removal of cholesterol, i.e. reverse cholesterol efflux, as the natural apolipoprotein A-I. In one embodiment the apolipoprotein A-I mimic comprises at least one amphiphilic alpha-helix with positively charged amino acid residues clustered at a hydrophobic-hydrophilic interface and negatively-charged amino acid residues clustered at a center of a hydrophilic face. In order to mimic the function of apolipoprotein A-I the apolipoprotein mimic comprise a repeat polypeptide of from 15 to 29 amino acid residues, in one embodiment of 22 amino acid residues (PVLDEFREKLNEELEALKQKLK (SEQ ID NO: 04); PVLDLFRELLNELLEAL KQKLLK (SEQ ID NO: 05)).

The term „cardiovascular disease“ in general denotes a disease or condition with respect to heart or blood vessels, such as arteriosclerosis, coronary heart disease, cerebrovascular disease, aortoiliac disease, ischemic heart disease or peripheral vascular disease. Such a disease may not be discovered prior to an adverse event as a result of the disease, such as myocardial infarct, stroke, angina pectoris, transient ischemic attacks, congestive heart failure, aortic aneurysm, mostly resulting in death of the subject.

The term "cholate" denotes 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid or a salt thereof, especially the sodium salt.

The term “critical micelle concentration” and its abbreviation “CMC”, which can be used interchangeably, denote the concentration of surfactants or detergents above which individual detergent molecules (monomers) aggregate spontaneously to micelles (micelles, round rods, lamellar structures etc.).

The term „conservative amino acid modification“ denotes modifications of the amino acid sequence which do not affect or alter the characteristics of the lipid particle or the apolipoprotein according to the invention. Modifications can be
introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid modifications include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g. lysine, arginine, histidine), acidic side chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), non-polar side chains (e.g. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g. threonine, valine, isoleucine), and aromatic side chains (e.g. tyrosine, phenylalanine, tryptophan, histidine). A “variant” protein, refers therefore herein to a molecule which differs in amino acid sequence from a “parent” protein’s amino acid sequence by up to ten, in one embodiment from about two to about five, additions, deletions, and/or substitutions.


The homology and identity of different amino acid sequences may be calculated using well known algorithms such as BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, or BLOSUM 90. In one embodiment the algorithm is BLOSUM 30.

The formation of lipid particles may be performed by incubating the apolipoprotein with detergent solubilized lipids at their respective transition temperature. The term “detergent” denotes a surface active chemical substance. A “detergent” is generally an amphiphatic molecule with a non-polar, hydrophobic part and a polar, hydrophilic part. The term “zwitterionic detergent” denotes a surface active chemical compound that has overall zero charge and at the same time comprises at least one positively charged moiety and at least one negatively charged moiety. In one embodiment the detergent is selected from sugar-based detergents, polyoxyalkylene-based detergents, bile-salt based detergents, synthetic detergents or a combination thereof. The term “sugar-based detergent” denotes a detergent selected from n-octyl-beta-D-glucopyranoside, n-nonyl-beta-D-glucopyranoside, n-dodecyl-beta-D-maltopyranoside, or 5-cyclohexylpentyl-beta-D-maltopyranoside, and derivatives thereof. The term “bile-salt based detergent” denotes a detergent selected from sodium cholate, potassium cholate, lithium cholate,
3-[(3-chloramidopropyl) dimethylammonio]-propane sulfonate (CHAPS), 3-[(3-chloramidopropyl) dimethylammonio]-2-hydroxy propane sulfonate (CHAPSO), and derivatives thereof. The term „polyoxyalkylene-based detergent“ denotes a detergent selected from Tween 20, Triton X-100, Pluronic F68, and a derivatives thereof. The term „synthetic detergents“ denotes a detergent selected from Zwittergent 3-6, Zwittergent 3-8, Zwittergent 3-10, Zwittergent 3-12, and derivatives thereof.

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term „high density lipoprotein particle“ or its abbreviation „HDL particle“, which can be used interchangeably, denotes a lipid-protein-complex comprising as main proteinaceous compound apolipoprotein A-I.

The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

The term "increase lipid efflux" and grammatical equivalents thereof denotes an increased level and/or rate of lipid efflux, promoting lipid efflux, enhancing lipid efflux, facilitating lipid efflux, upregulating lipid efflux, improving lipid efflux, and/or augmenting lipid efflux from cells or plaques. In one embodiment, the lipid efflux comprises efflux of phospholipid, triglyceride, cholesterol, and/or cholesterol ester.

An “individual” or “subject” is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

The term „DPPC“ denotes the phospholipid 1,2-di-palmitoyl-sn-glycero-3-phosphatidyl choline also referred to as 1,2-dipalmitoyl-phosphatidyl choline.
The term "multimer" denotes a complex consisting of two or more monomers. A multimer is formed by non-covalent interactions between the monomers. Each monomer comprises a multimerization domain. In one embodiment the multimer comprises 2 or 3 monomers. In another embodiment the multimerization domains interact via non-covalent interactions between the individual multimerization domains comprised in each monomer. The term "multimerization domain" denotes amino acid sequences capable of covalently or non-covalently associating two or more monomeric molecules. A multimerization domain is capable of interacting with multimerization domains of different, similar, or identical amino acid sequence. In one embodiment the multimerization domain is the tetranectin trimerising structural element or a derivative thereof that has an amino acid sequence that is at least 68 % identical with the consensus amino acid sequence of SEQ ID NO: 53. In one embodiment the cysteine residue at position 50 of SEQ ID NO: 53 is substituted by a different amino acid residue, in another embodiment by a serine residue, or a threonine residue, or a methionine residue. Polypeptides comprising a multimerization domain can associate with one or more other polypeptides also comprising a multimerization domain. The multimer formation can be initiated simply by mixing the polypeptides under suitable conditions. In another embodiment the multimerization domain has the amino acid sequence of SEQ ID NO: 53 wherein from 1 to 10 residues have been deleted from or added to the N- or C-terminus of the amino acid sequence. In a further embodiment the multimerization domain has an amino acid sequence of SEQ ID NO: 53 wherein six or nine amino acid residues have been deleted from the N-terminus of the amino acid sequence. In still another embodiment the multimerization domain has an amino acid sequence of SEQ ID NO: 53 wherein the N-terminal amino acid residue L or the N-terminal amino acid residues C and L have been deleted. In one embodiment the multimerization domain is the tetranectin trimerising structural element and has the amino acid sequence of SEQ ID NO: 54. The multimer is in one embodiment a homomer.

The multimers may be homomers or heteromers, since different apolipoproteins comprising a multimerization domain can be combined to be incorporated into the multimer. In one embodiment the multimer is a trimeric homomer.

According to one embodiment the multimerization domain is obtained from tetranectin. In one embodiment the multimerization domain comprises the tetranectin trimerising structural element that has an amino acid sequence of SEQ ID NO: 54. The trimerising effect of the tetranectin trimerising structural element is
caused by a coiled coil structure which interacts with the coiled coil structure of two other tetranectin trimerising structural elements to form a trimer. The tetranectin trimerising structural element may be obtained from human tetranectin, from rabbit tetranectin, from murine tetranectin, or from C-type lectin of shark cartilage. In one embodiment the tetranectin trimerising structural element comprises a sequence having at least 68%, or at least 75%, or at least 81%, or at least 87%, or at least 92% identity with the consensus sequence of SEQ ID NO: 53.

The term “non-covalent interactions” denotes non-covalent binding forces such as ionic interaction forces (e.g. salt bridges), non-ionic interaction forces (e.g. hydrogen-bonds), or hydrophobic interaction forces (e.g. van-der-Waals forces or π-stacking interactions).

“Percent (%) amino acid sequence identity” with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.
In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program’s alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

The term “phosphatidylcholine” denotes a molecule consisting of one glycerol moiety, two carboxylic acid moieties and one phosphocholine moiety, wherein the glycerol moiety is covalently bound to the other moieties each by a ester bond, i.e. two carboxylic ester bonds and one phosphoric ester bond, whereby the phosphoric ester bond is either to the 1-hydroxyl group or the 3-hydroxyl group of the glycerol moiety. The term “carboxylic acid moiety” denotes an organic moiety comprising at least one acyl group (R-C(O)O). The phosphatidylcholine may be of any kind or source. In one embodiment the phosphatidylcholine is selected from egg phosphatidylcholine, soybean phosphatidylcholine, dipalmityl phosphatidylcholine, dimyristoyl phosphatidylcholine, distearoyl phosphatidylcholine, dilauryl phosphatidylcholine, dipalmitoyl phosphatidylcholine.

All phospholipids as used herein may be derived from any source, i.e. (where appropriate) from soybean, milk, egg or even inner organs of animals excluding humans, they may be derived from natural origin, or semi-synthetic or even fully synthetic.

The term „POPC“ denotes the phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl choline also referred to as 1-palmitoyl-2-oleoyl-phosphatidyl choline.

As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

The term “variant” includes also variants of an apolipoprotein or an apolipoprotein mimic as reported herein wherein in the variants the amino acid sequence of the respective apolipoprotein or apolipoprotein mimic comprises one or more amino acid substitution, addition or deletion. The modification may increase or decrease the affinity of the apolipoprotein for an apolipoprotein receptor or an apolipoprotein converting enzyme, or may increase the stability of the apolipoprotein variant compared to the respective apolipoprotein, or may increase the solubility of the apolipoprotein variant compared to the respective apolipoprotein in aqueous solutions, or may increase the recombinant production of the apolipoprotein variant compared to the respective apolipoprotein in/by host cells.
Lipid particle

Herein is reported a lipid particle comprising
   a) a tetranectin-apolipoprotein A-I,
   b) a phosphatidylcholine, and
   c) a further lipid.

In one embodiment the lipid particle comprises a tetranectin-apolipoprotein A-I, a first phosphatidylcholine and a second phosphatidylcholine. In one embodiment the first phosphatidylcholine and the second phosphatidylcholine differ in one or two carboxylic acid moieties or carboxylic acid moiety derivatives esterified to the phospho-glycerol backbone of the phosphatidylcholine. In one embodiment the first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC. In one embodiment the tetranectin-apolipoprotein A-I, the phosphatidylcholine, and the in the lipid particle are non-covalently associated. In one embodiment the tetranectin-apolipoprotein A-I is a recombinantly produced tetranectin-apolipoprotein A-I.

The choice of the combination of lipids determines the efficacy and liver safety of lipid particles comprising apolipoprotein. In vivo studies of DMPC containing lipid particles using rabbits it has been found that rabbits treated with 30 mg/kg showed severe side effects but survived whereas rabbits treated with 100 mg/kg died.

In vitro functional tests confirmed that a lipid particle containing a single phosphatidylcholine such as DPPC or POPC activate LCAT.

It was also shown that cholesterol efflux was higher when the lipid particle comprised a combination of different phospholipids.
Table 1: Phospholipid combinations differing in their lipid composition prepared for in vivo rabbit studies.

<table>
<thead>
<tr>
<th>phospholipid molar ratio used for producing the lipid particle</th>
<th>LCAT substrate</th>
<th>cholesterol efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>POPC:DPPC 3:1</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>POPC:DPPC 1:1</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>POPC:DPPC 1:3</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>DPPC</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

These results were also confirmed by in vivo data demonstrating cholesterol mobilization for all combinations. However, for lipid particles containing only the single phosphatidylcholine DPPC, or the combination of DPPC and sphingomyelin (SM) an increase in liver enzymes can be determined (Figure 1).

From the technical point of view the formation of lipid particles with pure DPPC is more convenient compared to the formation with pure POPC. The risk of precipitate formation is reduced by using a combination of different phospholipids. Also the phase transition temperature of 41 °C for pure DPPC makes it easier to prepare the lipid particle compared to pure POPC that has a phase transition temperature of 4 °C. Also the obtained product is more homogeneous. This can be confirmed by lipid particle analysis via SEC-MALLS, an analytical tool which also allows the determination of the protein-lipid composition (protein-conjugate analysis). In Figure 2 a chromatogram of samples resolved in a size-exclusion chromatography (UV280 detection) is shown. An inhomogeneity of a sample can be seen by the occurrence of multiple separated or semi-detached peaks.

The number of POPC molecules per apolipoprotein monomer in the lipid particle when pure POPC is used for producing the lipid particle is in one embodiment of from 40 to 85, in one embodiment of from 50 to 80, in one embodiment of from 54 to 75.
The number of DPPC molecules per apolipoprotein monomer in the lipid particle when pure DPPC is used for producing the lipid particle is in one embodiment of from 50 to 150, in one embodiment of from 65 to 135, in one embodiment of from 76 to 123, and in one embodiment of from 86 to 102.

The number of phospholipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 1:3 is used for producing the lipid particle is in one embodiment of from about 50 to about 120, in one embodiment of from about 65 to about 105, and in one embodiment of from about 72 to about 96.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 1:1 is used for producing the lipid particle is in one embodiment of from 50 to 120, in one embodiment of from 60 to 100, in one embodiment of from 71 to 92, and in one embodiment of from 71 to 85.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 50 to 105.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 60 to 95.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 60 to 90.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 60 to 88.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 62 to 80.

The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment of from 66 to 86.
The number of lipid molecules per apolipoprotein monomer in the lipid particle when a mixture of POPC and DPPC at a molar ratio of 3:1 is used for producing the lipid particle is in one embodiment about 66.

For the production of a lipid particle comprising apolipoprotein and POPC a molar ratio of apolipoprotein to POPC in one embodiment of from 1:40 to 1:100 is employed, in one embodiment a molar ratio of from 1:40 to 1:80 is employed, and in one embodiment a molar ratio of about 1:60 is employed.

For the production of a lipid particle comprising apolipoprotein and DPPC a molar ratio of apolipoprotein to DPPC in one embodiment of from 1:70 to 1:100 is employed, in one embodiment a molar ratio of from 1:80 to 1:90 is employed, and in one embodiment a molar ratio of about 1:80 is employed.

For the production of a lipid particle comprising apolipoprotein, POPC and DPPC a molar ratio of apolipoprotein to POPC and DPPC with POPC and DPPC at a 1:3 molar ratio in one embodiment of from 1:60 to 1:100 is employed, in one embodiment a molar ratio of from 1:70 to 1:90 is employed, and in one embodiment a molar ratio of about 1:80 is employed.

For the production of a lipid particle comprising apolipoprotein, DPPC and POPC a molar ratio of apolipoprotein to POPC and DPPC with POPC and DPPC at a 1:1 molar ratio in one embodiment of from 1:60 to 1:100 is employed, in one embodiment a molar ratio of from 1:60 to 1:80 is employed, and in one embodiment a molar ratio of about 1:70 is employed.

For the production of a lipid particle comprising apolipoprotein, DPPC and POPC a molar ratio of apolipoprotein to POPC and DPPC with POPC and DPPC at a 3:1 molar ratio in one embodiment of from 1:60 to 1:100 is employed, in one embodiment a molar ratio of from 1:50 to 1:70 is employed, and in one embodiment a molar ratio of about 1:60 is employed.

In one embodiment if a mixture of lipids is used for producing the lipid particle the mixture has a phase transition temperature of from 4 °C to 45 °C, in one embodiment of from 10 °C to 38 °C, and in one embodiment of from 15 °C to 35 °C.
The lipid particle comprises in one embodiment an average number of from 1 to 10 apolipoprotein molecules per lipid particle, in one embodiment of from 1 to 8 apolipoprotein molecules per lipid particle, and in one embodiment of from 1 to 4 apolipoprotein molecules per lipid particle.

In one embodiment the lipid particle comprises an average number of at least 1, or 2, or 3, or 4, or 5, or 6, or 7, or 8, or 9, or 10 apolipoprotein molecules per lipid particle. In one embodiment the average number is 1.

In one embodiment the lipid particle comprises one or more further polypeptides beside the apolipoprotein.

Without limitation the lipid particle may serve as an enzymatic co-factor and/or a carrier for taking up lipids, especially cholesterol.

One or more detergents can be present in the lipid particle as reported herein. Such a detergent can be any detergent, i.e. a pharmaceutically acceptable detergent or other detergents at non-toxic concentrations, such as a non-ionic or ionic detergent.

The non-ionic detergent can be an alkylene oxide derivative of an organic compound which contains one or more hydroxyl groups. In one embodiment the non-ionic detergent is selected from ethoxylated and/or propoxylated alcohol or ester compounds or mixtures thereof. In another embodiment the ester is selected from esters of sorbitol and fatty acids, such as sorbitan monooleate or sorbitan monopalmitate, oily sucrose esters, polyoxyethylene sorbitane fatty acid esters, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene fatty acid esters, polyoxyethylene alkyl ethers, polyoxyethylene sterol ethers, polyoxyethylene-polypropoxy alkyl ethers, block polymers and cethyl ether, polyoxyethylene castor oil or hydrogenated castor oil derivatives and polyglycerine fatty acid esters. In one embodiment the non-ionic detergent is selected from Pluronic®, Poloxamer®, Span®, Tween®, Polysorbate®, Tyloxapol®, Emulphor® or Cremophor®.

The ionic detergent can be a bile duct agent. In one embodiment the ionic detergent is selected from cholic acid or deoxycholic acid, or their salts and derivatives, or from free fatty acids, such as oleic acid, linoleic acid and others.

In one embodiment the ionic detergent is selected from cationic lipids like C_{10}-C_{24} alkylamine or alkanolamine and cationic cholesterol esters.

In one embodiment the lipid particle comprises less than 0.75 % by weight detergent.
In one embodiment the lipid particle comprises less than 0.3 % by weight detergent.

In one embodiment the detergent is selected from sugar-based detergents, polyoxyalkylene-based detergents, bile-salt based detergents, synthetic detergents, or a combination thereof. In one embodiment the detergent is cholic acid.

The efficiency at which cholesterol is mobilized into the blood can be determined by comparing the respective excursion of total cholesterol with apolipoprotein concentrations after administration of apolipoprotein in vivo. For a quantitative assessment, the quotient of the baseline corrected area under the concentration–time curve (AUC) of total cholesterol and the area under the concentration–time curve of apolipoprotein was calculated.

The lipid particle as reported herein, especially a lipid particle comprising a tetranectin-apolipoprotein of SEQ ID NO: 01 and POPC and DPPC at a molar ratio of 3:1, shows enhanced cholesterol mobilization in vivo.

Tetranectin-apolipoprotein A-I

Beside the lipid particle as outlined above is herein reported also a tetranectin-apolipoprotein A-I.

