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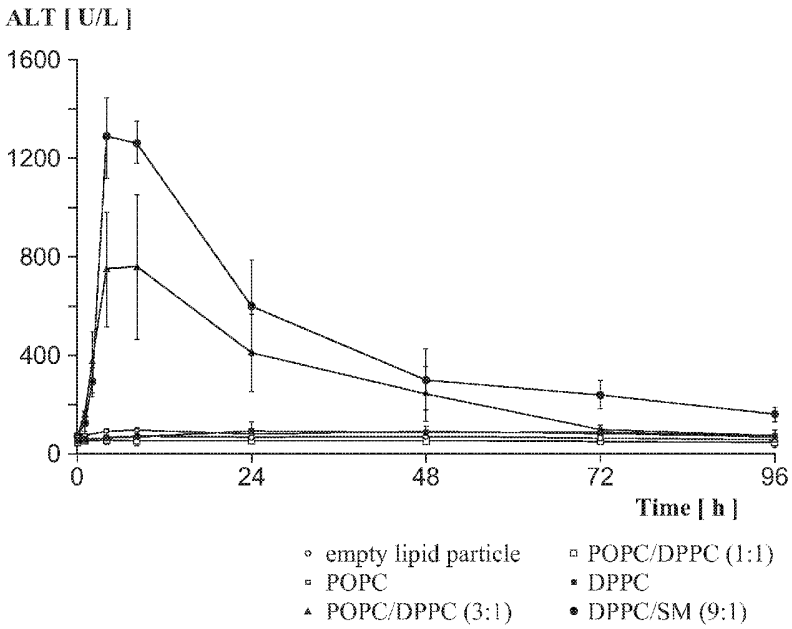
[Continued on next page]

(54) Title: METHOD FOR PRODUCING A LIPID PARTICLE, THE LIPID PARTICLE ITSELF AND ITS USE

Fig. 1



(57) Abstract: Herein is reported a method for producing a lipid particle comprising the following steps i) providing a first solution comprising denatured apolipoprotein, ii) adding the first solution to a second solution comprising at least two lipids and a detergent but no apolipoprotein, and iii) removing the detergent from the solution obtained in step ii) and thereby producing a lipid particle.

WO 2012/028524 A2

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Method for producing a lipid particle, the lipid particle itself and its use

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

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.

Cholesterol in the human body, especially in circulating body fluids such as blood, is not present as isolated molecule but in form of a complex with certain proteins (lipoproteins). The major fraction of the cholesterol is complexed with low density lipoprotein (LDL) or with high density lipoprotein (HDL). LDL particles comprise apolipoprotein B as major proteinaceous compound whereas HDL particles comprise apolipoprotein A-I as major proteinaceous compound.

Cholesterol taken up by HDL particles is esterified by the enzyme lecithin-cholesterol-acyl-transferase (LCAT). The cholesterol ester has an increased hydrophobicity and diffuses towards the core of the HDL particle. The HDL-cholesterol-ester particle may be delivered to the liver and removed from circulation.

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 suitable for sequestering cholesterol and other molecules comprising a core comprising an inorganic material are reported. Methods for producing nanoscale bound bilayers comprising the depletion of detergents from intermediate mixtures within about one hour of obtaining the mixture are reported in WO 2009/097587. 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 in reported in US 2002/10142953. In WO 2005/084642 an apoprotein-cochelate composition is reported. In WO 2007/137400 a method and compound for the treatment of valvular stenosis is reported. Pharmaceutical formulations, methods and dosing regimens for the treatment and prevention of acute coronary syndromes are reported in WO 2005/041866.

In US 6,287,590 a peptide/lipid complex formation by co-lyophilization is reported. Apolipoprotein A-I agonists and their use to treat dislipidemic disorders is reported in US 6,037,323.

In WO 2009/097587 nanoscale bound bilayers, methods of use and production are reported. The formulations of hydrophobic proteins in an immunogenic composition having improved tolerability is reported in WO 2005/065708. In WO 2006/069371 a method of plasma lipidation tom prevent, inhibit and/or reverse atherosclerosis is reported. The formation of proteoliposomes comprising membrane proteins is reported in FR 2 915 490.