Tetranectin-apolipoprotein A-I is a fusion protein of the human tetranectin trimerising structural element and the wild-type human apolipoprotein A-I. The amino acid sequence of the human tetranectin part can be shortened by the first 9 amino acids starting with the isoleucine residue of position 10, a naturally occurring truncation site. As a consequence of this truncation the O-glycosylation site at threonine residue of position 4 has been deleted. Between the tetranectin trimerising structural element and the human apolipoprotein A-I the five amino acid residues “SLKGS” (SEQ ID NO: 03) were removed.

For improved expression and purification a construct can be generated comprising an N-terminal purification tag, e.g. a hexahistidine-tag, and an IgA protease cleavage site. As a result of the specific cleavage two amino acids – as first alanine or glycine or serine or proline and as second proline – are maintained at the N-terminus of the tetranectin-apolipoprotein A-I. The tetranectin-apolipoprotein A-I can have the amino acid sequence of SEQ ID NO: 01.
The tetranection trimerising structural element provides for a domain that allows for the formation of a trimeric tetranection-apolipoprotein A-I multimer that is constituted by non-covalent interactions between each of the individual tetranection-apolipoprotein A-I monomers.

By using an alternative purification method, the purification-tag and the IgA protease cleavage site can be omitted resulting in a tetranection-apolipoprotein A-I of the amino acid sequence of SEQ ID NO: 02.

In one embodiment the apolipoprotein can be a variant comprising conservative amino acid substitutions.

Apolipoprotein A-I can be determined enzymatically, via NMR spectroscopy, or by using monoclonal or polyclonal anti-apolipoprotein-A-I antibodies. Other aspects as reported herein are therefore polyclonal and monoclonal antibodies specifically binding the tetranection-apolipoprotein A-I as reported herein. Such antibodies can be obtained with methods known to a person skilled in the art. Also the labeling of the antibodies for use in immunoassays can be performed with methods known to a person of skill in the art.

In one embodiment the apolipoprotein can be a variant comprising conservative amino acid substitutions, or an apolipoprotein A-I mimic. In one embodiment the tetranection-apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 02, or SEQ ID NO: 66, or SEQ ID NO: 67, wherein X is selected from SEQ ID NO: 68 to SEQ ID NO: 105.

Thus, in one embodiment the tetranection-apolipoprotein A-I has the amino acid sequence of

IVNAKKDVNTKMFEEKLKSRDLTDLAQEVALLKEQQALQTVDDEPPQPSPWDR
VKDLATVYVDVLKDSGRDYVSQFEQSGALGKQLNLKLLDNWDSVTSTFSK
LREQLGPVTQEFWDNLEKEETEQLRQEMSDKDL EEVKAQVQPYLDFQKKW
QEMELYRQKVEPLRQALQEGARQKLHELQEKLSPLGEEMRDRARAHVD
ALRTHLAPYSDELRQRLAARLEALKENGGARLAEMYHAKATEHLSLSEKAKP
PAEGLRQRQGLPVLEFSKFVSFLSALEYT九龙LNTQ (SEQ ID NO: 02).

In one embodiment the tetranection-apolipoprotein A-I has the amino acid sequence of

(A,G,S,T)IVNAKKDVNTKMFEEKLKSRDLTDLAQEVALLKEQQALQTVD
PQSPWDRVKDLATVYVDVLKDSGRDYVSQFEQSGALGKQLNLKLLDNWDS
In one embodiment the tetranectin-apolipoprotein A-I has the amino acid sequence of

(M)HHHHTHXXXXVNAVXKDDVVMELKSRLDQETEPLLKEQQALQTV
DEPQPQSPWDRVKDLATVYVDLKDSDKRQVYFSQFGSALGKQLNLKILD
WDSVTSTFSKLREQQLGPVTQEFWDNLEKEGETLRQEMSKDLLEEVKAVQP
YLDDFQKKWQEEMELRYKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDR
ARAHVDALRTHLAPYSDELQRLAARLEVAKENNGARLAELYHAKATEHL
EHLSTLSEKAFAKPALEDLRQGLLPVESFKVSFLSALEYTKKLNTQ (SEQ ID NO: 67),

wherein X can be any of the following amino acid sequences A, G, S, P, AP, GP,
SP, PP, GSAP (SEQ ID NO: 68), GSAP (SEQ ID NO: 69), GSPP (SEQ ID NO: 70),
GSPP (SEQ ID NO: 71), GGGS (SEQ ID NO: 72), GGGS (SEQ ID NO: 73),
GGSGGGGS (SEQ ID NO: 74), GGGSGGGGS (SEQ ID NO: 75),
GGGGGGGGGGS (SEQ ID NO: 76), GGGGSGGGGGGGS (SEQ ID NO: 77),
GGGGSP (SEQ ID NO: 78), GGGSAP (SEQ ID NO: 79), GGGSSP (SEQ ID NO:
80), GGGSSPP (SEQ ID NO: 81), GGGSSPAP (SEQ ID NO: 82), GGGSSGSP
(SEQ ID NO: 83), GGGSSGSP (SEQ ID NO: 84), GGGSSPP (SEQ ID NO: 85),
GGGGGGGSSAP (SEQ ID NO: 86), GGGGGGGGSP (SEQ ID NO: 87),
GGGGGGGGSPP (SEQ ID NO: 88), GGGGGGGSSPP (SEQ ID NO: 89),
GGGGGGGSSGSGSAP (SEQ ID NO: 90), GGGGSGGGSGGGSP (SEQ ID NO:
91), GGGGSGGGGSGGSSP (SEQ ID NO: 92), GGGGSGGGGSSGSSP (SEQ ID
NO: 93), GGGGSSAP (SEQ ID NO: 94), GGGGSSP (SEQ ID NO: 95), GGGGGSSP
(SEQ ID NO: 96), GGGGGSSP (SEQ ID NO: 97), GGGGGGGGSSAP (SEQ ID
NO: 98), GGGGGGGGGSAP (SEQ ID NO: 99), GGGGSGGGGSPP (SEQ ID
NO: 100), GGGGGGGGGSPP (SEQ ID NO: 101), GGGGSGGGGGSGGGGSSAP
(SEQ ID NO: 102), GGGGSGGGGGSGGGGSPP (SEQ ID NO: 103),
GGGGGGGGGGGGGSSPP (SEQ ID NO: 104), and GGGGSGGGGGGGGSSPP
(SEQ ID NO: 105).

It has to be noted that if a polypeptide is recombinantly produced in E.coli strains
the N-terminal methionine residue is usually not efficiently cleaved off by E.coli.
proteases. Thus, the N-terminal methionine residue is partially present in the produced polypeptide.

**Properties:**

The tetranectin-apolipoprotein A-I as reported herein or the lipid particle as reported herein can be used for the treatment and/or diagnosis of a disease or condition characterized by non-normal lipid levels or a deposition of lipids within body components, such as plaques in blood vessels.

In order to determine the capacity of the lipid particle as reported herein to support LCAT catalyzed cholesterol esterification cholesterol can be incorporated in the lipid particle by addition of an ethanolic cholesterol solution. Lipid particles containing pure POPC are better LCAT substrates than complexes containing DPPC independent of their apolipoprotein constituent, such as wild-type apolipoprotein A-I or tetranectin-apolipoprotein A-I (Figure 3).

Initial velocity of cholesterol esterification in lipid particles comprising different mixtures of POPC and DPPC show that mixtures are better LCAT substrates than a single pure phosphatidylcholine. This can be seen from the initial velocities of cholesterol esterification (see Table 2 and Figure 4).

**Table 2:** Initial velocities of cholesterol esterification in lipid particles comprising different mixtures of phospholipids.

<table>
<thead>
<tr>
<th>phospholipid molar ratio used for producing the lipid particle</th>
<th>$K_m$ [μM]</th>
<th>$V_{max}$ [nmol ester/h/μg LCAT]</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>4.6</td>
<td>1.6</td>
</tr>
<tr>
<td>POPC:DPPC 3:1</td>
<td>0.4</td>
<td>1.9</td>
</tr>
<tr>
<td>POPC:DPPC 1:1</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>POPC:DPPC 1:3</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>DPPC</td>
<td>0.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Macrophage like human THP1 cells obtained by exposing THP-1 monocytic leukemia cells to phorbol myristate acetate and loaded with a radioactive labeled cholesterol tracer can be exposed to cholesterol acceptor test compounds.
Efflux velocity induced by acceptor test compounds can be calculated as the ratio of cholesterol radioactivity in the supernatant to the sum of the radioactivity in the cells plus their supernatant and compared to cells exposed to medium containing no acceptors and analyzed by linear fit. Parallel experiments can be performed using cells exposed and not exposed to a RXR-LXR agonist which is known to upregulate mainly ABCA-1 and bias efflux toward ABCA-1 mediated transport.

In cells not pre-treated with RXR-LXR lipid particles a higher increase in cholesterol efflux compared to the efflux obtained with non lipidated tetranectin-apolipoprotein A-I can be seen. Only a small influence of the lipid mixture on efflux can be observed in the tested series (Figure 5). In cells pre-treated with RXR-LXR a comparable increase in cholesterol efflux can be seen using a non-lipidated tetranectin-apolipoprotein A-I. The overall increase was higher as compared to that observed with not pre-treated cells. Only a small influence of the lipid mixture on efflux can be observed in the tested series (Figure 6).

Different lipid particles were tested in vivo in rabbits. The lipid particle was applied as intravenous infusion and serial blood sampling was performed over 96 h after application. Values of liver enzymes, cholesterol, and cholesterol ester were determined. Plasma concentrations are comparable for all tested lipid particles comprising an initial distribution phase followed by log-linear decline of plasma concentrations (Figure 7). As can be seen from Table 3 pharmacokinetic parameters are similar for all tested compounds. The observed half-lives are close to 1.5 days.

**Table 3:** Determined pharmacokinetic parameters.

<table>
<thead>
<tr>
<th>phospholipid molar ratio used for producing the lipid particle</th>
<th>$C_L$ [ml/h/kg]</th>
<th>$V_{ss}$ [ml/kg]</th>
<th>$T_{1/2}$ [h]</th>
<th>$C_{max}$ [mg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>0.89 ± 0.22</td>
<td>45.0 ± 2.5</td>
<td>36.9 ± 8.2</td>
<td>2.40 ± 0.19</td>
</tr>
<tr>
<td>POPC:DPCC 3:1</td>
<td>0.82 ± 0.06</td>
<td>37.8 ± 5.6</td>
<td>34.2 ± 4.5</td>
<td>2.65 ± 0.28</td>
</tr>
<tr>
<td>POPC:DPCC 1:1</td>
<td>0.85 ± 0.14</td>
<td>43.1 ± 5.9</td>
<td>38.6 ± 10.6</td>
<td>2.34 ± 0.31</td>
</tr>
<tr>
<td>DPPC</td>
<td>0.96 ± 0.10</td>
<td>37.8 ± 4.9</td>
<td>30.2 ± 7.7</td>
<td>2.29 ± 0.19</td>
</tr>
<tr>
<td>DPPC:SM 9:1</td>
<td>1.28 ± 0.62</td>
<td>50.7 ± 8.7</td>
<td>31.3 ± 8.2</td>
<td>1.91 ± 0.33</td>
</tr>
</tbody>
</table>
As can be seen from Figure 8 cholesterol is mobilized and esterified in plasma. Plasma cholesterol ester levels do continue to increase even after the concentration of tetranectin-apolipoprotein A-I is already decreasing. When plasma tetranectin-apolipoprotein A-I levels have decreased to about 0.5 mg/ml (about 50\% of normal wild-type apolipoprotein A-I) increased cholesterol ester levels can still be detected.

Lipid particles comprising tetranectin-apolipoprotein A-I do not induce liver enzymes in rabbits as well as in mice as can be seen from Figures 1 and 9. Also no hemolysis can be determined in plasma samples obtained two hours after intravenous application (Figure 10).

Therefore aspects as reported herein are a pharmaceutical composition and a diagnostic composition comprising a lipid particle as reported herein, or a tetranectin-apolipoprotein A-I as reported herein.

The lipid particle as reported herein has improved in vivo properties compared to non-lipidated apolipoprotein and other lipid particles as shown in the following Table 4.

**Table 4:** In vivo properties of different apolipoproteins and lipid particles.

<table>
<thead>
<tr>
<th>protein</th>
<th>lipid particle comprising</th>
<th>applied to</th>
<th>highest applied dose</th>
<th>acute liver toxicological effect</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>apolipoprotein A-I mutants</td>
<td>no particle</td>
<td>rat</td>
<td>orally, 1 g/kg</td>
<td>no toxic effect up to 500 mg/kg</td>
<td>US 2005/0287636</td>
</tr>
<tr>
<td>A-I, tetranectin-apolipoprotein A-I</td>
<td>DMPC</td>
<td>mouse</td>
<td>i.v. 1 to 1.2 mg/mouse</td>
<td>not described</td>
<td>WO 2002/38609; Graversen, (2008)</td>
</tr>
<tr>
<td>pro apolipoprotein A-I</td>
<td>SM</td>
<td>not reported</td>
<td>not reported</td>
<td>injection, toxic at dose of 200 mg/kg</td>
<td>WO 2003/096983</td>
</tr>
<tr>
<td>apolipoprotein A-I</td>
<td>PG/SM</td>
<td>rabbit</td>
<td>i.v. 15 mg/kg</td>
<td>not described</td>
<td>WO 2006/100567</td>
</tr>
<tr>
<td>protein</td>
<td>lipid particle comprising</td>
<td>applied to</td>
<td>highest applied dose</td>
<td>acute liver toxicological effect</td>
<td>reference</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---------------------------</td>
<td>------------</td>
<td>----------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>apolipoprotein A-I</td>
<td>PC (soybean)</td>
<td>human</td>
<td>80 mg/kg</td>
<td>treatment group was discontinued early because of liver function test abnormalities (10-fold increase in alanine aminotransferase)</td>
<td>WO 2007/137400</td>
</tr>
<tr>
<td>apolipoprotein A-I Milano variant</td>
<td>POPC</td>
<td>human</td>
<td>45 mg/kg</td>
<td>one patient withdrawn due to development of an elevated aspartate aminotransferase level (3x upper limit of normal)</td>
<td>Nissen, S.E., et al., JAMA 290 (2003) 2292-2300</td>
</tr>
<tr>
<td>tetranectin-apolipoprotein A-I</td>
<td>DMPC</td>
<td>rabbit</td>
<td>100 mg/kg</td>
<td>lethal after 3-4 hours in all animals tested</td>
<td></td>
</tr>
<tr>
<td>tetranectin-apolipoprotein A-I</td>
<td>POPC/DPP C</td>
<td>rabbit</td>
<td>100 mg/kg</td>
<td>increase not observed</td>
<td></td>
</tr>
<tr>
<td>tetranectin-apolipoprotein A-I</td>
<td>POPC/DPP C</td>
<td>rat</td>
<td>i.v. 500 mg/kg</td>
<td>increase not observed</td>
<td></td>
</tr>
<tr>
<td>tetranectin-apolipoprotein A-I</td>
<td>POPC/DPP C</td>
<td>cynomolgus monkey</td>
<td>i.v. 200 mg/kg</td>
<td>increase not observed</td>
<td></td>
</tr>
</tbody>
</table>

Formation of lipid particles

For the formation of lipid particles as reported herein different methods are known, such as freeze-drying, freeze-thawing, detergent solubilization followed by dialysis, microfluidization, sonification, and homogenization.

For example aqueous mixtures of phospholipids with detergents can be incubated with purified apolipoprotein. The apolipoprotein can be added in native form. The detergent is afterwards removed by dialysis or diafiltration. The formation of lipid
particles comprising tetranection-apolipoprotein A-I can be achieved by incubating tetranection-apolipoprotein A-I in monomeric or multimeric form with detergent solubilized lipids at their respective transition temperature. Removal of the detergent by dialysis results in the formation of lipid particles. A common method for the formation of lipid particles containing an apolipoprotein is based on the cholate method as described e.g. in Jonas, A., Methods Enzymol. 128 (1986) 553-582 or Experimental Lung Res. 6 (1984) 255-270. Removal of the detergent by dialysis results in the formation of lipid particles.

The main points which have to be considered for the lipid particle formation are i) the requirements for biological activity, and ii) technical requirements directed to the manufacturability of the lipid particle. For the formation of lipid particles comprising an apolipoprotein these requirements point in opposite directions.

From a technical point of view saturated phospholipids containing carboxylic acid moieties with a chain of 16 carbon atoms and shorter would be chosen (e.g. dipalmitoyl-sn-glycero-3-phosphocholine, DPPC; dimyristoyl-sn-glycero-3-phosphocholine, DMPC etc.). In contrast thereto from biological data it can be assumed that non-saturated phospholipids containing carboxylic acid moieties with a chain of at least 16 carbon-atoms (e.g. palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC; stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, SOPC) are more effective and non-liver toxic.

The phosphatidylcholines DPPC and POPC and mixtures thereof can be used for the formation of lipid particles containing an apolipoprotein. These exemplary phosphatidylcholines differ in one carboxylic acid moiety and have one identical carboxylic acid moiety esterified to the phosphoglycerol backbone. The manufacture of lipid particles was easier when DPPC was used. In contrast POPC was more effective in in vitro functional assays, particularly as substrate for the activation of the lecithin cholesterol acetyl transferase (LCAT) enzyme which is necessary for the conversion of the mobilized cholesterol into cholesterol ester. It has been found that lipid particles comprising mixtures of two phosphatidylcholines, as e.g. POPC and DPPC, in different molar ratios have improved properties compared to lipid particles comprising only one phosphatidylcholine (see e.g. Figure 4).

Different methods to reconstitute lipid particles from recombinant apolipoprotein or delipidated apolipoprotein derived from human HDL particles have been reported (HDL = high density lipoprotein). For example aqueous mixtures of
phospholipids with detergents are incubated with purified apolipoprotein. The apolipoprotein is added in native form. The detergent is afterwards removed by dialysis or diafiltration. The formation of lipid particles comprising tetranection-apolipoprotein A-I can be achieved by incubating tetranection-apolipoprotein A-I or a multimer thereof with detergent solubilized lipids at their respective transition temperature. Removal of the detergent by dialysis results in the formation of lipid particles.

The lipid particle can be purified by a combination of precipitation and/or chromatography steps. For example excess detergent, i.e. detergent not part of the lipid particle, can be removed in a hydrophobic adsorption chromatography step. The lipid particle can be recovered from the hydrophobic adsorption material with a detergent-free solution.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Description of the Sequence Listing**

SEQ ID NO: 01  Tetranectin-apolipoprotein A-I (1).
SEQ ID NO: 02  Tetranectin-apolipoprotein A-I (2).
SEQ ID NO: 03  Excised peptide.
SEQ ID NO: 04  Apolipoprotein A-I mimetic (1).
SEQ ID NO: 05  Apolipoprotein A-I mimetic (2).
SEQ ID NO: 06  Human apolipoprotein A-I.
SEQ ID NO: 07  Human apolipoprotein A-II.
SEQ ID NO: 08  Human apolipoprotein A-IV.
SEQ ID NO: 09  Human apolipoprotein A-V.
SEQ ID NO: 10  Human apolipoprotein C-I.
SEQ ID NO: 11  Human apolipoprotein C-II.
SEQ ID NO: 12  Human apolipoprotein C-III.
SEQ ID NO: 13  Human apolipoprotein C-IV.
SEQ ID NO: 14  Human apolipoprotein D.
SEQ ID NO: 15  Human apolipoprotein E.
SEQ ID NO: 16  Human apolipoprotein F.
SEQ ID NO: 17  Human apolipoprotein H.
SEQ ID NO: 18  Human apolipoprotein L-I.
SEQ ID NO: 19  Human apolipoprotein L-II.
SEQ ID NO: 20  Human apolipoprotein L-III.
SEQ ID NO: 21  Human apolipoprotein L-IV.
SEQ ID NO: 22  Human apolipoprotein L-V.
SEQ ID NO: 23  Human apolipoprotein L-VI.
SEQ ID NO: 24  Human apolipoprotein M.
SEQ ID NO: 25  Human apolipoprotein O.
SEQ ID NO: 26  Human apolipoprotein OL.
SEQ ID NO: 27  Human apolipoprotein clus.
SEQ ID NO: 28 to 52  Apolipoprotein.
SEQ ID NO: 53  Human tetranectin trimerization domain.
SEQ ID NO: 54  Shortened human tetranectin trimerization domain.
SEQ ID NO: 55  Human interferon fragment.
SEQ ID NO: 56  Hexahistidine tag.
SEQ ID NO: 57  Fusion protein.
SEQ ID NO: 58  Primer N1.
SEQ ID NO: 59  Primer N2.
SEQ ID NO: 60 to 65  IgA protease cleavage site.
SEQ ID NO: 66  Tetranectin-apolipoprotein A-I.
SEQ ID NO: 67  Tetranectin-apolipoprotein A-I with his-tag.
SEQ ID NO: 68 to 105  Linker.

Description of the Figures

Figure 1  Results of in vivo rabbit studies conducted with five lipid particles differing in their lipid composition. Top: cholesterol mobilization and, thus, efficacy could be shown for all prepared batches. Bottom: Increase of liver enzyme was noticed for lipid particles generated by the use of DPPC as single phospholipid.

Figure 2  SEC-MALLS analysis of lipid particles of POPC and apolipoprotein according to the current invention; molar ratios 1:20 to 1:160.

Figure 3  Impact of DPPC and POPC on LCAT activity.

Figure 4  Initial velocity of cholesterol esterification in lipid particles containing POPC and/or DPPC.

Figure 5  Cholesterol efflux to THP-1 derived foam cells in cells not primed with a RXR-LXR agonist.
Figure 6  Cholesterol efflux to THP-1 derived foam cells after ABCA-I pathway activation using an RXR-LXR agonist.

Figure 7  Time dependent plasma concentration of different apolipoprotein compositions.

Figure 8  Time and concentration course of cholesterol mobilization and esterification in plasma.

Figure 9  Comparison of liver enzyme release by different compositions comprising apolipoprotein according to the invention in mice after a single i.v. injection of 100 mg/kg.

Figure 10  In vivo rabbit study – spontaneous hemolysis in plasma.

Figure 11  Analytical SEC of lipid particles using 250 mM Tris-HCl, 140 mM NaCl, pH 7.5.

Figure 12  Analytical SEC of lipid particles using 50 mM K₂HPO₄, 250 mM arginine hydrochloride, 7.5% trehalose at pH 7.5.

Figure 13  Native PAGE of lipid particles of POPC and tetranection-apolipoprotein A-I in molar ratios of from 1:20 to 1:320 (lane 1: native Marker, lane 2: molar ratio 1 : 320; lane 3: molar ratio 1 : 160; lane 4: molar ratio 1 : 80; lane 5: molar ratio 1 : 80 (f/t); lane 6: molar ratio 1 : 40; lane 7: molar ratio 1 : 20; lane 8: apolipoprotein (forming hexamers)).

Figure 14  SEC-MALLS analysis of lipid particles of POPC and tetranection-apolipoprotein A-I in molar ratios of from 1:20 to 1:160.

Figure 15  Superposition of SEC chromatograms (UV280 signal) of lipid particle of POPC and tetranection-apolipoprotein A-I.

Figure 16  SEC-MALLS analysis of a lipid particle of POPC and tetranection-apolipoprotein A-I obtained at a molar ratio of 1:40.

Figure 17  Native PAGE of lipid particles of DPPC and tetranection-apolipoprotein A-I obtained with molar ratios of from 1:20 to 1:100 (1: molecular weight marker; 2: tetranection-apolipoprotein A-I without lipid; 3: 1:20; 4: 1:40; 5: 1:60; 6: 1:80; 7: 1:100).

Figure 18  SEC-MALLS analysis (UV280 signal) of a lipid particle of a mixture of POPC:DPPC = 3:1 and tetranection-apolipoprotein A-I obtained at molar ratios of from 1:60 (uppermost curve) to 1:100 (lowest curve).

Figure 19  Native PAGE SDS of a lipid particle of tetranection-apolipoprotein A-I using cholate, Zwittergent 3-8, 3-10 and 3-12. Lane 1 on each
gel: pure apolipoprotein; lane 2 on each gel: 0.1 x CMC cholate lipdated sample as references.

**Figure 20** SEC-MALLS protein conjugate analysis of lipid particle of tetranection-apolipoprotein A-I using 3 x CMC Zwittergent 3-8 and POPC (molar ratio apolipoprotein:phospholipid = 1:60).

**Figure 21** SEC-MALLS protein conjugate analysis of lipid particle of tetranection-apolipoprotein A-I using 2 x CMC Zwittergent 3-10 and POPC (molar ratio apolipoprotein:phospholipid = 1:60).

**Figure 22** SEC-MALLS protein conjugate analysis of lipid particle of tetranection-apolipoprotein A-I using POPC. Upper: lipid particle formed from native tetranection-apolipoprotein A-I; lower: lipid particle formed from denatured tetranection-apolipoprotein A-I.

**Figure 23** Results of in vivo rabbit studies performed with tetranection-apolipoprotein A-I lipided with DMPC (1:100) (di myristoyl phosphatidylcholine) (a) and not lipided in PBS (b).

**Figure 24** SE-HPLC chromatogram of lipid particles containing wild-type apolipoprotein A-I (A) and tetranection-apolipoprotein A-I as reported herein (B) stored at 5 °C and 40 °C.

**Materials and Methods**

20 Size-exclusion-HPLC:

The chromatography was conducted with a Tosoh Haas TSK 3000 SWXL column on an ASI-100 HPLC system (Dionex, Idstein, Germany). The elution peaks were monitored at 280 nm by a UV diode array detector (Dionex). After dissolution of the concentrated samples to 1 mg/ml the column was washed with a buffer consisting of 200 mM potassium dihydrogen phosphate and 250 mM potassium chloride pH 7.0 until a stable baseline was achieved. The analyzing runs were performed under isocratic conditions using a flow rate of 0.5 ml/min. over 30 minutes at room temperature. The chromatograms were integrated manually with Chromeleon (Dionex, Idstein, Germany). Aggregation in % was determined by comparing the area under the curve (AUC) of high molecular weight forms with the AUC of the monomer peak.

**Dynamic light scattering (DLS):**

DLS is a non-invasive technique for measuring particle size, typically in the sub-micron size range. In the current invention the Zetasizer Nano S apparatus
(Malvern Instruments, Worcestershire, UK) with a temperature controlled quartz cuvette (25 °C) was used for monitoring a size range between 1 nm and 6 μm. The intensity of the back scattered laser light was detected at an angle of 173°. The intensity fluctuates at a rate that is dependent upon the particle diffusion speed, which in turn is governed by particle size. Particle size data can therefore be generated from an analysis of the fluctuation in scattered light intensity (Dahneke, B.E. (ed.), Measurement of Suspended Particles by Quasielectric Light Scattering, Wiley Inc. (1983); Pecora, R., Dynamic Light Scattering: Application of Photon Correlation Spectroscopy, Plenum Press (1985)). The size distribution by intensity was calculated using the multiple narrow mode of the DTS software (Malvern). Experiments were conducted with undiluted samples.

SEC-MALLS:

SEC-MALLS is a combination of size exclusion chromatography with a three detector system: i) UV detection, ii) refraction index detection and iii) light scattering detection. For the separation by size a Superose 6 column 10/300 GL column from GE Healthcare is used. The method is run isocratically with a PBS buffer pH 7.4 applying a flow rate of 0.4 ml/min. Three detector systems are connected in series. The complete lipid particle (protein-lipid particle) signal is monitored by the refraction index detector whereas the UV absorbance determined at 280 nm determines the signal induced by the protein part. The proportion of the lipid fraction is obtained by a simple subtraction of the protein UV signal from the complete signal. Applying light scattering allows for the detection of the molecular mass of the respective species and, thus, a complete and detailed description of the lipid particle.

Detergent determination:

The determination of residual detergent was conducted by reversed-phase chromatography coupled with an evaporative light scattering detector (RP-ELSD). As column a Luna C18 4.6 x 150 mm, 5 μm, 100 Å from Phenomenex (Aschaffenburg, Germany) was used. After centrifugation through a 10 kDa membrane 90 μl of the flow-through were used for HPLC separation. Elution was performed under isocratic conditions with 74 % (v/v) methanol solution containing 0.1 % (v/v) trifluoro acetic acid. Column temperature was set to 30 °C. Detection was performed by an evaporative light scattering detector applying a nebulization temperature of 30 °C, an evaporating temperature of 80 °C and a gas flow of 1.0
l/min. Quantification of the residual detergent was conducted by the establishment of a calibration curve, in case of cholate in the range of 0.22 μg to 7.5 μg cholate.

**Protein determination:**

The protein concentration was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

**Recombinant DNA technique:**

Standard methods were used to manipulate DNA as described in Sambrook, J., et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

**Example 1**

**Making and description of the E. coli expression plasmids**

The tetranectin-apolipoprotein A-I fusion polypeptide was prepared by recombinant means. The amino acid sequence of the expressed fusion polypeptide in N- to C-terminal direction is as follows:

- the amino acid methionine (M),
- a fragment of an interferon sequence that has the amino acid sequence of CDLPQTHSL (SEQ ID NO: 55),
- a GS linker,
- a hexa-histidine tag that has the amino acid sequence of HHHHHHH (SEQ ID NO: 56),
- a GS linker,
- an IgA protease cleavage site that has the amino acid sequence of VVAPPAP (SEQ ID NO: 60), and
- a tetranectin-apolipoprotein A-I that has the amino acid sequence of SEQ ID NO: 02.

The tetranectin-apolipoprotein A-I fusion polypeptides as described above are precursor polypeptides from which the tetranectin-apolipoprotein A-I fusion polypeptides was released by enzymatic cleavage in vitro using IgA protease.

The precursor polypeptide encoding fusion gene was assembled with known recombinant methods and techniques by connection of appropriate nucleic acid
segments. Nucleic acid sequences made by chemical synthesis were verified by DNA sequencing. The expression plasmid for the production of tetranectin-apolipoprotein A-I of SEQ ID NO: 01 encoding a fusion protein of SEQ ID NO: 31 was prepared as follows.

**Making of the E.coli expression plasmid**

Plasmid 4980 (4980-pBRori-URA3-LACI-SAC) is an expression plasmid for the expression of core-streptavidin in E. coli. It was generated by ligation of the 3142 bp long EcoRI/CelII-vector fragment derived from plasmid 1966 (1966-pBRori-URA3-LACI-T-repeat; reported in EP-B 1 422 237) with a 435 bp long core-streptavidin encoding EcoRI/CelIII-fragment.

The core-streptavidin E.coli expression plasmid comprises the following elements:

- the origin of replication from the vector pBR322 for replication in E. coli (corresponding to bp position 2517-3160 according to Sutcliffe, G., et al., Quant. Biol. 43 (1979) 77-90),
- the URA3 gene of Saccharomyces cerevisiae coding for orotidine 5’-phosphate decarboxylase (Rose, M. et al. Gene 29 (1984) 113-124) which allows plasmid selection by complementation of E.coli pyrF mutant strains (uracil auxotrophy),
- the core-streptavidin expression cassette comprising
  - the core-streptavidin gene,
- two bacteriophage-derived transcription terminators, the λ-T0 terminator (Schwarz, E., et al., Nature 272 (1978) 410-414) and the fd-terminator (Beck E. and Zink, B. Gene 1-3 (1981) 35-58),
- the lacI repressor gene from E. coli (Farabaugh, P.J., Nature 274 (1978) 765-769).

The final expression plasmid for the expression of the tetranectin-apolipoprotein A-I precursor polypeptide was prepared by excising the core-streptavidin structural gene from vector 4980 using the singular flanking EcoRI and CelII restriction endonuclease cleavage site and inserting the EcoRII/CelII restriction site flanked
nucleic acid encoding the precursor polypeptide into the 3142 bp long EcoRI/CelII-4980 vector fragment.

**Example 2**

**Expression of tetranection-apolipoprotein A-I**

For the expression of the fusion protein as described in example 1 there was employed an E.coli host/vector system which enables an antibiotic-free plasmid selection by complementation of an E.coli auxotrophy (PyrF) (EP 0 972 838 and US 6,291,245).

The E.coli K12 strain CSPZ-2 (leuB, proC, trpE, th-l, ΔpyrF) was transformed by electroporation with the expression plasmid p(IFN-His6-IgA-tetranection-apolipoprotein A-I). The transformed E.coli cells were first grown at 37 °C on agar plates.

**Fermentation protocol 1:**

For pre-fermentation a M9 medium according to Sambrook et al (Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press; 2nd edition (December 1989) supplemented with about 1 g/l L-leucine, about 1 g/l L-proline and about 1 mg/l thiamine-HCl has been used.

For pre-fermentation 300 ml of M9-medium in a 1000 ml Erlenmeyer-flask with baffles was inoculated with 2 ml out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 13 hours at 37 °C until an optical density (578 nm) of 1-3 was obtained.

For fermentation a batch medium according to Riesenb et al. was used (Riesenb, D., et al., J. Biotechnol. 20 (1991) 17-27): 27.6 g/l glucose*H₂O, 13.3 g/l KH₂PO₄, 4.0 g/l (NH₄)₂HPO₄, 1.7 g/l citrate, 1.2 g/l MgSO₄*7 H₂O, 60 mg/l iron(III)citrate, 2.5 mg/l CoCl₂*6 H₂O, 15 mg/l MnCl₂*4 H₂O, 1.5 mg/l CuCl₂*2 H₂O, 3 mg/l H₃BO₃, 2.5 mg/l Na₂MoO₄*2 H₂O, 8 mg/l Zn(CH₃COO)₂*2 H₂O, 8.4 mg/l Tiritplex III, 1.3 ml/l Synperonic 10 % anti foam agent. The batch medium was supplemented with 5.4 mg/l Thiamin-HCl and 1.2 g/l L-leucine and L-proline respectively. The feed 1 solution contained 700 g/l glucose supplemented with 19.7 g/l MgSO₄*7 H₂O. The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH₃ solution supplemented with 50 g/l L-leucine and 50 g/l L-proline respectively. All components were dissolved in deionized water.
The fermentation was carried out in a 10 l Biostat C DCU3 fermenter (Sartorius, Melsungen, Germany). Starting with 6.4 l sterile fermentation batch medium plus 300 ml inoculum from the pre-fermentation the batch fermentation was performed at 37 °C, pH 6.9 ± 0.2, 500 mbar and an aeration rate of 10 l/min. After the initially supplemented glucose was depleted the temperature was shifted to 28 °C and the fermentation entered the fed-batch mode. Here the relative value of dissolved oxygen (pO2) was kept at 50 % (DO-stat, see e.g. Shay, L.K., et al., J. Indus. Microbiol. Biotechnol. 2 (1987) 79-85) by adding feed 1 in combination with constantly increasing stirrer speed (550 rpm to 1000 rpm within 10 hours and from 1000 rpm to 1400 rpm within 16 hours) and aeration rate (from 10 l/min to 16 l/min in 10 hours and from 16 l/min to 20 l/min in 5 hours). The supply with additional amino acids resulted from the addition of the alkaline solution, when the pH reached the lower regulation limit (6.70) after approximately 8 hours of cultivation. The expression of recombinant therapeutic protein was induced by the addition of 1 mM IPTG at an optical density of 70.

At the end of fermentation the cytoplasmatic and soluble expressed tetranectin-apolipoprotein A-I is transferred to insoluble protein aggregates, the so called inclusion bodies, with a heat step where the whole culture broth in the fermenter is heated to 50 °C for 1 or 2 hours before harvest (see e.g. EP-B 1 486 571). Thereafter, the content of the fermenter was centrifuged with a flow-through centrifuge (13,000 rpm, 13 l/h) and the harvested biomass was stored at -20 °C until further processing. The synthesized tetranectin-apolipoprotein A-I precursor proteins were found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

The synthesized fusion protein was found exclusively in the insoluble cell debris fraction in the form of insoluble protein aggregates, so-called inclusion bodies (IBs).

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{Target} = 5) are resuspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 300 µL and to each pellet (= insoluble) fraction
400 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under shaking to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µl molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µl, 0.6 µl and 0.9 µl) quantification standard with known product protein concentration (0.1 µg/µl) are positioned on the gel.

The electrophoresis was run for 60 Minutes at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 minutes with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

Fermentation protocol 2:

For pre-fermentation a M9 medium according to Sambrook et al. (Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press; 2nd edition (December 1989)) supplemented with about 1 g/l L-leucine, about 1 g/l L-proline and about 1 mg/l thiamine-HCl has been used.

For pre-fermentation 300 ml of modified M9-medium in a 1000 ml Erlenmeyer-flask with baffles was inoculated from agar plate or with 1-2 ml out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 13 hours at 37 °C until an optical density (578 nm) of 1-3 was obtained.