Summary of the Invention

Herein is reported a method for producing a lipid particle comprising a protein. It has been found that lipid particles can be formed starting from a solution comprising the denatured protein by rapid dilution into a solution comprising at least one lipid and a detergent. In this step the concentration of the detergent is reduced below the CMC. With this method a preceding naturation step can be omitted and, thus, with the method as reported herein a faster production of lipid particles is possible.

In one embodiment the dilution is about 1:3 (v:v) to about 1:20 (v:v).

In one embodiment the dilution is about 1:5 (v:v) to about 1:10 (v:v).

In one embodiment the dilution is about 1:5 (v:v).

In one embodiment the detergent is diluted at at least a factor of about 3. In one embodiment the detergent is diluted at at least a factor of about 5.

One aspect as reported herein is a method for producing a lipid particle comprising the following steps:

i) providing a first solution comprising denatured protein,
ii) adding the first solution to a second solution comprising at least one lipid and a detergent but which does not comprise the protein, and
iii) removing the detergent from the solution obtained in step ii) and thereby producing a lipid particle.

In one embodiment the first solution is free of lipids.

In one embodiment the protein is a recombinantly produced protein.

In one embodiment the protein is an apolipoprotein. In another embodiment the apolipoprotein is a purified apolipoprotein.

In one embodiment the apolipoprotein has the amino acid sequence selected from the amino acid sequences of SEQ ID NO: 01, 02, and 04 to 52, and 66 to 67 or comprises at least a contiguous fragment comprising at least 80 % of the amino acid sequence of SEQ ID NO: 01, 02, and 04 to 52, and 66 and 67.

In one embodiment the apolipoprotein has an amino acid sequence or is at least a contiguous fragment of at least 80 % of an amino acid sequence selected from SEQ ID NO: 01, 02, and 04 to 52, and 66 and 67.

In one embodiment the apolipoprotein is an apolipoprotein A-I. In one embodiment the apolipoprotein A-I is human apolipoprotein A-I. In a further embodiment the apolipoprotein 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: 66, or SEQ ID NO: 67.

In one embodiment the apolipoprotein has the amino acid sequence of SEQ ID NO: 06 with a mutation selected from R151C and R197C.

In one embodiment the second solution has a volume that is at least two-times the volume of the first solution.

In one embodiment the second solution has about 3-times to about 20-times the volume of the first solution. In one embodiment the second solution has about 5-times to about 10-times the volume of the first solution.

In one embodiment the at least one lipid is selected from phospholipids, fatty acids and steroid lipids.

In one embodiment the at least one lipid is at least two lipids, optionally selected independently of each other from phospholipids, fatty acids and steroid lipids. In another embodiment the at least one lipid is of from one to four lipids, i.e. it is selected from the group comprising one lipid, two lipids, three lipids, and four lipids.

In one embodiment the second solution comprises a phospholipid, a lipid, and a detergent.

In one embodiment the second solution is consisting of a phospholipid, a lipid, a detergent and a buffer salt.

In one embodiment the lipids are two different phospholipids. In another embodiment the lipids are two different phosphatidylcholines. In another embodiment 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 first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC.

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 another embodiment the detergent is selected from cholic acid, Zwittergent or a salt thereof.

In one embodiment of the methods as reported herein the first solution is substantially free of lipid particles.

In one embodiment the method comprises after step ii) and prior to step iii) the following step iia) incubating the solution obtained in step ii). In one embodiment the incubating and/or removing is at a temperature of from 4 °C to 45 °C.

In one embodiment the polypeptide is incubated with the detergent for about 0.5 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 0.5 hours to about 20 hours. In one embodiment the polypeptide is incubated with the detergent for about 2 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 12 hours to about 20 hours. In one embodiment the polypeptide is incubated with the detergent for about 16 hours.

In one embodiment the detergent is a detergent with a high CMC. In another embodiment the detergent is a detergent with a CMC of at least 5 mM. In another embodiment the detergent is a detergent with a CMC of at least 10 mM.

In one embodiment the concentration of the detergent is at least 0.5 x CMC in the second solution.

In one embodiment the removing is by diafiltration or dialysis or adsorption. The adsorption is in one embodiment selected from affinity or hydrophobic chromatography. In one embodiment the removing is by dialysis.

In one embodiment the first solution has a first volume, the second solution has a second volume, the protein in the first solution has a defined concentration, and the lipids and the detergent in the second solution each have a defined concentration, and in step ii) the concentration of the apolipoprotein, of the lipids and of the detergent is changed/reduced allowing the formation of a lipid particle.

In one embodiment the method comprises the following step:

iv) purifying the lipid particle and thereby producing a lipid particle.

One aspect as reported herein is a lipid particle obtained by a method as reported herein.

One aspect as reported herein is a pharmaceutical composition comprising a lipid particle comprising apolipoprotein obtained with a method as reported herein as well as the use of a lipid particle as reported herein for the manufacture of a medicament for the treatment of arteriosclerosis.

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 KQKLK (SEQ ID NO: 05)).

The term "at least one" denotes one, two, three, four, five, six, seven, eight, nine, ten or more. The term "at least two" denotes two, three, four, five, six, seven, eight, nine, ten or more.

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 formation of lipid particles may be performed by incubating the apolipoprotein with detergent solubilized lipids at their respective transition temperature.

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 Amino acid sequence modifications can be performed by mutagenesis based on molecular modeling as described by Riechmann, L., et al., Nature 332 (1988) 323-327, and Queen, C., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033.

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]-yl-propane sulfonate (CHAPS), 3-[(3-chloramidopropyl) dimethylammonio]-2-hydroxyl 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.

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 term „immunoassay" denotes standard solid-phase immunoassays with monoclonal antibodies involving the formation of a complex between an antibody adsorbed/immobilized on a solid phase (capture antibody), the antigen, and an antibody to another epitope of the antigen conjugated with an enzyme (tracer antibody). Thus, a sandwich is formed: solid phase-capture antibody-antigen-tracer antibody. In the reaction catalyzed by the sandwich, the activity of the antibody-conjugated enzyme is proportional to the antigen concentration in the incubation medium. The standard sandwich method is also called double antigen bridging immunoassay because capture and tracer antibodies bind to different epitopes of the antigen. Other types of assays are radioimmunoassay, fluorescence immunoassays and enzyme-linked immunoassays. Methods for carrying out such assays as well as practical applications and procedures are known to a person of skill in the art. The immunoassays can be performed as homogeneous or heterogeneous immunoassay.

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.

The term „DMPC" denotes the phospholipid dimyristoyl phosphatidylcholine.

The term „DPPC" denotes the phospholipid 1,2-di-palmitoyl-sn-glycero-3-phosphatidylcholine also referred to as 1,2-dipalmitoyl-phosphatidylcholine.

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 of 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).

The term "phase transition temperature" denotes the temperature required to induce a change in the lipid physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid. The formation of the lipid particles may be carried out at or above the phase transition temperature of the phospholipids / phospholipid mixtures used. The phase transition temperature of some phosphatidylcholines and mixtures thereof are listed in the following Table 1.

Table 1: Transition temperatures of pure phosphatidylcholines and phosphatidylcholine mixtures.

phospholipid molar ratio	**phase transition temperature**
POPC	4 °C (-3 °C)
DPPC	41 °C
DPPC:POPC 3:1	34 °C
DPPC:POPC 1:1	27 °C
DPPC:POPC 1:3	18 °C

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, dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine, distearoyl phosphatidylcholine, dilauryl phosphatidylcholine, dipalmitoyl phosphatidylcholine, 1-myristoyl-2-palmitoyl phosphatidylcholine, 1-palmitoyl-2-myristoyl phosphatidylcholine, 1-palmitoyl-2-stearoyl phosphatidylcholine, 1-stearoyl-2-palmitoyl phosphatidylcholine, dioleoyl phosphatidylcholine, 1-palmitoyl-2-oleoyl phosphatidylcholine, 1-oleoyl-2-palmitoyl phosphatidylcholine, and an analogues and derivatives thereof.