For fermentation and high yield expression of tetranectin-apolipoprotein A-I the following batch medium and feeds were used:

8.85 g/l glucose, 63.5 g/l yeast extract, 2.2 g/l NH₄Cl, 1.94 g/l L-leucine, 2.91 g/l L-proline, 0.74 g/l L-methionine, 17.3 g/l KH₂PO₄*H₂O, 2.02 g/l MgSO₄*7 H₂O, 25.8 mg/l Thiamin-HCl, 1.0 ml/l Synperonic 10 % anti foam agent. The feed 1 solution contained 333 g/l yeast extract and 333 g/l 85%-glycerol supplemented with 1.67 g/l L-methionine and 5 g/l L-leucine and L-proline each. The feed 2 was a solution of 600 g/l L-Proline. The alkaline solution for pH regulation was a 10 % (w/v) KOH solution and as acid a 75 % glucose solution was used. All components were dissolved in deionized water.
The fermentation was carried out in a 10 l Biostat C DCU3 fermenter (Sartorius, Melsungen, Germany). Starting with 5.15 l sterile fermentation batch medium plus 300 ml inoculum from the pre-fermentation the fed-batch fermentation was performed at 25 °C, pH 6.7 ± 0.2, 300 mbar and an aeration rate of 10 l/min. Before the initially supplemented glucose was depleted the culture reached an optical density of 15 (578 nm) and the fermentation entered the fed-batch mode when feed 1 was started with 70 g/h. Monitoring the glucose concentration in the culture the feed 1 was increased to a maximum of 150 g/h while avoiding glucose accumulation and keeping the pH near the upper regulation limit of 6.9. At an optical density of 50 (578 nm) feed 2 was started with a constant feed rate of 10 ml/h. The relative value of dissolved oxygen (pO₂) was kept above 50 % by increasing stirrer speed (500 rpm to 1500 rpm), aeration rate (from 10 l/min to 20 l/min) and pressure (from 300 mbar to 500 mbar) in parallel. The expression of recombinant therapeutic protein was induced by the addition of 1 mM IPTG at an optical density of 90.

Seven samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_target = 5) are resuspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 μL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble) fraction 300 μL and to each pellet (= insoluble) fraction 200 μL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95 °C under shaking to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 μL of each sample are transferred to a 10% Bis-Tris polyacrylamide gel (Novagen). Additionally 5 μl molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 μl, 0.6 μl and 0.9 μl) quantification standard with known product protein concentration (0.1 μg/μl) are positioned on the gel.

The electrophoresis was run for 35 minutes at 200 V and then the gel was stained with Coomassie Brilliant Blue R dye, destained with heated water and transferred to an optical densitometer for digitalization (GS710, Bio-Rad). Gel images were analyzed using Quantity One 1-D analysis software (Bio-Rad). With the three standards a linear regression curve is calculated with a coefficient of >0.98 and thereof the concentrations of target protein in the original sample was calculated.
At the end of fermentation the cytoplasmatic and soluble expressed tetranection-apolipoprotein A-I is transferred to insoluble protein aggregates, the so called inclusion bodies (IBs), with a heat step where the whole culture broth in the fermenter is heated to 50 °C for 1 or 2 hours before harvest (see e.g. EP-B 1 486 571). After the heat step the synthesized tetranection-apolipoprotein A-I precursor proteins were found exclusively in the insoluble cell debris fraction in the form of IBs.

The contents of the fermenter are cooled to 4-8 °C, centrifuged with a flow-through centrifuge (13,000 rpm, 13 l/h) and the harvested biomass is stored at -20 °C until further processing. The total harvested biomass yield ranged between 39 g/l and 90 g/l dry matter depending on the expressed construct.

**Example 3**

**Preparation of tetranection-apolipoprotein A-I**

Inclusion body preparation was carried out by resuspension of harvested bacteria cells of example 2 in a potassium phosphate buffer solution or Tris buffer solution (0.1 M, supplemented with 1 mM MgSO₄, pH 6.5). After the addition of DNAse the cell were disrupted by homogenization at a pressure of 900 bar. A buffer solution comprising 1.5 M NaCl and 60 mM EDTA was added to the homogenized cell suspension. After the adjustment of the pH value to 5.0 with 25 % (w/v) HCl the final inclusion body slurry was obtained after a further centrifugation step. The slurry was stored at -20 °C in single use, sterile plastic bags until further processing.

The inclusion body slurry (about 15 kg) was solubilized in a guanidinium hydrochloride solution (150 l, 6.7 M). After clarification of the solubilisate by depth filtration, the solution was applied to a Zn-chelate affinity chromatography material. The fusion polypeptide was purified by Zn-chelate chromatography material and cleaved by IgA protease. Thereafter the polypeptide was further purified with an anion exchange chromatography and a cation exchange chromatography step. These steps were performed in a urea containing solution (7 M), i.e. under denaturing conditions. These steps were used for the removal of polypeptide fragments, endotoxins, and further impurities. A diafiltration into 6.7 M guanidinium hydrochloride containing solution was carried out. The obtained final solution contains denatured tetranection-apolipoprotein A-I.
Example 4
Refolding and lipidation of tetraneclin-apolipoprotein A-I

In the following the tetraneclin-apolipoprotein A-I as produced in the previous examples 1 to 3 of SEQ ID NO: 01 was used.

a) General method

Pure crystalline POPC or DPPC (Lipoid, Switzerland) have been dissolved in an aqueous buffer (lipidation buffer) containing cholate in a molar ratio phospholipid:cholate of 1:1.35. The mixtures have been incubated under nitrogen atmosphere and protected from light at room temperature (POPC) or at 55 °C (DPPC) until a clear solution has been obtained. The clear lipid-cholate solution is cooled to 4 °C (POPC) or stored at 41 °C (DPPC). Purified tetraneclin-apolipoprotein A-I has been added at 4 °C (POPC) or 41 °C (DPPC) at a defined apolipoprotein:phospholipid ratio. For lipid particle formation the reaction mixture was incubated over night at 4 °C (POPC) or 41 °C (DPPC) under nitrogen atmosphere and protected from light. Finally, cholate was removed by extensive dialysis (4 °C/41 °C) against lipidation buffer. Finally samples were centrifuged to remove precipitated material.

Cholate solubilized lipid solutions containing pure POPC or pure DPPC have been prepared as described above. Lipid mixtures were prepared by combining the lipid solutions at the desired ratio followed by storage at the respective T_m (T_m = phase transition temperature). Lipid particle formation of tetraneclin-apolipoprotein A-I was performed as described for pure lipid solutions but at the respective T_m of the lipid mixture chosen.

The following lipidation buffers have been tested:

1. 50 mM potassium phosphate buffer supplemented with 250 mM arginine hydrochloride, 7.5 % sucrose at pH 7.5
2. 50 mM dipotassium hydrogen phosphate buffer supplemented with 250 mM arginine hydrochloride, 7.5 % sucrose, 10 mM methionine at pH 7.5
3. 250 mM tris-hydroxylamino methane (TRIS) supplemented with 140 mM NaCl, 10 mM methionine at pH 7.5
4. 50 mM dipotassium hydrogen phosphate buffer supplemented with 250 mM arginine hydrochloride, 7 % trehalose, 10 mM methionine at pH 7.5.
The homogeneity of the lipid particles formed from tetranection-apolipoprotein A-I samples has been assessed by analytical SEC (Figures 11 and 12). Overall, the choice of the lipidation buffer has only a minor effect compared to the choice of phospholipid. DPPC-lipid particles elute as one main peak, whereas POPC-lipid particles shows a two peak pattern. The choice of lipidation buffer was influenced by the purification process of the apolipoprotein and the supply of stabilized lipid-free apolipoprotein. Lipid particle formation was shown to be feasible irrespective of the lipidation buffer. Among various buffers tested the most appropriate lipidation buffer was identified to be 250 mM Tris, 140 mM NaCl, 10 mM methionine, pH 7.5.

Lipidation mixtures contained a defined amount of apolipoprotein each and the amount of phospholipid, e.g., POPC, was calculated accordingly. All calculations of the molar amount of lipid were based on the tetranection-apolipoprotein A-I monomer.

b) POPC and cholate

Table 5: Lipid particle formation with tetranection-apolipoprotein A-I as example using pure POPC. Molar ratios apolipoprotein:phospholipid are calculated for the protein monomer. Controls: apolipoprotein incubated without addition of lipid (pure Apo) and lipid without apolipoprotein (no Apo).

<table>
<thead>
<tr>
<th>molar ratio apolipoprotein:phospholipid</th>
<th>observation after overnight incubation</th>
<th>protein conc. before dialysis [mg/ml]</th>
<th>protein conc. after dialysis [mg/ml]</th>
<th>observation after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:320</td>
<td>clear</td>
<td>0.67</td>
<td>n.d.</td>
<td>turbid</td>
</tr>
<tr>
<td>1:160</td>
<td>clear</td>
<td>1.34</td>
<td>1.47</td>
<td>clear</td>
</tr>
<tr>
<td>1:80</td>
<td>clear</td>
<td>2.68</td>
<td>2.6</td>
<td>clear</td>
</tr>
<tr>
<td>1:40</td>
<td>clear</td>
<td>5.36</td>
<td>4.87</td>
<td>clear</td>
</tr>
<tr>
<td>1:20</td>
<td>turbid</td>
<td>10.73</td>
<td>5.02</td>
<td>turbid*</td>
</tr>
<tr>
<td>only Apo</td>
<td>turbid</td>
<td>2.68</td>
<td>0.51</td>
<td>turbid*</td>
</tr>
<tr>
<td>no Apo</td>
<td>clear</td>
<td>-</td>
<td>-</td>
<td>clear</td>
</tr>
</tbody>
</table>

*clear after centrifugation
The molar ratios from 1:40 to 1:160 remain clear during the whole process. Neither turbidity through excess phospholipid nor protein precipitation was observed.

Lipid particle samples have been analyzed by native PAGE (see Figure 13). The most homogeneous band pattern was found with the sample 1:80 (lane 4). In addition 1 x freeze/thaw (-80 °C) did not alter appearance of the sample (lane 5). The band patterns of samples 1:320 and 1:160 indicate an inhomogeneous product resulting in multiple bands (lane 2 and 3). Samples 1:40 and also 1:20 have additional bands below the main product band (lane 6 and 7). The migration pattern of pure tetraneclin-apolipoprotein A-I is shown in lane 8 of Figure 13.

SEC-MALLS analysis was used to gain more detailed information on the homogeneity of the lipid particles and their apolipoprotein-phospholipid composition (protein-conjugate analysis). Figure 14 shows the chromatogram of SEC resolved samples (UV/280 detection). Here the 1:160 sample is divided into three separated peaks. The 1:80 sample appeared to contain at least two species of different size as displayed as double peak. The peak obtained from sample 1:20 shows the most homogeneous product.

The experiment was carried out using tetraneclin-apolipoprotein A-I (3.84 mg/ml; 10 mg per sample) and the molar ratio apolipoprotein:phospholipid was increased from 1:40 to 1:80 in steps of 5. At molar ratios below 1:40 the lipid particle formation is incomplete. Molar ratios above 1:80 are excluded experimentally: after removal of cholate by dialysis the samples became turbid. Moreover the lipid particles became more inhomogeneous at higher lipid ratios.

**Table 6:** Lipid particle formation of tetraneclin-apolipoprotein A-I using pure POPC. Molar ratio apolipoprotein:phospholipid has been calculated based on the tetraneclin-apolipoprotein A-I monomer.

<table>
<thead>
<tr>
<th>molar ratio apolipoprotein:phospholipid</th>
<th>protein conc. before dialysis [mg/ml]*</th>
<th>protein conc. after dialysis [mg/ml]*</th>
<th>yield [%]</th>
<th>observation after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:40</td>
<td>3.5</td>
<td>2.67</td>
<td>76</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:45</td>
<td>3.5</td>
<td>2.74</td>
<td>78</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:50</td>
<td>3.5</td>
<td>2.94</td>
<td>84</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:55</td>
<td>3.5</td>
<td>3.05</td>
<td>87</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:60</td>
<td>3.5</td>
<td>3.19</td>
<td>91</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:65</td>
<td>3.5</td>
<td>3.34</td>
<td>95</td>
<td>precipitation</td>
</tr>
<tr>
<td>1:70</td>
<td>3.5</td>
<td>3.52</td>
<td>100**</td>
<td></td>
</tr>
</tbody>
</table>
During incubation at the transition temperature of -3 °C all samples remained optically clear. After removal of cholate by dialysis increasing turbidity of the samples 1:40 to 1:65 was observed. Precipitate could be removed by centrifugation and the samples remained clear afterwards.

SEC-MALLS analysis was used to gain detailed information on the homogeneity of the formed lipid particles and their apolipoprotein-phospholipid composition (protein-conjugate analysis). All lipid particles were comparably homogeneous on analytical size exclusion chromatography (SEC; Figure 15) displaying a minor post peak which is more pronounced at lower molar ratios. In addition, there is a noticeable shift in the peak pattern at higher molar ratios towards higher molecular weights. The respective retention times are given in Table 7.

Table 7: Summary of size exclusion chromatography results; percentages were calculated by integration of the area under the curve (AUC).

<table>
<thead>
<tr>
<th>UV280</th>
<th>retention time main peak [min.]</th>
<th>main peak [%]</th>
<th>post peak [%]</th>
<th>total area [mAU*min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC 1:40</td>
<td>56.2</td>
<td>89.3</td>
<td>10.7</td>
<td>322.3</td>
</tr>
<tr>
<td>POPC 1:45</td>
<td>55.9</td>
<td>89.7</td>
<td>10.4</td>
<td>331.3</td>
</tr>
<tr>
<td>POPC 1:50</td>
<td>55.8</td>
<td>90.0</td>
<td>10.0</td>
<td>333.2</td>
</tr>
<tr>
<td>POPC 1:55</td>
<td>55.7</td>
<td>91.0</td>
<td>9.1</td>
<td>342.5</td>
</tr>
<tr>
<td>POPC 1:60</td>
<td>55.6</td>
<td>90.8</td>
<td>9.2</td>
<td>331.7</td>
</tr>
<tr>
<td>POPC 1:65</td>
<td>55.3</td>
<td>90.9</td>
<td>9.2</td>
<td>337.2</td>
</tr>
<tr>
<td>POPC 1:70</td>
<td>55.2</td>
<td>91.1</td>
<td>8.9</td>
<td>326.5</td>
</tr>
<tr>
<td>POPC 1:75</td>
<td>55.1</td>
<td>91.3</td>
<td>8.7</td>
<td>347.1</td>
</tr>
<tr>
<td>POPC 1:80</td>
<td>54.8</td>
<td>92.0</td>
<td>8.0</td>
<td>347.8</td>
</tr>
</tbody>
</table>

The protein-conjugate analysis (summarized in Table 8) enables the calculation of the total molecular weight of the protein (MW protein) and the lipid component (MW lipid) for each lipid particle eluted from the SEC column. Based on the
molecular weights of tetranection-apolipoprotein A-I monomer (32.7 kDa) and POPC (760 Da) the composition of the lipid particle can be calculated (n protein and n POPC). The molecular weight of the apolipoprotein component found in the lipid particle main peak at all molar ratios was approximately 100 kDa corresponding to a tetranection-apolipoprotein A-I trimer per lipid particle. The ratio n(POPC)/n(protein monomer) gives the number of POPC molecules per tetranection-apolipoprotein A-I monomer in the lipid particle. The number of POPC molecules per tetranection-apolipoprotein A-I monomer varies between 54 and 75 though molar ratios from 1:40 up to 1:80 have been applied. The value % protein is a parameter for the degree of lipidation. The lower the percentage of the protein in the lipid particle, the higher the degree of lipidation.

Table 8: Summary of protein conjugate analysis of lipid particles of POPC and tetranection-apolipoprotein A-I as shown in Figure 16.

<table>
<thead>
<tr>
<th></th>
<th>MW total [kDa]</th>
<th>MW Protein [kDa]</th>
<th>n (monomer)</th>
<th>MW lipid [kDa]</th>
<th>n (POPC)</th>
<th>n(POPC)/n(Protein)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:40</td>
<td>Main peak</td>
<td>238</td>
<td>104</td>
<td>3.3</td>
<td>135</td>
<td>178</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>230</td>
<td>148</td>
<td>4.6</td>
<td>81</td>
<td>107</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>238</td>
<td>101</td>
<td>3.2</td>
<td>138</td>
<td>182</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>184</td>
<td>118</td>
<td>3.7</td>
<td>66</td>
<td>87</td>
<td>24</td>
</tr>
<tr>
<td>1:50</td>
<td>Main peak</td>
<td>244</td>
<td>100</td>
<td>3.1</td>
<td>143</td>
<td>188</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>187</td>
<td>118</td>
<td>3.7</td>
<td>70</td>
<td>92</td>
<td>25</td>
</tr>
<tr>
<td>1:55</td>
<td>Main peak</td>
<td>247</td>
<td>99</td>
<td>3.1</td>
<td>148</td>
<td>195</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>182</td>
<td>107</td>
<td>3.3</td>
<td>75</td>
<td>99</td>
<td>30</td>
</tr>
<tr>
<td>1:60</td>
<td>Main peak</td>
<td>248</td>
<td>98</td>
<td>3.1</td>
<td>150</td>
<td>197</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>183</td>
<td>106</td>
<td>3.3</td>
<td>76</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>1:65</td>
<td>Main peak</td>
<td>255</td>
<td>97</td>
<td>3.0</td>
<td>158</td>
<td>208</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>191</td>
<td>103</td>
<td>3.2</td>
<td>88</td>
<td>116</td>
<td>36</td>
</tr>
<tr>
<td>1:70</td>
<td>Main peak</td>
<td>260</td>
<td>97</td>
<td>3.0</td>
<td>163</td>
<td>214</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>196</td>
<td>100</td>
<td>3.1</td>
<td>95</td>
<td>125</td>
<td>40</td>
</tr>
<tr>
<td>1:75</td>
<td>Main peak</td>
<td>266</td>
<td>99</td>
<td>3.1</td>
<td>168</td>
<td>221</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>208</td>
<td>118</td>
<td>3.7</td>
<td>91</td>
<td>120</td>
<td>32</td>
</tr>
<tr>
<td>1:80</td>
<td>Main peak</td>
<td>275</td>
<td>99</td>
<td>3.1</td>
<td>176</td>
<td>232</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>215</td>
<td>112</td>
<td>3.5</td>
<td>103</td>
<td>136</td>
<td>39</td>
</tr>
</tbody>
</table>
c) DPPC and cholate

Prior to lipidation the tetractin-apolipoprotein A-I was dialyzed against 50 mM KH₂PO₄, 250 mM arginine hydrochloride, 7% trehalose, 10 mM methionine at pH 7.5. Tetractin-apolipoprotein A-I (3.84 mg/ml, 3 mg per sample) has been lipidated using molar ratios from 1:60 to 1:100 increasing lipid concentrations in steps of 5. The lipidation buffer was 250 mM Tris-HCl, 140 mM NaCl, 10 mM methionine, pH 7.5.

Table 9: Sample overview of lipid particles of apolipoprotein with DPPC.

<table>
<thead>
<tr>
<th>molar ratio apolipoprotein: phospholipid*</th>
<th>observation after o/n incubation</th>
<th>yield based on protein [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>clear</td>
<td>85</td>
</tr>
<tr>
<td>1:40</td>
<td>clear</td>
<td>88</td>
</tr>
<tr>
<td>1:60</td>
<td>clear</td>
<td>89</td>
</tr>
<tr>
<td>1:80</td>
<td>clear</td>
<td>91</td>
</tr>
<tr>
<td>1:100</td>
<td>clear</td>
<td>94</td>
</tr>
<tr>
<td>only Apo</td>
<td>clear</td>
<td>86</td>
</tr>
<tr>
<td>no Apo</td>
<td>clear</td>
<td>DPPC precipitated</td>
</tr>
</tbody>
</table>

* calculated for protein monomer

During lipid particle formation neither precipitation of protein nor turbidity through excess lipid was observed. The yield of tetractin-apolipoprotein A-I in the final product was higher the more DPPC was used for lipidation.

Residual lipid-free apolipoprotein was found in the 1:20 sample on native PAGE (lane 3, Figure 17). The 1:40 and 1:60 sample look most homogeneous (lanes 4 and 5) on native PAGE whereas the 1:80 and 1:100 samples contain additional higher molecular bands above the main lipid particle band (lanes 6 and 7).

SEC-MALLS protein conjugate analysis was used to characterize the composition of the lipid particles obtained after DPPC lipid particle formation (MW DPPC: 734 Da). Homogeneous SEC peaks were obtained at molar ratios of 1:80 and below. At higher lipid ratios a pre-peak emerged (see e.g. 1:90 sample in Table 10).
Table 10: Summary SEC-MALLS protein conjugate analysis of lipid particles of DPPC and tetranectin-apolipoprotein A-I.

<table>
<thead>
<tr>
<th>molar ratio</th>
<th>peak</th>
<th>MW total [kDa]</th>
<th>MW protein [kDa]</th>
<th>n (protein)</th>
<th>MW lipid [kDa]</th>
<th>n (DPPC)</th>
<th>n (protein)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:60</td>
<td>1</td>
<td>724</td>
<td>298</td>
<td>9.0</td>
<td>425</td>
<td>193</td>
<td>41.2</td>
<td></td>
</tr>
<tr>
<td>1:65</td>
<td>1</td>
<td>281</td>
<td>109</td>
<td>3.3</td>
<td>171</td>
<td>77</td>
<td>38.9</td>
<td></td>
</tr>
<tr>
<td>1:70</td>
<td>1</td>
<td>273</td>
<td>103</td>
<td>3.1</td>
<td>169</td>
<td>76</td>
<td>37.9</td>
<td></td>
</tr>
<tr>
<td>1:75</td>
<td>1</td>
<td>286</td>
<td>103</td>
<td>3.1</td>
<td>183</td>
<td>83</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td>1</td>
<td>295</td>
<td>100</td>
<td>3.0</td>
<td>194</td>
<td>88</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td>1:85</td>
<td>1</td>
<td>307</td>
<td>99</td>
<td>3.0</td>
<td>207</td>
<td>94</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>1:90</td>
<td>1</td>
<td>361</td>
<td>117</td>
<td>3.5</td>
<td>244</td>
<td>110</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>319</td>
<td>101</td>
<td>3.0</td>
<td>217</td>
<td>98</td>
<td>31.8</td>
<td></td>
</tr>
<tr>
<td>1:95</td>
<td>1</td>
<td>397</td>
<td>134</td>
<td>4.0</td>
<td>262</td>
<td>118</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>327</td>
<td>100</td>
<td>3.0</td>
<td>226</td>
<td>102</td>
<td>30.8</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>1</td>
<td>405</td>
<td>132</td>
<td>4.0</td>
<td>273</td>
<td>123</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>344</td>
<td>101</td>
<td>3.0</td>
<td>243</td>
<td>110</td>
<td>29.3</td>
<td></td>
</tr>
</tbody>
</table>

The highest degree of lipidation (lowest percentage of protein) is found with the 1:80 to 1:90 molar ratios. In addition DLS revealed most homogeneous particle formation at ratios 1:80 to 1:90 (> 98 %) at a particle size of 14-17 nm.

d) 75 % DPPC / 25 % POPC

The lipid particle formation was carried out accordingly as reported in items a) to c) of this example with the following parameters:

- Protein: tetranectin-apolipoprotein A-I at 3.84 mg/ml, 3 mg per sample
- Lipidation buffer: 250 mM Tris-HCl, 140 mM NaCl, 10 mM methionine pH 7.5
- Lipidation: at 34 °C
- Dialysis: at 4 °C
- Molar ratios tested: 1:60 to 1:100 with increasing the lipid in steps of 5

Lipid particle formation was straight forward and comparable to the process using pure lipids. All samples remained clear during the process and dialysis. The yield
of lipid particles was similar for all ratios tested (~85%). SEC-MALLS analysis showed that the molar ratio of 1:80 resulted in the most homogeneous lipid particles with 90.9% main peak, no pre-peak and 9.1% post-peak. Protein conjugate analysis revealed the presence of one tetranectin-apolipoprotein A-I trimer per lipid particle in the main species of all samples (see Figure 18 and Tables 11 and 12).

**Table 11:** Summary of SEC results; percentages were calculated by integration of the AUC.

<table>
<thead>
<tr>
<th>UV280</th>
<th>Retention time</th>
<th>Main peak</th>
<th>Pre peak [%]</th>
<th>Main peak [%]</th>
<th>Post peak [%]</th>
<th>total [mAU* min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>75/25 DPPC/POPC 1:60</td>
<td>58.3</td>
<td>-</td>
<td>89.7</td>
<td>10.3</td>
<td>360.5</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:65</td>
<td>58.3</td>
<td>-</td>
<td>89.2</td>
<td>10.8</td>
<td>383.7</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:70</td>
<td>58.3</td>
<td>-</td>
<td>89.5</td>
<td>10.5</td>
<td>376.8</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:75</td>
<td>58.4</td>
<td>-</td>
<td>90.3</td>
<td>9.7</td>
<td>367.0</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:80</td>
<td>58.3</td>
<td>-</td>
<td>90.9</td>
<td>9.1</td>
<td>383.5</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:85</td>
<td>58.2</td>
<td>10.4</td>
<td>79.5</td>
<td>10.1</td>
<td>356.4</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:90</td>
<td>58.3</td>
<td>10.2</td>
<td>81.5</td>
<td>8.3</td>
<td>344.6</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:95</td>
<td>58.0</td>
<td>16.9</td>
<td>74.9</td>
<td>8.2</td>
<td>377.4</td>
<td></td>
</tr>
<tr>
<td>75/25 DPPC/POPC 1:100</td>
<td>58.0</td>
<td>21.0</td>
<td>70.4</td>
<td>7.7</td>
<td>365.0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 12:** Summary protein-conjugate analysis of 75% DPPC/25% POPC and tetranectin-apolipoprotein A-I lipid particles.

<table>
<thead>
<tr>
<th></th>
<th>MW total</th>
<th>MW protein [kDa]</th>
<th>n (protein monomer)</th>
<th>MW lipid [kDa]</th>
<th>n (lipid)</th>
<th>n(lipid)/n(prototype)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:60</td>
<td>Main peak</td>
<td>257</td>
<td>96</td>
<td>3.0</td>
<td>161</td>
<td>217</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>92</td>
<td>75</td>
<td>2.3</td>
<td>17</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>1:65</td>
<td>Main peak</td>
<td>263</td>
<td>95</td>
<td>3.0</td>
<td>167</td>
<td>226</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>116</td>
<td>102</td>
<td>3.2</td>
<td>14</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>1:70</td>
<td>Main peak</td>
<td>268</td>
<td>95</td>
<td>3.0</td>
<td>173</td>
<td>234</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>93</td>
<td>83</td>
<td>2.6</td>
<td>10</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>1:75</td>
<td>Main peak</td>
<td>275</td>
<td>95</td>
<td>3.0</td>
<td>180</td>
<td>243</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>98</td>
<td>82</td>
<td>2.6</td>
<td>16</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>1:80</td>
<td>Main peak</td>
<td>279</td>
<td>95</td>
<td>3.0</td>
<td>184</td>
<td>248</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>97</td>
<td>86</td>
<td>2.7</td>
<td>11</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>MW total</td>
<td>MW protein [kDa]</td>
<td>n (protein monomer)</td>
<td>MW lipid [kDa]</td>
<td>n (lipid)</td>
<td>n(lipid)/n(monomer)</td>
<td>% protein</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>------------------</td>
<td>---------------------</td>
<td>-----------------</td>
<td>-----------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1:85</td>
<td>Pre peak</td>
<td>329</td>
<td>104</td>
<td>3.3</td>
<td>224</td>
<td>302</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>291</td>
<td>96</td>
<td>3.0</td>
<td>195</td>
<td>263</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>129</td>
<td>107</td>
<td>3.3</td>
<td>22</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td>1:90</td>
<td>Pre peak</td>
<td>443</td>
<td>107</td>
<td>3.3</td>
<td>237</td>
<td>320</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>293</td>
<td>95</td>
<td>3.0</td>
<td>197</td>
<td>266</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>126</td>
<td>102</td>
<td>3.2</td>
<td>25</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>1:95</td>
<td>Pre peak</td>
<td>384</td>
<td>110</td>
<td>3.4</td>
<td>274</td>
<td>370</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>303</td>
<td>96</td>
<td>3.0</td>
<td>207</td>
<td>280</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>130</td>
<td>103</td>
<td>3.2</td>
<td>27</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>1:100</td>
<td>Pre peak</td>
<td>398</td>
<td>111</td>
<td>3.5</td>
<td>287</td>
<td>388</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>310</td>
<td>96</td>
<td>3.0</td>
<td>213</td>
<td>288</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>122</td>
<td>86</td>
<td>2.7</td>
<td>36</td>
<td>49</td>
<td>18</td>
</tr>
</tbody>
</table>

**e) 50 % DPPC / 50 % POPC**

The lipid particle formation was carried out accordingly as reported in items a) to c) of this example with the following parameters:

5.
- **Protein:** tetranectin-lipoprotein A-I at 3.84 mg/ml, 3 mg per sample
- **Lipidation buffer:** 250 mM Tris-HCl, 140 mM NaCl, 10 mM methionine, pH 7.5
- **Lipidation:** at 27 °C
- **Dialysis:** at room temperature
- **Molar ratios tested:** 1:60 to 1:100 with increasing lipid in steps of 5

All samples remained clear during the process and dialysis. The yield of lipid particles was similar for all ratios tested.
Table 13: Summary of SEC results; percentages were calculated by integration of the AUC.

<table>
<thead>
<tr>
<th>UV280</th>
<th>Retention time</th>
<th>Main peak [min]</th>
<th>Pre peak [%]</th>
<th>Main peak [%]</th>
<th>Post peak [%]</th>
<th>total [mAU*min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/50 DPPC/POPC 1:60</td>
<td>58.2</td>
<td>-</td>
<td>88.9</td>
<td>11.1</td>
<td>341.3</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:65</td>
<td>58.3</td>
<td>-</td>
<td>89.3</td>
<td>10.7</td>
<td>349.6</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:70</td>
<td>58.3</td>
<td>-</td>
<td>89.9</td>
<td>10.1</td>
<td>336.9</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:75</td>
<td>58.2</td>
<td>6.1</td>
<td>84.3</td>
<td>9.6</td>
<td>347.4</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:80</td>
<td>58.1</td>
<td>8.5</td>
<td>82.2</td>
<td>9.3</td>
<td>356.9</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:85</td>
<td>58.0</td>
<td>11.3</td>
<td>79.8</td>
<td>8.9</td>
<td>352.7</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:90</td>
<td>58.0</td>
<td>14.4</td>
<td>77.1</td>
<td>8.5</td>
<td>356.5</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:95</td>
<td>58.0</td>
<td>19.3</td>
<td>72.6</td>
<td>8.1</td>
<td>367.0</td>
<td></td>
</tr>
<tr>
<td>50/50 DPPC/POPC 1:100</td>
<td>57.9</td>
<td>36.6</td>
<td>65.8</td>
<td>7.6</td>
<td>365.3</td>
<td></td>
</tr>
</tbody>
</table>

Using a lipid mixture of 50% DPPC and 50% POPC for lipid particle formation of tetranectin-apolipoprotein A-I the most homogeneous product was obtained at a molar ratio of 1:70 (see Table 14). The product was 89.9% pure with respect to the main peak and contained one single tetranectin-apolipoprotein A-I trimer (see Table 14).

Table 14: Summary protein conjugate analysis of lipid particles with 50% DPPC/50% POPC and tetranectin-apolipoprotein A-I.

<table>
<thead>
<tr>
<th></th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n (monomer)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:60 Main peak</td>
<td>331</td>
<td>124</td>
<td>3.9</td>
<td>207</td>
<td>277</td>
<td>71</td>
<td>38</td>
</tr>
<tr>
<td>Post peak</td>
<td>131</td>
<td>106</td>
<td>3.3</td>
<td>24</td>
<td>32</td>
<td>10</td>
<td>81</td>
</tr>
<tr>
<td>1:65 Main peak</td>
<td>264</td>
<td>95</td>
<td>2.9</td>
<td>169</td>
<td>226</td>
<td>78</td>
<td>36</td>
</tr>
<tr>
<td>Post peak</td>
<td>127</td>
<td>112</td>
<td>3.5</td>
<td>16</td>
<td>21</td>
<td>6</td>
<td>88</td>
</tr>
<tr>
<td>1:70 Main peak</td>
<td>273</td>
<td>96</td>
<td>3.0</td>
<td>178</td>
<td>238</td>
<td>79</td>
<td>35</td>
</tr>
<tr>
<td>Post peak</td>
<td>258</td>
<td>213</td>
<td>6.7</td>
<td>45</td>
<td>60</td>
<td>9</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>MW total</td>
<td>MW protein</td>
<td>n (protein monomer)</td>
<td>MW lipid</td>
<td>n(lipid)</td>
<td>n(lipid)/ n (monomer)</td>
<td>% protein</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>------------</td>
<td>---------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1:75</td>
<td>Pre peak</td>
<td>319</td>
<td>108</td>
<td>3.4</td>
<td>211</td>
<td>83</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>271</td>
<td>93</td>
<td>2.9</td>
<td>178</td>
<td>82</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>126</td>
<td>106</td>
<td>3.3</td>
<td>20</td>
<td>8</td>
<td>84</td>
</tr>
<tr>
<td>1:80</td>
<td>Pre peak</td>
<td>333</td>
<td>108</td>
<td>3.4</td>
<td>225</td>
<td>89</td>
<td>32</td>
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<tr>
<td></td>
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<td>278</td>
<td>95</td>
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<td>184</td>
<td>85</td>
<td>34</td>
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<tr>
<td></td>
<td>Post peak</td>
<td>122</td>
<td>100</td>
<td>3.1</td>
<td>21</td>
<td>9</td>
<td>83</td>
</tr>
<tr>
<td>1:85</td>
<td>Pre peak</td>
<td>359</td>
<td>109</td>
<td>3.4</td>
<td>250</td>
<td>98</td>
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</tr>
<tr>
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<td>284</td>
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<td>87</td>
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<td>Post peak</td>
<td>132</td>
<td>118</td>
<td>3.7</td>
<td>14</td>
<td>5</td>
<td>89</td>
</tr>
<tr>
<td>1:90</td>
<td>Pre peak</td>
<td>373</td>
<td>109</td>
<td>3.4</td>
<td>264</td>
<td>104</td>
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<td>286</td>
<td>94</td>
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<td>192</td>
<td>89</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>133</td>
<td>110</td>
<td>3.4</td>
<td>23</td>
<td>9</td>
<td>83</td>
</tr>
<tr>
<td>1:95</td>
<td>Pre peak</td>
<td>390</td>
<td>111</td>
<td>3.5</td>
<td>278</td>
<td>106</td>
<td>29</td>
</tr>
<tr>
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<td>33</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>162</td>
<td>136</td>
<td>4.3</td>
<td>26</td>
<td>8</td>
<td>84</td>
</tr>
<tr>
<td>1:100</td>
<td>Pre peak</td>
<td>404</td>
<td>113</td>
<td>3.5</td>
<td>291</td>
<td>111</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>293</td>
<td>94</td>
<td>2.9</td>
<td>199</td>
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<td>32</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>142</td>
<td>107</td>
<td>3.3</td>
<td>35</td>
<td>14</td>
<td>75</td>
</tr>
</tbody>
</table>

**f) 25 % DPPC / 75 % POPC**

The lipid particle formation was carried out accordingly as reported in items a) to c) of this example with the following parameters:

- **Protein:** tetranectin-apolipoprotein A-I at 3.84 mg/ml, 3 mg per sample
- **Lipidation buffer:** 250 mM Tris-HCl, 140 mM NaCl, 10 mM methionine, pH 7.5
- **Lipidation:** at 18 °C
- **Dialysis:** at room temperature
- **Molar ratios tested:** 1:60 to 1:100 with increasing lipid in steps of 5

Lipid particle formation was straightforward and comparable to the process using pure lipids. All samples remained clear during the process and dialysis.
Table 15: Summary of SEC results; percentages were calculated by integration of the AUC.

<table>
<thead>
<tr>
<th>UV280</th>
<th>Retention time</th>
<th>Main peak</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
<th>Total [nA/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25/75 DPPC/POPC</td>
<td>58.2</td>
<td>-</td>
<td>90.2</td>
<td>9.8</td>
<td>342.6</td>
<td></td>
</tr>
<tr>
<td>1:60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>58.2</td>
<td>4.6</td>
<td>85.9</td>
<td>9.4</td>
<td>345.6</td>
<td></td>
</tr>
<tr>
<td>1:65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>58.1</td>
<td>8.8</td>
<td>82.3</td>
<td>8.9</td>
<td>353.2</td>
<td></td>
</tr>
<tr>
<td>1:70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>58.0</td>
<td>9.0</td>
<td>82.4</td>
<td>8.6</td>
<td>357.5</td>
<td></td>
</tr>
<tr>
<td>1:75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>57.9</td>
<td>10.8</td>
<td>81.2</td>
<td>8.0</td>
<td>356.7</td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>57.9</td>
<td>21.2</td>
<td>71.0</td>
<td>7.8</td>
<td>366.3</td>
<td></td>
</tr>
<tr>
<td>1:85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>57.8</td>
<td>26.1</td>
<td>66.4</td>
<td>7.5</td>
<td>357.8</td>
<td></td>
</tr>
<tr>
<td>1:90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>57.7</td>
<td>32.7</td>
<td>60.5</td>
<td>6.8</td>
<td>365.9</td>
<td></td>
</tr>
<tr>
<td>1:95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/75 DPPC/POPC</td>
<td>57.6</td>
<td>36.1</td>
<td>57.5</td>
<td>6.4</td>
<td>373.4</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Using a lipid mixture of 25 % DPPC and 75 % POPC for lipid particle formation of tetranectin-apolipoprotein A-I the most homogeneous product was obtained at a molar ratio of 1:60 (see Table 16). The product was 90.2 % pure with respect to the main peak and contained one single tetranectin-apolipoprotein A-I trimer (see Table 15).