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.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as “proteins”. A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

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

The term “rapid” denotes a process that is completed within at most 10 hours. A rapid dilution is a process in which a first solution is added to a second solution in at most 10 hours. In one embodiment the process is completed in at most 5 hours, in a further embodiment in at most 2 hours.

The term "substantially free" denotes that a solution comprising a protein an one or more lipids contains less than 5 % (w/w) lipid particles, less than 2.5 % lipid particles, less than 1 % lipid particles, or less than 0.5 % lipid particles.

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.

Reported herein

It has been found that lipid particles and be formed directly from a solution containing a denatured protein but no detergent and no lipid by rapid dilution into a solution containing a detergent and at least one lipid but no protein. The generally required naturation step can be omitted, thus, providing for more simple and robust method for the production of lipid particles. Additionally a more homogeneous lipid particle is formed.

Method for the production of lipid particles

Herein is reported a method for producing a lipid particle, which comprises a protein, comprising the following steps:

i) providing a first solution comprising denatured protein,
ii) adding the first solution to a second solution, which comprises a lipid and a detergent but no protein, i.e. which is free of the protein, and
iii) removing the detergent from the solution obtained in step ii) and thereby producing a lipid particle.

In one embodiment the method for producing a lipid particle, which comprises an apolipoprotein, comprises the following steps:

i) providing a first solution comprising denatured apolipoprotein,

ii) adding the first solution to a second solution, which comprises a lipid and a detergent but no apolipoprotein, and

iii) removing the detergent from the solution obtained in step ii) and thereby producing a lipid particle.

In one embodiment the second solution has a volume that is at least two-times the volume of the first solution.

In one embodiment the second solution has about 3-times to about 20-times the volume of the first solution. In one embodiment the second solution has about 5-times to about 10-times the volume of the first solution.

In one embodiment the second solution comprises at least two different lipids independently of each other selected from phospholipids, fatty acids and steroid lipids. In another embodiment the at least two different lipids are two different phosphatidylcholines. In one embodiment the first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC.

In one embodiment the detergent is selected from cholic acid, Zwittergent or a salt thereof.

A number of different methods for the production of lipid particles from naturally occurring or recombinantly produced polypeptides, such as e.g. apolipoprotein A-I or delipidated apolipoprotein A-I derived from human HDL particles, have been reported. Therein, for example, an aqueous mixture of phospholipids such as palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine with detergents such as sodium cholate are incubated with purified apolipoprotein A-I, wherein the apolipoprotein A-I is employed in native, i.e. non-denatured, form. The detergent is removed after the formation of the lipid particle by dialysis or diafiltration.

The method as reported herein allows to refold and to lipidate completely denatured protein in a single step. By using a method as reported herein a lipid particle with improved product quality can be obtained, the time consuming preconditioning of the protein can be omitted and a large scale processing for biopharmaceutical production is possible for the first time.

The method as reported herein allows to refold and lipidate completely denatured apolipoprotein A-I in a single step. By using a method as reported herein a lipid particle with improved product quality can be obtained, the time consuming

preconditioning of the apolipoprotein A-I can be omitted and a large scale processing for biopharmaceutical production is possible for the first time.

The main points which have to be considered for the lipid particle formation process development are i) the requirements for biological activity, and ii) technical requirements directed to the manufacturability of the lipid particle. For example, 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 choice of the combination of lipids determines the efficacy and liver safety of lipid particles comprising an apolipoprotein. In 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. Results clearly indicated that lipidation is needed for cholesterol mobilization and consequently for the efficacy of the molecule (Figure 23).

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 for a combination of different phospholipids.

Table 2: **Phospholipid** combinations differing in their lipid composition prepared for in vivo rabbit studies.

phospholipid molar ratio used for producing the lipid particle	**LCAT substrate**	**cholesterol efflux**
POPC	yes	yes
POPC:DPPC 3:1	yes	yes
POPC:DPPC 1:1	yes	yes
POPC:DPPC 1:3	no	yes
DPPC	no	yes

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).

Thus, also an aspect is a lipid particle obtained by a method as reported herein.

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 inhomogeniety 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 of from 64 to 70.