Table 16: Summary protein conjugate analysis of lipid particles of 25 % DPPC/75 % POPC and tetranectin-apolipoprotein A-I.

<table>
<thead>
<tr>
<th></th>
<th>MW total</th>
<th>MW protein</th>
<th>n{protein monomer}</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n{protein monomer}</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:60</td>
<td>Main peak</td>
<td>254</td>
<td>100</td>
<td>3.1</td>
<td>153</td>
<td>203</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>127</td>
<td>110</td>
<td>3.4</td>
<td>17</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>1:65</td>
<td>Pre peak</td>
<td>272</td>
<td>132</td>
<td>4.1</td>
<td>141</td>
<td>187</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>259</td>
<td>100</td>
<td>3.1</td>
<td>159</td>
<td>211</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>183</td>
<td>131</td>
<td>4.1</td>
<td>7</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>1:70</td>
<td>Pre peak</td>
<td>280</td>
<td>121</td>
<td>3.8</td>
<td>159</td>
<td>211</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>264</td>
<td>99</td>
<td>3.1</td>
<td>165</td>
<td>219</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>119</td>
<td>105</td>
<td>3.3</td>
<td>14</td>
<td>19</td>
<td>6</td>
</tr>
</tbody>
</table>

5
<table>
<thead>
<tr>
<th>1:75</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW total</td>
<td>291</td>
<td>268</td>
<td>120</td>
</tr>
<tr>
<td>MW protein</td>
<td>109</td>
<td>98</td>
<td>101</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
<td>3.4</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td>MW lipid</td>
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<td>170</td>
<td>19</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>243</td>
<td>226</td>
<td>25</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>71</td>
<td>73</td>
<td>8</td>
</tr>
<tr>
<td>% protein</td>
<td>37</td>
<td>37</td>
<td>84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1:80</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW total</td>
<td>311</td>
<td>276</td>
<td>137</td>
</tr>
<tr>
<td>MW protein</td>
<td>114</td>
<td>96</td>
<td>127</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
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<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>MW lipid</td>
<td>197</td>
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<td>10</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>261</td>
<td>234</td>
<td>13</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>73</td>
<td>78</td>
<td>3</td>
</tr>
<tr>
<td>% protein</td>
<td>37</td>
<td>36</td>
<td>93</td>
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</table>

<table>
<thead>
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<th>1:85</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW total</td>
<td>331</td>
<td>278</td>
<td>139</td>
</tr>
<tr>
<td>MW protein</td>
<td>115</td>
<td>98</td>
<td>117</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
<td>3.6</td>
<td>3.1</td>
<td>3.7</td>
</tr>
<tr>
<td>MW lipid</td>
<td>216</td>
<td>180</td>
<td>22</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>287</td>
<td>239</td>
<td>29</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>80</td>
<td>77</td>
<td>8</td>
</tr>
<tr>
<td>% protein</td>
<td>35</td>
<td>35</td>
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<table>
<thead>
<tr>
<th>1:90</th>
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<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
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<td>285</td>
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</tr>
<tr>
<td>MW protein</td>
<td>113</td>
<td>98</td>
<td>110</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
<td>3.5</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>MW lipid</td>
<td>232</td>
<td>187</td>
<td>33</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>308</td>
<td>248</td>
<td>44</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>88</td>
<td>80</td>
<td>13</td>
</tr>
<tr>
<td>% protein</td>
<td>33</td>
<td>34</td>
<td>77</td>
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</table>

<table>
<thead>
<tr>
<th>1:95</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
<tr>
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<td>363</td>
<td>292</td>
<td>155</td>
</tr>
<tr>
<td>MW protein</td>
<td>115</td>
<td>97</td>
<td>122</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
<td>3.6</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MW lipid</td>
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<td>194</td>
<td>33</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>329</td>
<td>257</td>
<td>44</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>91</td>
<td>86</td>
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</tr>
<tr>
<td>% protein</td>
<td>32</td>
<td>33</td>
<td>79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1:100</th>
<th>Pre peak</th>
<th>Main peak</th>
<th>Post peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW total</td>
<td>377</td>
<td>298</td>
<td>160</td>
</tr>
<tr>
<td>MW protein</td>
<td>117</td>
<td>98</td>
<td>114</td>
</tr>
<tr>
<td>n(protein/monomer)</td>
<td>3.7</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>MW lipid</td>
<td>260</td>
<td>200</td>
<td>46</td>
</tr>
<tr>
<td>n(lipid)</td>
<td>345</td>
<td>265</td>
<td>61</td>
</tr>
<tr>
<td>n(lipid)/n(prot)(monomer)</td>
<td>93</td>
<td>86</td>
<td>17</td>
</tr>
<tr>
<td>% protein</td>
<td>31</td>
<td>33</td>
<td>71</td>
</tr>
</tbody>
</table>

g) Lipid particle formation using Zwittergent

The lipid particle formation was carried out accordingly as reported in items a) to c) of this example with the following parameters and the exception that cholate was replaced by the synthetic detergent Zwittergent:

- **Protein:** tetranectin-apolipoprotein A-I at 23.5 mg/ml
- **Buffer:** 50 mM Tris-HCl, 7.2 M guanidinium hydrochloride, 10 mM Methionine, pH 8
- **Lipidation buffer:** 250 mM Tris-HCl, 140 mM NaCl, pH 7.5

100 % POPC, molar ratio apolipoprotein:phospholipid = 1:60
Table 17: Sample overview of various approaches and observations / parameters of lipid particle formation.

<table>
<thead>
<tr>
<th>sample</th>
<th>detergent [%]</th>
<th>turbidity</th>
<th>volume after dialysis [ml]</th>
<th>c after dialysis [μg/ml]</th>
<th>mg TN-Apo A-I</th>
<th>yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dissolved lip</td>
<td>lipidation</td>
<td>after dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zwittergent 3-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 x CMC</td>
<td>0.8</td>
<td>+++</td>
<td>+++</td>
<td>2.1</td>
<td>2230.18</td>
<td>4.68</td>
</tr>
<tr>
<td>0.5 x CMC</td>
<td>4.2</td>
<td>++</td>
<td>++</td>
<td>2.9</td>
<td>1536.81</td>
<td>4.46</td>
</tr>
<tr>
<td>1 x CMC</td>
<td>8.4</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>1475.07</td>
<td>4.43</td>
</tr>
<tr>
<td>2 x CMC</td>
<td>16.7</td>
<td>-</td>
<td>-</td>
<td>4.3</td>
<td>1081.27</td>
<td>4.65</td>
</tr>
<tr>
<td>3 x CMC</td>
<td>25.1</td>
<td>-</td>
<td>-</td>
<td>5.5</td>
<td>839.85</td>
<td>4.62</td>
</tr>
<tr>
<td>Zwittergent 3-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 x CMC</td>
<td>0.1</td>
<td>+++</td>
<td>+++</td>
<td>2</td>
<td>2361.56</td>
<td>4.72</td>
</tr>
<tr>
<td>0.5 x CMC</td>
<td>0.6</td>
<td>+++</td>
<td>++</td>
<td>2</td>
<td>2221.38</td>
<td>4.44</td>
</tr>
<tr>
<td>1 x CMC</td>
<td>1.2</td>
<td>++</td>
<td>+</td>
<td>2.1</td>
<td>2267.16</td>
<td>4.76</td>
</tr>
<tr>
<td>2 x CMC</td>
<td>2.5</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>2082.18</td>
<td>4.79</td>
</tr>
<tr>
<td>5 x CMC</td>
<td>6.2</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>1941.61</td>
<td>4.85</td>
</tr>
<tr>
<td>10 x CMC</td>
<td>12.3</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>1073.92</td>
<td>4.30</td>
</tr>
<tr>
<td>Zwittergent 3-12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 x CMC</td>
<td>0.01</td>
<td>+++</td>
<td>+++</td>
<td>2</td>
<td>2722.85</td>
<td>5.45</td>
</tr>
<tr>
<td>1 x CMC</td>
<td>0.1</td>
<td>+++</td>
<td>+++</td>
<td>2</td>
<td>2158.81</td>
<td>4.32</td>
</tr>
<tr>
<td>2 x CMC</td>
<td>0.2</td>
<td>+++</td>
<td>++</td>
<td>2</td>
<td>2636</td>
<td>5.27</td>
</tr>
<tr>
<td>20 x CMC</td>
<td>1.9</td>
<td>+</td>
<td>+</td>
<td>2.1</td>
<td>2525.69</td>
<td>5.30</td>
</tr>
<tr>
<td>100 x CMC</td>
<td>9.4</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>1567.85</td>
<td>5.49</td>
</tr>
<tr>
<td>300 x CMC</td>
<td>28.1</td>
<td>-</td>
<td>-</td>
<td>5.6</td>
<td>1069.04</td>
<td>5.99</td>
</tr>
</tbody>
</table>
Lipid particles comprising tetranectin-apolipoprotein A-I have been analyzed on native PAGE. Lipid-free tetranectin-apolipoprotein A-I migrates at 140 kDa (lanes 1 in Figure 19), whereas lipid particles show a characteristic shift to a higher molecular weight between 232 kDa and 440 kDa.

Lipid-free tetranectin-apolipoprotein A-I but no lipid particles were detected in all samples prepared with only 0.1 x CMC of the respective detergent (Figure 19, lanes 2, 8, 13, and 19). However, a detergent concentration of 0.5 x CMC was sufficient for Zwittergent 3-8 and 3-10 to enable the lipid particle formation with tetranectin-apolipoprotein A-I (lanes 3, 9, and 14). With Zwittergent 3-12 lipid particle formation did not occur until a concentration of 2.0 x CMC was reached (lane 21).

Figure 20 shows the SEC-MALLS chromatogram of lipid particles comprising tetranectin-apolipoprotein A-I using 3x CMC Zwittergent 3-8 and POPC (molar ratio apolipoprotein:phospholipid = 1:60). Results of the protein conjugate analysis are summarized in Table 18. The lipid particle fraction consists of two different species as displayed in two overlapping peaks in the SEC chromatogram. However, these two species are very similar, differentiating mainly in the number of
tetranectin-apolipoprotein A-I molecules per particle (4.2 for peak 1 and 3.5 for peak 2).

Table 18: Summary of protein-conjugate analysis of lipid particles formed in the presence of Zwittergent 3-8.

<table>
<thead>
<tr>
<th>x CMC</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n (monomer)</th>
<th>% protein</th>
<th>Rh (w) (QELS) [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Pre peak</td>
<td>345</td>
<td>147</td>
<td>4.6</td>
<td>198</td>
<td>261.5</td>
<td>57</td>
<td>42.5</td>
<td>7.7</td>
</tr>
<tr>
<td>2 Main peak</td>
<td>268</td>
<td>113</td>
<td>3.6</td>
<td>154</td>
<td>203.2</td>
<td>56</td>
<td>42.4</td>
<td>6.5</td>
</tr>
<tr>
<td>3 Pre peak</td>
<td>323</td>
<td>134</td>
<td>4.2</td>
<td>188</td>
<td>249.9</td>
<td>60</td>
<td>41.6</td>
<td>7.4</td>
</tr>
<tr>
<td>3 Main peak</td>
<td>257</td>
<td>110</td>
<td>3.5</td>
<td>146</td>
<td>192.9</td>
<td>55</td>
<td>43.0</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Figure 21 shows the chromatogram of SEC-MALLS analysis and Table 19 the summary of the protein conjugate analysis for lipid particles comprising tetranectin-apolipoprotein A-I using 2 x CMC Zwittergent 3-10 and POPC (molar ratio apolipoprotein:phospholipid = 1:60). Both peaks contain lipid particles comprising 3.5 and 5 tetranectin-apolipoprotein A-I molecules, respectively.

Table 19: Summary of protein-conjugate analysis of lipid particles formed in the presence of Zwittergent 3-10.

<table>
<thead>
<tr>
<th>x CMC</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n (monomer)</th>
<th>% protein</th>
<th>Rh (w) (QELS) [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Pre peak</td>
<td>373</td>
<td>161</td>
<td>5.0</td>
<td>211</td>
<td>278.7</td>
<td>56</td>
<td>43.2</td>
<td>7.8</td>
</tr>
<tr>
<td>2 Main peak</td>
<td>272</td>
<td>112</td>
<td>3.5</td>
<td>159</td>
<td>210.3</td>
<td>60</td>
<td>41.4</td>
<td>6.6</td>
</tr>
<tr>
<td>5 Pre peak</td>
<td>345</td>
<td>150</td>
<td>4.7</td>
<td>195</td>
<td>256.6</td>
<td>55</td>
<td>43.6</td>
<td>7.5</td>
</tr>
<tr>
<td>5 Main peak</td>
<td>263</td>
<td>112</td>
<td>3.5</td>
<td>151</td>
<td>199.1</td>
<td>57</td>
<td>42.6</td>
<td>6.6</td>
</tr>
<tr>
<td>10 Pre peak</td>
<td>405</td>
<td>151</td>
<td>4.7</td>
<td>253</td>
<td>334.1</td>
<td>71</td>
<td>37.4</td>
<td>7.9</td>
</tr>
<tr>
<td>10 Main peak</td>
<td>265</td>
<td>110</td>
<td>3.3</td>
<td>154</td>
<td>203.2</td>
<td>58</td>
<td>41.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>
The results of lipid particle formation comprising tetraneceptin-apolipoprotein A-I using Zwittergent 3-12 and POPC (molar ratio apolipoprotein:phospholipid = 1:60) are summarized in Table 20. The lipid particle fraction consists of two different species as displayed in two overlapping peaks in the SEC chromatogram. However, these two species are very similar, differentiating mainly in the number of tetraneceptin-apolipoprotein A-I molecules per particle.

**Table 20:** Summary of protein-conjugate analysis of lipid particles formed in the presence of Zwittergent 3-12.

<table>
<thead>
<tr>
<th>x CMC</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n (monomer)</th>
<th>% protein</th>
<th>Rh (w) (QELS) [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>Main peak</td>
<td>487</td>
<td>342</td>
<td>10.7</td>
<td>145</td>
<td>191.3</td>
<td>18</td>
<td>70.2</td>
</tr>
<tr>
<td>300</td>
<td>Main peak</td>
<td>241</td>
<td>208</td>
<td>6.5</td>
<td>32</td>
<td>43.3</td>
<td>7</td>
<td>86.4</td>
</tr>
</tbody>
</table>

The results of lipid particle formation comprising tetraneceptin-apolipoprotein A-I using cholate and POPC (molar ratio apolipoprotein:phospholipid = 1:60) are summarized in Table 21. The lipid particle fraction consists of two different species as displayed in two overlapping peaks in the SEC chromatogram. However, these two species are very similar, differentiating mainly in the number of tetraneceptin-apolipoprotein A-I molecules per particle.
Table 21: Summary of protein-conjugate analysis of lipid particles formed in
the presence of cholate.

<table>
<thead>
<tr>
<th>CMC</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipid</th>
<th>n(lipid)</th>
<th>n(lipid)/n (monomer)</th>
<th>% protein</th>
<th>Rh (w) (QELS) [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Pre peak</td>
<td>1295</td>
<td>461</td>
<td>14.5</td>
<td>829</td>
<td>273</td>
<td>75</td>
<td>35.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>361</td>
<td>153</td>
<td>4.8</td>
<td>207</td>
<td>273</td>
<td>57</td>
<td>42.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>283</td>
<td>115</td>
<td>3.6</td>
<td>168</td>
<td>221</td>
<td>62</td>
<td>40.6</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>1050</td>
<td>414</td>
<td>12.9</td>
<td>623</td>
<td>836</td>
<td>65</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>337</td>
<td>154</td>
<td>4.8</td>
<td>182</td>
<td>240</td>
<td>50</td>
<td>45.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>284</td>
<td>121</td>
<td>3.8</td>
<td>162</td>
<td>214</td>
<td>56</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td>Post peak</td>
<td>332</td>
<td>143</td>
<td>4.5</td>
<td>188</td>
<td>248</td>
<td>55</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>269</td>
<td>111</td>
<td>3.5</td>
<td>158</td>
<td>209</td>
<td>60</td>
<td>41.2</td>
</tr>
<tr>
<td>1</td>
<td>Pre peak</td>
<td>314</td>
<td>143</td>
<td>4.5</td>
<td>171</td>
<td>225</td>
<td>50</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>278</td>
<td>118</td>
<td>3.7</td>
<td>158</td>
<td>208</td>
<td>56</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td>Main peak</td>
<td>292</td>
<td>135</td>
<td>4.2</td>
<td>156</td>
<td>206</td>
<td>50</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>271</td>
<td>115</td>
<td>3.6</td>
<td>155</td>
<td>204</td>
<td>57</td>
<td>42.6</td>
</tr>
</tbody>
</table>

Example 5

Rapid dilution method for refolding and lipid particle formation

In the following the tetranectin-apolipoprotein A-I as produced in the previous
examples 1 to 3 of SEQ ID NO: 01 was used.

a) POPC and sodium cholate

Tetranectin-apolipoprotein A-I was expressed in E. coli and purified according to
Examples 1 to 3 (protocol 1). After purification, the buffer was exchanged by
diafiltration to a solution containing 250 mM Tris, 140 mM NaCl, 6.7 M
guanidinium hydrochloride, pH 7.4. The protein concentration was adjusted to 28
mg/ml.

A lipid stock solution was prepared by dissolving 100 moles/l of POPC in a buffer
containing 250 mM Tris-HCl, 140 mM NaCl, 135 mM sodium cholate, pH 7.4 at
room temperature. The lipid stock solution was incubated for 2 hours at room
temperature. Refolding buffer was prepared by diluting 77 ml of the lipid stock mixture into 1478 ml of 250 mM Tris-HCl, 140 mM NaCl, pH 7.4. This buffer was stirred for an additional 7 hours at room temperature.

Refolding and lipid particle formation was initiated by the addition of 162 ml tetranection-apolipoprotein A-I in 250 mM Tris, 140 mM NaCl, 6.7 M guanidinium hydrochloride, pH 7.4 to refolding buffer. This results in a 1:10 dilution of the guanidinium hydrochloride. The solution was incubated at room temperature for 16 hours while constantly stirring. The removal of the detergent was carried out by diafiltration.

Table 22: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with POPC.

<table>
<thead>
<tr>
<th>Peak</th>
<th>MW total [kDa]</th>
<th>MW protein [kDa]</th>
<th>n (protein monomer)</th>
<th>MW lipid [kDa]</th>
<th>n (lipid)</th>
<th>n (lipid)/n (protein)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Peak</td>
<td>347</td>
<td>141</td>
<td>4.4</td>
<td>207</td>
<td>272</td>
<td>62</td>
<td>41</td>
</tr>
<tr>
<td>Main Peak</td>
<td>269</td>
<td>111</td>
<td>3.5</td>
<td>159</td>
<td>209</td>
<td>60</td>
<td>41</td>
</tr>
</tbody>
</table>

Tetranection-apolipoprotein A-I was expressed in E. coli and purified according to Examples 1 to 3 (protocol 2). After purification, the buffer was exchanged by diafiltration to a solution containing 50 mM Tris, 10 mM L-methionine, 6.7 M guanidinium hydrochloride, pH 7.4. The protein concentration was adjusted to 20.4 mg/ml.

A lipid stock solution was prepared by dissolving 100 moles/l of phospholipid (POPC:DPPC in a ratio 3:1) in a buffer containing 250 mM Tris-HCl, 140 mM NaCl, 10 mM L-methionine, 135 mM sodium cholate, pH 7.4 at room temperature.

Refolding buffer was prepared by diluting 3.7 ml of the lipid stock solution into 35.6 ml of 250 mM Tris-HCl, 140 mM NaCl, pH 7.4. This buffer was stirred for an additional 2 hours at room temperature.

Refolding and lipid particle formation was initiated by the addition of 9.8 ml tetranection-apolipoprotein A-I in 50 mM Tris, 10 mM L-methionine, 6.7 M
guanidinium hydrochloride, pH 8.0 to refolding buffer. This results in a 1:5 dilution of the guanidinium hydrochloride. The solution was incubated at room temperature over night while constantly stirring. The removal of the detergent was carried out by diafiltration.

Table 23: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with a POPC/DPPC/cholate mixture.

<table>
<thead>
<tr>
<th>Peak</th>
<th>MW total [kDa]</th>
<th>MW Protein [kDa]</th>
<th>n Protein (APO-Monomer)</th>
<th>MW Lipid [kDa]</th>
<th>n Lipid</th>
<th>n Lipid / n Protein</th>
<th>% Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Peak</td>
<td>419</td>
<td>167</td>
<td>5.2</td>
<td>251</td>
<td>333</td>
<td>64</td>
<td>41</td>
</tr>
<tr>
<td>Main Peak</td>
<td>252</td>
<td>101</td>
<td>3.2</td>
<td>151</td>
<td>200</td>
<td>63</td>
<td>41</td>
</tr>
</tbody>
</table>

b) POPC and DPPC and sodium cholate

Tetranectin-apolipoprotein A-I was expressed in E. coli and purified according to Examples 1 to 3. After purification, the buffer was exchanged by diafiltration into a solution containing 250 mM Tris, 140 mM NaCl, 6.7 M guanidinium hydrochloride, pH 7.4. The protein concentration was adjusted to 30 mg/ml.

Two separate lipid stock solutions were prepared. Solution A was prepared by dissolving 100 moles/l of POPC in a buffer containing 250 mM Tris-HCl, 140 mM NaCl, 135 mM sodium cholate, pH 7.4 at room temperature. Solution B was prepared by dissolving 100 moles/l of DPPC in 250 mM Tris-HCl, 140 mM NaCl, 135 mM sodium cholate, pH 7.4 at 41°C. Lipid stock solutions A and B were mixed in a ratio of 3:1 and incubated for 2 hours at room temperature. Refolding buffer was prepared by diluting 384 ml of the lipid stock mixture into 6365 ml of 250 mM Tris-HCl, 140 mM NaCl, pH 7.4. This buffer was stirred for an additional 24 hours at room temperature.

Refolding and lipid particle formation was initiated by the addition of 750 ml tetranectin-apolipoprotein A-I solution in 250 mM Tris, 140 mM NaCl, 6.7 M guanidinium hydrochloride, pH 7.4 to the refolding buffer. This results in a 1:10 dilution of the guanidinium hydrochloride. The solution was incubated at room
temperature for at least 12 hours while constantly stirring. Detergent removal was carried out by diafiltration.

**Table 24:** Summary protein conjugate analysis of lipid particle obtained by rapid dilution with POPC:DPPC = 1:1.

<table>
<thead>
<tr>
<th>Peak</th>
<th>MW total [kDa]</th>
<th>MW protein [kDa]</th>
<th>n (protein monomer)</th>
<th>MW lipid [kDa]</th>
<th>n (lipid)</th>
<th>n (lipid)/n (protein)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main peak</td>
<td>263</td>
<td>102</td>
<td>3.2</td>
<td>161</td>
<td>214</td>
<td>67</td>
<td>39</td>
</tr>
<tr>
<td>Post peak</td>
<td>182</td>
<td>85</td>
<td>2.7</td>
<td>97</td>
<td>129</td>
<td>48</td>
<td>47</td>
</tr>
</tbody>
</table>

c) Different guanidinium hydrochloride concentrations

Tetranectin-apolipoprotein A-I according to the invention was expressed in E. coli and purified over a metal chelate affinity chromatographic process from inclusion bodies (see Examples 1 to 3). After purification, the buffer was exchanged by diafiltration into a solution containing 250 mM Tris, 140 mM NaCl, 6.7 M guanidinium hydrochloride, pH 7.4. The protein concentration was adjusted to 28 mg/ml.

A lipid stock solution was prepared by dissolving 100 moles/l of POPC in a buffer containing 250 mM Tris-HCl, 140 mM NaCl, 135 mM sodium cholate, pH 7.4 at room temperature. The lipid stock solution was incubated for 2 hours at room temperature. Refolding buffer was prepared by diluting lipid stock solution into 250 mM Tris-HCl, 140 mM NaCl, pH 7.4. This buffer was stirred for an additional 12 hours at room temperature. Varying amounts of tetranectin-apolipoprotein A-I were diluted into refolding buffer: 1:5, 1:7.5, 1:10, 1:12.5. This results in different residual concentrations of guanidinium hydrochloride in the refolding buffer. The solution was allowed to stir at room temperature o/n to initiate refolding and lipid particle formation. Detergent removal was carried out by dialysis.
Table 25: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with different dilution ratios.

<table>
<thead>
<tr>
<th>dilution</th>
<th>Peak</th>
<th>MW total [kDa]</th>
<th>MW protein [kDa]</th>
<th>n (protein monomer)</th>
<th>MW lipid [kDa]</th>
<th>n (lipid)</th>
<th>n (lipid) / n (protein)</th>
<th>% protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>Main</td>
<td>273</td>
<td>103</td>
<td>3,2</td>
<td>170</td>
<td>226</td>
<td>70</td>
<td>38</td>
</tr>
<tr>
<td>1:7.5</td>
<td>Main</td>
<td>272</td>
<td>100</td>
<td>3,1</td>
<td>173</td>
<td>230</td>
<td>73</td>
<td>37</td>
</tr>
<tr>
<td>1:10</td>
<td>Main</td>
<td>266</td>
<td>106</td>
<td>3,3</td>
<td>160</td>
<td>212</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>1:12.5</td>
<td>Main</td>
<td>281</td>
<td>101</td>
<td>3,2</td>
<td>180</td>
<td>239</td>
<td>76</td>
<td>36</td>
</tr>
</tbody>
</table>

d) POPC and sodium cholate in the presence of urea

Tetranectin-apolipoprotein A-I is expressed in E. coli and purified according to Examples 1 to 3. After purification, the buffer is exchanged by diafiltration to a solution containing 250 mM Tris, 140 mM NaCl, 6.7 M urea, pH 7.4. The protein concentration is adjusted to 28 mg/ml.

A lipid stock solution is prepared by dissolving 100 moles/l of POPC in a buffer containing 250 mM Tris-HCl, 140 mM NaCl, 135 mM sodium cholate, pH 7.4 at room temperature. The lipid stock solution is incubated for 2 hours at room temperature. Refolding buffer is prepared by diluting 77 ml of the lipid stock mixture into 1478 ml of 250 mM Tris-HCl, 140 mM NaCl, pH 7.4. This buffer is stirred for an additional 7 hours at room temperature.

Refolding and lipid particle formation is initiated by the addition of 162 ml tetranectin-apolipoprotein A-I solution in 250 mM Tris, 140 mM NaCl, 6.7 M urea, pH 7.4 to refolding buffer. This results in a 1:10 dilution of the urea. The solution is incubated at room temperature for 16 hours while constantly stirring. The removal of the detergent is carried out by diafiltration.

e) POPC and sodium cholate and wild-type apolipoprotein A-I

In another exemplary second method human apolipoprotein A-I (wild-type apolipoprotein A-I) in 6.7 M guanidinium hydrochloride, 50 mM Tris, 10 mM methionine, at pH 8.0 was diluted 1:5 (v/v) into lipidation buffer resulting in a protein concentration of 0.6 mg/ml. The lipidation buffer was consisting of 7 mM
cholate, 4 mM POPC and 1.3 mM DPPC corresponding to a lipid to protein ratio of 240:1. SEC-MALLS was employed to analyze complex formation. Approximately two apolipoprotein molecules were found in a complex consisting of around 200 lipid molecules.

Table 26: Summary of protein conjugate analysis.

<table>
<thead>
<tr>
<th>Starting material</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipids</th>
<th>Number of lipids</th>
<th>Ratio lipid:protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>denatured</td>
<td>Mainpeak</td>
<td>235</td>
<td>71</td>
<td>163</td>
<td>216</td>
<td>1:97</td>
</tr>
</tbody>
</table>

Example 6
Lipid particle formation starting from denatured or native protein

In the following, the tetranectin-apolipoprotein A-I as produced in the previous examples 1 to 3 of SEQ ID NO: 01 was used.

The method as reported in Example 4 (first method) requires native apolipoprotein for lipid particle formation whereas the method reported in Example 5 (second method) starts with fully denatured apolipoprotein for lipid particle formation.

In an exemplary first method denatured tetranectin-apolipoprotein A-I in 6.7 M guanidinium hydrochloride, 50 mM Tris, 10 mM methionine, at pH 8.0 was extensively dialyzed against a buffer consisting of 250 mM Tris, 140 mM NaCl, 10 mM methionine, at pH 7.5 at a protein concentration of 3.46 mg/ml. A mixture of POPC and cholate was then added to yield a final concentration of 6 mM POPC and 8 mM cholate in the solution. This corresponds to a ratio of 60 molecules of POPC per molecule of tetranectin-apolipoprotein A-I monomer (60:1). The detergent was subsequently removed by diafiltration. Analysis of formed protein-lipid complexes was by SEC-MALLS. Using this method a heterogeneous product was formed wherein approximately 60% of the formed species comprised more than three tetranectin-apolipoprotein A-I monomers.

In an exemplary second method denatured tetranectin-apolipoprotein A-I in 6.7 M guanidinium hydrochloride, 50 mM Tris, 10 mM methionine, at pH 8.0 was directly diluted 1:10 (v/v) into lipidation buffer resulting in a protein concentration of 2.5 mg/ml. The lipidation buffer was consisting of 6 mM cholate and 4.5 mM
POPC corresponding to a lipid to protein ratio of 60:1. Using this method a homogenous product was formed comprising more than 90% of a single formed species wherein 60 molecules of lipid where bound per molecule of tetractein-apolipoprotein A-I (see Figure 22).

Table 27: Summary of protein conjugate analysis.

<table>
<thead>
<tr>
<th>Starting material</th>
<th>MW total</th>
<th>MW protein</th>
<th>n (protein monomer)</th>
<th>MW lipids</th>
<th>Number of lipids</th>
<th>Ratio lipid:protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>321</td>
<td>131</td>
<td>4.1</td>
<td>190</td>
<td>250</td>
<td>61</td>
</tr>
<tr>
<td>Prepeak (60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mainpeak (40%)</td>
<td>269</td>
<td>107</td>
<td>3.3</td>
<td>162</td>
<td>213</td>
<td>65</td>
</tr>
<tr>
<td>denatured</td>
<td>269</td>
<td>111</td>
<td>3.5</td>
<td>159</td>
<td>209</td>
<td>60</td>
</tr>
<tr>
<td>Mainpeak (&gt;90%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Example 7

Lipidation of Insulin-F with cholate- and Zwittergent-solubilized POPC/DPPC

The protein chosen for lipid particle formation is commercially available Insulin (Humalog®, Insulin Lispro, Lilly). The molecular weight of the protein is 5808 Da. To increase the detection limit for insulin in the lipid particle the protein has been labeled with NHS-fluorescein (6-[fluorescein-5(6)-carboxamido] hexanoic acid N-hydroxysuccinimide ester, Sigma Aldrich # 46940-5MG-F).

Zwittergent- and cholate-mediated lipidation of NHS-Fluorescein-labeled Insulin (Insulin-F) were carried out as reported in Example 4 using a 1:1 mixture of POPC and DPPC. A 0.5 mM lipid mixture was dissolved in either 1 x CMC cholate, 2 x CMC Zwittergent 3-8 or 5 x CMC Zwittergent 3-10 in PBS pH 7.4. Solubilization of the lipids was achieved at 45 °C for 1 h in an ultrasonic bath. Insulin-F was added to the solubilized lipid at a molar ratio protein:lipid of 1:2 (Zwittergent 3-8) or 1:1.2 (Zwittergent 3-10 and cholate). The lipidation mixtures were incubated for one hour at room temperature followed by extensive dialysis against PBS pH 7.4 to remove the detergent.

The formed lipid particles and control samples were analyzed on SE-HPLC using fluorescence detection (494 nm ext., 521 nm em.) and UV280 absorption. Three
different samples per lipidation approach were analyzed on SE-HPLC: Insulin-F dissolved in PBS, liposomes without Insulin F in PBS and lipid particles comprising Insulin-F. Non-lipidated Insulin-F elutes from the column at about 40 min. elution time and the peak is detected by fluorescence and UV280 detection. Lipidated Insulin-F samples elute from the column as two separate peaks detected by fluorescence and UV280. The late peak (peak maximum at approx. 40 min.) co-migrates with the Insulin-F control sample. The early peak at 15 min. elution time has a higher molecular weight than pure Insulin-F and consists of lipidated Insulin-F. Protein free lipid particles elute at 15 min. elution time.

Example 8
Application of apolipoprotein

a) Impact of DPPC and POPC on LCAT activity

Lipid particles comprising either palmitoyl oleoyl phosphatidylcholine (POPC) or dipalmitoyl phosphatidylcholine (DPPC) and either recombinant wild-type apolipoprotein A-I or tetranectin-apolipoprotein A-I were examined for their ability to support cholesterol esterification by LCAT.

Tritiated cholesterol (4 %; relative to the phosphatidylcholine content on a molar basis) was incorporated in the lipid particle by addition of an ethanolic cholesterol solution. The capacity of the resulting protein-lipid complex to support LCAT catalyzed cholesterol esterification was tested in presence of 0.2 µg/ml recombinant LCAT enzyme (ROAR biochemical) in 125 µl (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaN₃; pH 7.4; 2 mg/ml HuFAF Albumin; 4 mM Beta mercapto ethanol) for 1 hour at 37 °C. The reaction was stopped by addition of chloroform:methanol (2:1) and lipids were extracted. “Percent” esterification was calculated after cholesterol – cholesteryl ester separation by TLC and scintillation counting. As less than 20 % of the tracer was incorporated into the formed ester, the reaction rate could be considered constant under the experimental conditions. Data were fitted to the Michaelis Menten equation using XLfit software (IDBS). For a visualization of the results see Figure 3.

b) Impact of DPPC/POPC mixtures on LCAT activity

Lipid particles were prepared using cholate as detergent by mixing recombinant wild-type apolipoprotein A-I with ³H cholesterol, a DPPC/POPC mixture, and
cholate in 1:4.80:113 molar ratios. DPPC/POPC mixtures contained either 100% POPC; 75% POPC; 50% POPC; 25% POPC.

After cholate removal by dialysis, the capacity of the resulting protein-lipid complex to support LCAT catalyzed cholesterol esterification was tested. $^3$H cholesterol (4 %, relative to the phosphatidylcholine content on a molar basis) was incorporated in the lipid particle by addition of an ethanolic cholesterol solution. The capacity of the resulting protein-lipid complex to support LCAT catalyzed cholesterol esterification was tested in presence of 0.2 $\mu$g/ml recombinant LCAT enzyme (ROAR biochemical) in 125 $\mu$l (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM Na$_3$; pH 7.4; 2 mg/ml HuFAF Albumin; 4 mM beta mercaptoethanol) for 1 hour at 37 °C. The reaction was stopped by addition of chloroform:methanol (2:1) and lipids were extracted. “Percent” esterification was calculated after cholesterol – cholesteryl ester separation by TLC and scintillation counting. As less than 20 % of the tracer was incorporated into esters, the reaction rate could be considered as constant in the experimental conditions. Data were fitted to the Michaelis Menten equation using XLFit software (IDBS) and are shown in Figure 4.

Table 2a: Apparent kinetic parameters.

<table>
<thead>
<tr>
<th>substrate [% POPC]</th>
<th>$K_m$ [nM]</th>
<th>$V_{max}$ [n mole ester/h/U LCAT]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>4.6</td>
<td>1.6</td>
</tr>
<tr>
<td>75</td>
<td>0.4</td>
<td>1.9</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>25</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>0</td>
<td>6.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

c) Cholesterol efflux to THP-1 derived foam cells

Macrophage like human THP-1 cells, were obtained by exposing THP-1 monocytic leukemia cells to phorbol myristate acetate. Subsequently cells were loaded by further culture in the presence of acetylated LDL containing $^3$H Cholesterol tracer. These model foam cells were then exposed for 4h - 8h to cholesterol acceptor test compounds (see below).

Cell culture supernatants were harvested and cells lysed in 5 % NP40. Fractional efflux was calculated as the ratio of cholesterol radioactivity in the supernatant relative to the sum of the radioactivity in the cells plus supernatant. Efflux from
cell exposed to medium containing no acceptors was subtracted and efflux velocity calculated by linear fit. Efflux velocity was standardized using efflux from cells to 10 μg/ml wild-type apolipoprotein A-I as reference (relative efflux velocity). Relative efflux velocities obtained in two separate experiments were plotted as function of cholesterol acceptor concentration and data fitted to the Michaelis Menten equation.