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 the polypeptide is incubated with the detergent for about 0.5 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 0.5 hours to about 20 hours. In one embodiment the

polypeptide is incubated with the detergent for about 2 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 12 hours to about 20 hours. In one embodiment the polypeptide is incubated with the detergent for about 16 hours.

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.

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

The lipid particle may comprise as in one embodiment an average number of from 1 to 10 apolipoprotein molecules, 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 may comprise 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 lipid, especially cholesterol, carrier for taking up lipids.

One or more detergents can be present in the lipid particle as reported herein. Such a detergent can be any pharmaceutically acceptable detergent, 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} alkylamines or alkanolamine and cationic cholesterol esters. In one embodiment the detergent is a detergent with a high CMC. In a further embodiment the detergent is a detergent with a CMC of at least 5 mM.

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

In one embodiment the lipid particle comprises less than 0.30 % by weight detergent.

In one embodiment the lipid particle comprises less than 0.1 % by weight detergent.

In one embodiment the lipid particle comprises less than 0.05 % 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 another embodiment the detergent is cholic acid or Zwittergent.

In one embodiment of the methods according to the invention the first solution is substantially free of lipid particles.

In one embodiment the method comprises after step ii) and prior to step iii) the following step iia) incubating the solution obtained in step ii). In one embodiment the polypeptide is incubated with the detergent for about 0.5 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 0.5 hours to about 20 hours. In one embodiment the polypeptide is incubated with

the detergent for about 2 hours to about 60 hours. In one embodiment the polypeptide is incubated with the detergent for about 12 hours to about 20 hours. In one embodiment the polypeptide is incubated with the detergent for about 16 hours.

In one embodiment the incubating and/or removing is at a temperature of from 4 °C to 45 °C.

In one embodiment the removing is by diafiltration or dialysis.

In one embodiment the first solution has a first volume, the second solution has a second volume, the protein, such as an apolipoprotein, in the first solution has a defined concentration, the lipids and the detergent in the second solution each have a defined concentration wherein in step ii) the concentration of the apolipoprotein, of the lipids and of the detergent is changed/reduced allowing the formation of a lipid particle. With the dilution of the apolipoprotein solution and the addition of lipids and detergent suited ratios of apolipoprotein to lipids on the one hand and also suited ratios of the lipids to the detergent on the other hand are adjusted allowing the formation of a lipid particle.

In one embodiment the method comprises the following step:

iv) purifying the lipid particle and thereby producing a lipid particle.

For example for the production of lipid particle comprising an apolipoprotein saturated phospholipids containing carboxylic acid moieties with a chain of 16 atoms and shorter would be chosen from a technical point of view (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 C-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 are advantageous compared to lipid particles comprising only one phosphatidylcholine (see e.g. Figure 4).

For example the lipid particle can contain only POPC. The number of POPC molecules per apolipoprotein monomer may vary between 54 and 75 when molar ratios from 1:40 up to 1:80 of apolipoprotein to lipid are used in the production of the lipid particle. In one embodiment the molar ratio of apolipoprotein to POPC is of from 1:40 to 1:80, in one embodiment the molar ratio is of from 1:50 to 1:70, in one embodiment the molar ratio is about 1:60.

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

For example the lipid particle can contain only DPPC. The number of DPPC molecules per apolipoprotein monomer may vary between 76 and 123 when molar ratios from 1:40 up to 1:80 of apolipoprotein to lipid are used in the production of the lipid particle. In one embodiment the molar ratio of apolipoprotein to DPPC is of from 1:70 to 1:100, in one embodiment the molar ratio is of from 1:75 to 1:90, in one embodiment the molar ratio is about 1:80.

For example the lipid particle can be produced starting from a mixture of POPC and DPPC at a molar ratio of 1:3. The number of phospholipid molecules per apolipoprotein monomer may vary between 72 and 112 when molar ratios from 1:60 up to 1:100 of apolipoprotein to lipid are used in the production of the lipid particle. In one embodiment the molar ratio of apolipoprotein to POPC and DPPC is of from 1:70 to 1:90, in one embodiment the molar ratio is of from 1:75 to 1:85, in one embodiment the molar ratio is about 1:80.