Parallel experiments were performed using cells exposed to a RXR-LXR agonist that is known to upregulate ABCA-1 transporters, and bias cholesterol transport toward ABCA-1 mediated efflux.

Only a modest influence of the lipid mixture was observed in the tested series (Figure 5 and Table 27).

Table 28: Different samples.

<table>
<thead>
<tr>
<th>tetranectin-apolipoprotein A-I with</th>
<th>molar ratio apolipoprotein: phospholipid</th>
<th>preparation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % POPC/ 0 % DPPC</td>
<td>1:60</td>
<td>cholate</td>
</tr>
<tr>
<td>75 % POPC/ 25 % DPPC</td>
<td>1:60</td>
<td>cholate</td>
</tr>
<tr>
<td>50 % POPC/ 50 % DPPC</td>
<td>1:70</td>
<td>cholate</td>
</tr>
<tr>
<td>0 % POPC/ 100 % DPPC</td>
<td>1:80</td>
<td>cholate</td>
</tr>
<tr>
<td>-</td>
<td>not</td>
<td>not</td>
</tr>
</tbody>
</table>

RXR-LXR pretreatment of the foam cells strongly increased efflux to the non-lipidated material with a six-fold increase of the maximal velocity over non treated cells. Impact on lipid particles was much less, with a two-fold increase, reflecting lower contribution of the ABCA-1 transporter to the cholesterol efflux (Figure 6).

**d) In vivo study**

Five lipid particle variants were studied:

i) only POPC

ii) only DPPC

iii) POPC:DPPC 3:1
iv) POPC:DPPC 1:1

v) DPPC:SM 9:1

Rabbits were intravenous infused over 0.5 h at 80 mg/kg (n = 3 rabbits/test compound) followed by serial blood sampling over 96 h post infusion.

Analysis of apolipoprotein levels with an ELISA:
- drug levels
- data on plasma values of liver enzymes, cholesterol, cholesterol ester.

Plasma concentrations are very similar for all tested compositions showing little pronounced initial "distribution" phase followed by log-linear decline of concentrations (Figure 7, Table 3).

**Table 3:** Pharmacokinetic data.

<table>
<thead>
<tr>
<th>Tetranectin-apolipoprotein A-I with</th>
<th>C_L [ml/h/kg]</th>
<th>V_ss [ml/kg]</th>
<th>T_1/2 [h]</th>
<th>C_max [mg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % POPC/0 % DPPC</td>
<td>0.897 ± 0.216</td>
<td>45.0 ± 2.5</td>
<td>36.9 ± 8.2</td>
<td>2.40 ± 0.19</td>
</tr>
<tr>
<td>0 % POPC/100 % DPPC</td>
<td>0.922 ± 0.098</td>
<td>37.8 ± 4.9</td>
<td>30.2 ± 7.7</td>
<td>2.29 ± 0.19</td>
</tr>
<tr>
<td>75 % POPC/25 % DPPC</td>
<td>0.815 ± 0.064</td>
<td>37.8 ± 5.6</td>
<td>34.2 ± 4.5</td>
<td>2.65 ± 0.28</td>
</tr>
<tr>
<td>50 % POPC/50 % DPPC</td>
<td>0.850 ± 0.135</td>
<td>43.1 ± 5.9</td>
<td>38.6 ± 10.6</td>
<td>2.34 ± 0.31</td>
</tr>
<tr>
<td>90 % DPPC/10 % SM</td>
<td>1.28 ± 0.62</td>
<td>50.7 ± 8.7</td>
<td>31.3 ± 8.2</td>
<td>1.91 ± 0.33</td>
</tr>
</tbody>
</table>

The determined pharmacokinetic (PK) parameters were similar for all tested compounds. Also a low inter-individual variability has been found. The determined half-lives are close to 1.5 days, i.e. increased compared to wild-type apolipoprotein A-I. The volume of distribution is similar to plasma volume (ca. 40 ml/kg in rabbits).

**f) Cholesterol mobilization**

Cholesterol is mobilized and esterified in plasma. Plasma cholesteryl ester levels do continue to increase even after tetranectin-apolipoprotein A-I is already decreasing. When plasma tetranectin-apolipoprotein A-I levels have decreased to
0.5 mg/ml (about 50% of normal wild-type apolipoprotein A-I) increased cholesterol ester levels are still detectable (Figure 8).

g) Liver enzyme release

Lipid particles comprising tetranectin-apolipoprotein A-I containing POPC do not induce liver enzyme release (Figure 1). Similar to the rabbit, a single i.v. injection of the tetranectin-apolipoprotein A-I according to the current invention containing POPC or POPC/DPPC mixtures are safe in mice. The apolipoprotein composition containing DPPC:POPC at a molar ratio of 1:3 was comparable to POPC alone (Figure 9).

No significant hemolysis was observed until two hours post infusion in any of the five preparations. Hemolysis was determined photometrically as red color in plasma samples obtained at two hours after i.v. application of tetranectin-apolipoprotein A-I. 100% hemolysis of whole blood (generated by 0.44% Triton X-100-final concentration) was used for calibration (Figure 10).

h) Anti-inflammatory effects of tetranectin-apolipoprotein A-I on human umbilical vein endothelial cells

Passage 5-10 HUVECs (human umbilical vein endothelial cells) were incubated in the respective tetranectin-apolipoprotein A-I preparations for 16h and stimulated with TNFα for the final 4 hours. VCAM1 surface expression was detected with specific antibodies by FACS.

Example 9

Lipid particle stability

Wild-type Apolipoprotein A-I containing an N-terminal histidine-tag and an IgA protease cleavage site was expressed in E. coli and purified by column chromatography as reported in the examples above. The histidine-tag was removed by IgA protease cleavage. Lipid particles (HDL particles) were assembled using a 1:150 ratio of protein to Loid S100 soybean phospholipid mixture. The particles were stored in a buffer containing 5 mM sodium phosphate and 1 % sucrose at pH value of 7.3. SE-HPLC revealed three distinct peaks upon incubation after lipidation and incubation for 10 days. After incubation at 40 °C, a predominant peak at retention time 10.8 minutes can be detected (47 % of total protein), which is absent in the sample stored at 5 °C. The 10.8 minutes peak indicates the
formation of soluble large molecular weight assemblies due to protein destabilization.

HDL particles containing tetranectin-apolipoprotein A-I as reported herein which were obtained starting from a POPC:DPPC mixture (ratio POPC to DPPC of 3:1) were also incubated at 5 °C and 40 °C. Incubation at elevated temperature lead to a slight degree of pre-peak formation, but no significant shift to high molecular weight assemblies at 10.8 minutes (< 2 % increase at 11 minutes). This indicates improved HDL particle stability compared to the particle containing wild-type apolipoprotein A-I.

**Example 10**

**Cholesterol mobilization**

The efficiency at which cholesterol is mobilized into the blood can be determined by comparing the respective excursion of total cholesterol with apolipoprotein concentrations after administration of apolipoprotein in vivo. For a quantitative assessment, the quotient of the baseline corrected area under the concentration–time curve (AUC) of total cholesterol and the area under the concentration–time curve of apolipoprotein was calculated.

In this experiment the following substances were analyzed:

- wild-type apolipoprotein A-I containing an N-terminal histidine-tag and an IgA protease cleavage site expressed in E. coli and purified by column chromatography as reported in the examples above; the histidine-tag was removed by IgA protease cleavage; lipid particles (HDL particles) were assembled using a 1:150 ratio of protein to Lipoid S100 soybean phospholipid mixture,

- apolipoprotein A-I Milano variant; lipid particles (HDL particles) were assembled using a 1:40 ratio of protein to POPC,

- tetranectin-apolipoprotein A-I as reported herein; lipid particles (HDL particles) were assembled using a 1:60 ratio of protein to POPC and DPPC (POPC and DPPC at a ratio of 3:1).

The three HDL particles were applied to rats. The values obtained for the respective AUC ratios are shown in Table 29.
Table 29: Cholesterol mobilization.

<table>
<thead>
<tr>
<th></th>
<th>lipids</th>
<th>AUC (time dependent concentration cholesterol in blood)</th>
<th>AUC (time dependent apolipoprotein A-I concentration in blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-apolipoprotein A-I</td>
<td>soybean phospholipid mixture</td>
<td>0.0002 (mmol/l)/(µg/ml)).</td>
<td></td>
</tr>
<tr>
<td>apolipoprotein A-I Milano variant</td>
<td>POPC</td>
<td>0.0004 (mmol/l)/(µg/ml)).</td>
<td></td>
</tr>
<tr>
<td>tetranectin-apolipoprotein A-I as reported herein</td>
<td>POPC:DPPC 3:1</td>
<td>0.0013 (mmol/l)/(µg/ml)</td>
<td></td>
</tr>
</tbody>
</table>
Patent Claims

1. A lipid particle comprising
   - an apolipoprotein A-I or a variant thereof, and
   - 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-
     phosphatidyl choline.

2. The lipid particle according to claim 1, characterized in that the molar ratio of
   the 1-palmitoyl-2-oleoyl-phosphatidyl choline to the 1,2-dipalmitoyl-
   phosphatidyl choline is of from 99:1 to 25:75.

3. The lipid particle according to any one of the preceding claims, characterized
   in that the apolipoprotein A-I or variant thereof is a multimer comprising
   three monomers.

4. The lipid particle according to any one of the preceding claims, characterized
   in that the apolipoprotein A-I is a tetranectin-apolipoprotein A-I that has the
   amino acid sequence of SEQ ID NO: 01, or SEQ ID NO: 02, or SEQ ID
   NO: 06, or SEQ ID NO: 66, or SEQ ID NO: 67.

5. The lipid particle according to any one of the preceding claims, characterized
   in that the amino acid sequence of the apolipoprotein A-I has one or more
   conservative amino acid modifications.

6. The lipid particle according to any one of the preceding claims, characterized
   in that the amino acid sequence of the apolipoprotein A-I is at least 70 %
   homologous to the amino acid sequence of SEQ ID NO: 01, or SEQ ID NO:
   02, or SEQ ID NO: 06, or SEQ ID NO: 66, or SEQ ID NO: 67.

7. The lipid particle according to any one of the preceding claims, characterized
   in binding to a receptor selected from the group consisting of cubulin,
   Scavenger receptor class B, type 1 (SR-BI), ATP-binding cassette 1 (ABCA-
   1), Lecithin-cholesterol acyltransferase (LCAT), Cholesteryl-ester transfer
   protein (CETP), or Phospholipid transfer protein (PLTP).

8. The lipid particle according to any one of the preceding claims, characterized
   in that the number of phospholipid molecules per apolipoprotein monomer in
   the lipid particle is of from 40 to 120.
9. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer is of from 50 to 110.

10. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer is of from 54 to 102.

11. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90.

12. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88.

13. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80.

14. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70.

15. The lipid particle according to any one of claims 1 to 12, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 66 to 86.

16. The lipid particle according to any one of the preceding claims, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is about 66.

17. Pharmaceutical composition comprising a lipid particle according to any one of claims 1 to 16.

18. Composition comprising the lipid particle according to any one of claims 1 to 16 wherein the lipid particle comprises a detectable label.

19. The lipid particle according to any one of claims 1 to 16 for use as a medicament.
20. The lipid particle according to any one of claims 1 to 16 for use as a medicament

- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or

21. Use of a lipid particle according to any one of claims 1 to 16 for the manufacture of a medicament.

22. Use of a lipid particle according to any one of claims 1 to 16 for the manufacture of a medicament

- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.

23. A method for the manufacture of a medicament

- for secondary prevention in patients with an acute coronary syndrome, or
- for the prevention or treatment of atherosclerosis wherein a lipid particle according any one of claims 1 to 16 is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.
24. A methods for

- secondary prevention in patients with an acute coronary syndrome, or
- the prevention or treatment of atherosclerosis wherein a lipid particle according to any one of claims 1 to 16 is comprised in an amount sufficient to induce reverse cholesterol transport and/or plaques pacification in a subject, or
- for inducing reverse cholesterol transport and/or plaques pacification, or
- for cleaning/dissolution/stabilization of atherosclerotic plaques in blood vessels of a subject or for redistributing cholesterol from the wall of arteries to the liver of a subject, or
- for preventing or treating a valvular stenosis in a subject, or
- for increasing the number of HDL particles in a subject, or
- for initiation of reverse cholesterol transport in a subject, or
- for the removal of endotoxins, or
- for the prevention of septic shock
- for the treatment of angina pectoris, or
- for the treatment of myocardial infarction, or
- for the treatment of unstable angina pectoris, or
- for the treatment of arterial stenoses such as peripheral artery diseases (PAD), carotis stenosis, cerebral arterial stenosis or coronary arterial stenosis, or
- for the treatment of vascular demencia, or
- for the treatment of amaurosis fugax.

25. The lipid particle according to any one of claims 1 to 16 for use in treating

- acute coronary syndrome, or
- atherosclerosis, or
- atherosclerotic plaques in blood vessels of a subject, or
- valvular stenosis in a subject, or
- septic shock, or
- angina pectoris, or
- myocardial infarction, or
- unstable angina pectoris, or
- arterial stenoses, or
- peripheral artery diseases (PAD), or
- carotis stenosis, or
- cerebral arterial stenosis, or
- coronary arterial stenosis, or
- vascular demencia, or
- amaurosis fugax.

26. The lipid particle according to any one of claims 1 to 16 for use in
- inducing reverse cholesterol transport, or
- inducing plaques pacification, or
- cleaning or dissolving or stabilizing atherosclerotic plaques, or
- redistributing cholesterol from the wall of arteries to the liver, or
- increasing the number of HDL particles, or
- removal of endotoxins.

27. Method of treating a disease or condition characterized by non-normal lipid levels or a lipid containing deposition within body components comprising

i) administering a therapeutically effective amount of a lipid particle according to any one of claims 1 to 16 to a subject in need of a treatment or an artificial system, and

ii) optionally monitoring the lipid level or the lipid containing deposition of a subject for a change.

28. A method of treating an individual having acute coronary syndrome, or atherosclerosis, or atherosclerotic plaques in blood vessels, or valvular stenosis, or septic shock, or angina pectoris, or myocardial infarction, or unstable angina pectoris, or arterial stenoses, or peripheral artery diseases (PAD), or carotis stenosis, or cerebral arterial stenosis, or coronary arterial stenosis, or vascular demencia, or amaurosis fugax comprising administering to the individual an effective amount of a lipid particle according to any one of claims 1 to 16.

29. A method of inducing reverse cholesterol transport, or inducing plaques pacification, or cleaning or dissolving or stabilizing atherosclerotic plaques, or redistributing cholesterol from the wall of arteries to the liver, or increasing the number of HDL particles, or removing endotoxins in an individual comprising administering to the individual an effective amount of comprising administering to the individual an effective amount of a lipid particle according to any one of claims 1 to 16 to induce reverse cholesterol
transport, or to induce plaques pacification, or to clean or dissolve or stabilize atherosclerotic plaques, or to redistribute cholesterol from the wall of arteries to the liver, or to increase the number of HDL particles, or to remove endotoxins.

30. The method according to claim 29, characterized in that the non-normal lipid level is in a body fluid.

31. The method according to claim 30, characterized in that the body fluid is whole blood or blood serum.

32. The method according to any one of claims 29 to 31, characterized in that the non-normal lipid level is an increased cholesterol level.

33. The method according to any one of claims 29 to 32, characterized in that the lipid containing deposition is a plaque in a blood vessel.

34. A method of treating a disease or condition characterized by non-normal lipid levels or a lipid containing deposition within body components comprising

   i) administering a therapeutically effective amount of a lipid particle according to any one of claims 1 to 16 to a subject in need of a treatment or an artificial system,

   ii) optionally monitoring the lipid level or the lipid containing deposition of a subject for a change.

35. A method for secondary prevention in patients with an acute coronary syndrome comprising administering to a subject in need thereof a lipid particle according to any one of claims 1 to 16.

36. A polypeptide that has the amino acid sequence of SEQ ID NO: 01 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 01.

37. A polypeptide that has the amino acid sequence of SEQ ID NO: 02 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 02.

38. A polypeptide that has the amino acid sequence of SEQ ID NO: 06 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 06.
39. A polypeptide that has the amino acid sequence of SEQ ID NO: 66 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 66.

40. A polypeptide that has the amino acid sequence of SEQ ID NO: 67 or is a variant thereof that has at least 70 % sequence identity with the amino acid sequence of SEQ ID NO: 67.

41. A lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and
- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 105, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 01.

42. A lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and
- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 105, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 02.

43. A lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and
- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 105, and
wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 06.

44. A lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and
- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 105, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 66.

45. A lipid particle comprising

- an apolipoprotein A-I or a variant thereof, and
- 1-palmitoyl-2-oleoyl-phosphatidyl choline and 1,2-dipalmitoyl-phosphatidyl choline,

wherein the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 50 to 105, and

wherein the apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 67.

46. The lipid particle according to any one of claims 41 to 45, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 95.

47. The lipid particle according to any one of claims 41 to 46, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 90.

48. The lipid particle according to any one of claims 41 to 47, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 60 to 88.
49. The lipid particle according to any one of claims 41 to 48, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 62 to 80.

50. The lipid particle according to any one of claims 41 to 48, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 66 to 86.

51. The lipid particle according to any one of claims 41 to 48, characterized in that the number of phospholipid molecules per apolipoprotein monomer in the lipid particle is of from 64 to 70.
Fig. 5

Relative efflux velocity

3.6  3.2  2.8  2.4  2.0  1.6  1.2  0.8  0.4

Tetranectin-apolipoprotein A-I [μg/ml]

- POPC:DPPC = 3:1
- POPC:DPPC = 1:1
- POPC
- DPPC
- Without phospholipid
Fig. 7

Conc. [ mg/ml ]

10

POPC:DPPC = 1:1
■ POPC
△ DPPC
PoPC:DPPC = 3:1
× DPPC:SM = 9:1

Time [ h ]

0 20 40 60 80 100

0.1
Fig. 20

![Graph showing molar mass over time for total, lipid, and protein components. The y-axis represents molar mass in g/mol, and the x-axis represents time in minutes. The graph shows a peak at around 58.0 minutes for the LS component.]
Fig. 21

- total
- lipid
- protein

molar mass [ g/mol ]

Time [ min ]
Fig. 22
Fig. 23

Cholesterol efflux [ mmol/l ]

a) 5
   4
   3
   2
   1
   0
   0 0.5 1.0 1.5 2.0 2.5 3.0
   Time [ h ]

100 mg/kg
30 mg/kg

Cholesterol efflux [ mmol/l ]

b) 5
   4
   3
   2
   1
   0
   0 0.5 1.0 1.5 2.0 2.5 3.0
   Time [ h ]

100 mg/kg
30 mg/kg
30 mg/kg