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

For example the lipid particle can be produced starting from a mixture of POPC and DPPC at a molar ratio of 1:1. The number of phospholipid molecules per apolipoprotein monomer may vary between 71 and 111 when molar ratios from 1:60 up to 1:100 of apolipoprotein to lipid are used in the production of the lipid particle. In one embodiment the molar ratio of apolipoprotein to POPC and DPPC is of from 1:60 to 1:80, in one embodiment the molar ratio is of from 1:65 to 1:75, in one embodiment the molar ratio is about 1:70.

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

For example the lipid particle can be produced starting from a mixture of POPC and DPPC at a molar ratio of 3:1. The number of phospholipid molecules per apolipoprotein monomer may vary between 46 and 93 when molar ratios from 1:60 up to 1:100 of apolipoprotein to lipid are used in the production of the lipid particle. In one embodiment the molar ratio of apolipoprotein to POPC and DPPC is of from 1:50 to 1:70, in one embodiment the molar ratio is of from 1:55 to 1:65, in one embodiment the molar ratio is about 1:60.

Thus, for the production of a lipid particle, which comprises apolipoprotein, DPPC and POPC, the molar ratio of apolipoprotein to POPC and DPPC, whereby POPC and DPPC are at a 3:1 molar ratio, is in one embodiment of from 1:60 to 1:100, in one embodiment the molar ratio is of from 1:50 to 1:70, and in one embodiment the molar ratio is about 1:60.

In one embodiment the apolipoprotein is provided as an aqueous solution of the apolipoprotein and can be obtained from downstream processing after recombinant production or any other source of apolipoprotein production and can comprise different concentrations of apolipoprotein with varying purity.

Basically lipid particle formation is achieved by incubating a polypeptide with detergent solubilized lipids at their respective transition temperature. Removal of the detergent by dialysis results in the formation of lipid particles consisting of a lipid bilayer.

Basically lipid particle formation can be achieved by incubating tetranectin-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 consisting of a lipid bilayer surrounded by the α-helical apolipoprotein.

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. In one embodiment a step of the method for purifying a lipid particle comprises a hydrophobic adsorption chromatography step. In another embodiment the chromatographic material for the hydrophobic adsorption step is selected from Extracti Gel D (available from Pierce Biotechnology, Rockford IL, USA), CALBIOSORB™ (available from Calbiochem, San Diego, CA, USA), SDR HyperD™ Solvent-Detergent Removal Chromatography Resin (available from PALL Corporation, Ann Arbor, MI, USA). The lipid particle is recovered from the hydrophobic adsorption material with a detergent-free solution.

In one embodiment dialysis is used to remove a detergent with a high CMC.

Pharmaceutical and diagnostic composition:

The lipid particle obtained by a method as reported herein can be used for the treatment and/or diagnosis of a disease or condition.

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 lipid within body components, such as plaques in blood vessels.

In order to determine the capacity of the resulting protein-lipid complex to support LCAT catalyzed cholesterol esterification cholesterol was incorporated in the lipid particle as reported herein by quick 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 shows that mixtures are better LCAT substrates than

any of the pure phosphatidylcholine as can be seen from the initial velocities of cholesterol esterification (see Table 3 and Figure 4).

Table 3: Initial velocities of cholesterol esterification in lipid particles comprising different mixtures of phospholipids.

phospholipid molar ratio used for producing the lipid particle	**K_m [µm]**	**V_{max} [nmol ester/h/µg LCAT]**
POPC	4.6	1.6
POPC:DPPC 3:1	0.4	1.9
POPC:DPPC 1:1	0.5	1.8
POPC:DPPC 1:3	1.0	1.7
DPPC	0.9	1.8

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 were 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 of lipid particles a non-lipidated tetranectin-apolipoprotein A-I can be seen. The overall increase was higher 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 4 pharmacokinetic parameters are similar for all tested compounds. The observed half-lives are close to 1.5 days.

Table 4: Determined pharmacokinetic parameters.

phospholipid molar ratio used for producing the lipid particle	**C_L [ml/h/kg]**	**V_{ss} [ml/kg]**	**$T_{1/2}$ [h]**	**C_{max} [mg/ml]**
POPC	0.89 ± 0.22	45.0 ± 2.5	36.9 ± 8.2	2.40 ± 0.19
POPC:DPPC 3:1	0.82 ± 0.06	37.8 ± 5.6	34.2 ± 4.5	2.65 ± 0.28
POPC:DPPC 1:1	0.85 ± 0.14	43.1 ± 5.9	38.6 ± 10.6	2.34 ± 0.31
DPPC	0.96 ± 0.10	37.8 ± 4.9	30.2 ± 7.7	2.29 ± 0.19
DPPC:SM 9:1	1.28 ± 0.62	50.7 ± 8.7	31.3 ± 8.2	1.91 ± 0.33

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 induced liver enzymes in rabbits as well as in mice as can be seen from Figure 1 and 9. Also no hemolysis can be determined in plasma samples obtained two hours after intravenous application (Figure 10).

Therefore aspects of the current invention are a pharmaceutical composition and a diagnostic composition comprising a lipid particle comprising apolipoprotein 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 5.

Table 5: In vivo properties of different apolipoproteins and lipid particles.

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

protein	lipid particle comprising	applied to	highest applied dose	acute liver toxicological effect	reference
tetranectin-apolipoprotein A-I	POPC/DPPC	rabbit	100 mg/kg	increase not observed	
tetranectin-apolipoprotein A-I	POPC/DPPC	rat	i.v. 500 mg/kg	increase not observed	
tetranectin-apolipoprotein A-I	POPC/DPPC	cynomolgus monkey	i.v. 200 mg/kg	increase not observed	

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 was generated comprising an N-terminal purification tag, e.g. a hexahistidine-tag, containing an IgA protease

cleavage site. As a result of the specific cleavage two amino acids – alanine and proline – remain at the N-terminus of the tetranectin-apolipoprotein A-I according to the current invention after purification and the tetranectin-apolipoprotein A-I has the amino acid sequence of SEQ ID NO: 01.

The tetranectin trimerising structural element provides for a domain that allows for the formation of a trimeric tetranectin-apolipoprotein A-I multimer that is constituted by non-covalent interactions between each of the individual tetranectin-apolipoprotein A-I monomers.

By using a different production method the purification-tag and the IgA protease cleavage site can be omitted resulting in a tetranectin-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 or an apolipoprotein A-I mimic.

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 tetranectin-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 tetranectin-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 tetranectin-apolipoprotein A-I has the amino acid sequence of
IVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVDEPPQSPWDR
VKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTSTFSK
LREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKW
QEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVD
ALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKA
KPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ (SEQ ID NO: 02).

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

(A,G,S,T)PIVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVDEP
PQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDS
VTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLD
DFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDR
ARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHL
STLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ (SEQ ID NO: 66).

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

(M)HHHHHHXIVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTV
DEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDN
WDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQP
YLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEM
RDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKAT
EHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ (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), GSGP (SEQ ID NO: 69), GSSP (SEQ ID NO: 70), GSPP (SEQ ID NO: 71), GGGS (SEQ ID NO: 72), GGGGS (SEQ ID NO: 73), GGGSGGGS (SEQ ID NO: 74), GGGGSGGGGS (SEQ ID NO: 75), GGGSGGGSGGGS (SEQ ID NO: 76), GGGGSGGGGSGGGGS (SEQ ID NO: 77), GGGSAP (SEQ ID NO: 78), GGGSGP (SEQ ID NO: 79), GGGSSP (SEQ ID NO: 80), GGGSPP (SEQ ID NO: 81), GGGGSAP (SEQ ID NO: 82), GGGGSGP (SEQ ID NO: 83), GGGGSSP (SEQ ID NO: 84), GGGGSPP (SEQ ID NO: 85), GGGSGGGSAP (SEQ ID NO: 86), GGGSGGGSGP (SEQ ID NO: 87), GGGSGGGSSP (SEQ ID NO: 88), GGGSGGGSPP (SEQ ID NO: 89), GGGSGGGSGGGSAP (SEQ ID NO: 90), GGGSGGGSGGGSGP (SEQ ID NO: 91), GGGSGGGSGGGSSP (SEQ ID NO: 92), GGGSGGGSGGGSPP (SEQ ID NO: 93), GGGGSAP (SEQ ID NO: 94), GGGGSGP (SEQ ID NO: 95), GGGGSSP (SEQ ID NO: 96), GGGGSPP (SEQ ID NO: 97), GGGGSGGGGSAP (SEQ ID NO: 98), GGGGSGGGGSGP (SEQ ID NO: 99), GGGGSGGGGSSP (SEQ ID NO: 100), GGGGSGGGGSPP (SEQ ID NO: 101), GGGGSGGGGSGGGGSAP

(SEQ ID NO: 102), GGGGSGGGGSGGGGSGP (SEQ ID NO: 103), GGGGSGGGGSGGGGSSP (SEQ ID NO: 104), and GGGGSGGGGSGGGGSPP (SEQ ID NO: 105).

If a heterologous polypeptide is produced in E.coli strains the amino-terminal methionine residue is usually not efficiently cleaved off by proteases, thus the amino-terminal methionine residue is partially present in the produced polypeptide.

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	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	Apolipoprotein.
SEQ ID NO: 29	Apolipoprotein.
SEQ ID NO: 30	Apolipoprotein.
SEQ ID NO: 31	Apolipoprotein.
SEQ ID NO: 32	Apolipoprotein.
SEQ ID NO: 33	Apolipoprotein.
SEQ ID NO: 34	Apolipoprotein.
SEQ ID NO: 35	Apolipoprotein.
SEQ ID NO: 36	Apolipoprotein.
SEQ ID NO: 37	Apolipoprotein.
SEQ ID NO: 38	Apolipoprotein.
SEQ ID NO: 39	Apolipoprotein.
SEQ ID NO: 40	Apolipoprotein.
SEQ ID NO: 41	Apolipoprotein.
SEQ ID NO: 42	Apolipoprotein.
SEQ ID NO: 43	Apolipoprotein.
SEQ ID NO: 44	Apolipoprotein.
SEQ ID NO: 45	Apolipoprotein.
SEQ ID NO: 46	Apolipoprotein.
SEQ ID NO: 47	Apolipoprotein.
SEQ ID NO: 48	Apolipoprotein.
SEQ ID NO: 49	Apolipoprotein.
SEQ ID NO: 50	Apolipoprotein.
SEQ ID NO: 51	Apolipoprotein.
SEQ ID NO: 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	IgA protease cleavage site.

SEQ ID NO: 61	IgA protease cleavage site.
SEQ ID NO: 62	IgA protease cleavage site.
SEQ ID NO: 63	IgA protease cleavage site.
SEQ ID NO: 64	IgA protease cleavage site.
SEQ ID NO: 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 K_2HPO_4, 250 mM arginine hydrochloride, 7.5 % trehalose at pH 7.5.

Figure 13 Native PAGE of lipid particles of POPC and tetranectin-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 tetranectin-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 tetranectin-apolipoprotein A-I.

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

Figure 17 Native PAGE of lipid particles of DPPC and tetranectin-apolipoprotein A-I obtained with molar ratios of from 1:20 to 1:100 (1: molecular weight marker; 2: tetranectin-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 tetranectin-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 tetranectin-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 lipidated sample as references.

Figure 20 SEC-MALLS protein conjugate analysis of lipid particle of tetranectin-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 tetranectin-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 tetranectin-apolipoprotein A-I using POPC. Upper: lipid particle formed from native tetranectin-apolipoprotein A-I; lower: lipid particle formed from denatured tetranectin-apolipoprotein A-I.

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

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

Materials and Methods

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. Colum 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 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.

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/CelII-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 T5 hybrid promoter (T5-PN25/03/04 hybrid promoter according to Bujard, H., et al. Methods. Enzymol. 155 (1987) 416-433 and Stueber, D., et al., Immunol. Methods IV (1990) 121-152) including a synthetic ribosomal binding site according to Stueber, D., et al. (see before),
  - 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 tetranectin-apolipoprotein A-I

For the expression of the fusion protein 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 0972838 and US 6,291,245).

The E.coli K12 strain CSPZ-2 (leuB, proC, trpE, th-1, ΔpyrF) was transformed by electroporation with the expression plasmid p(IFN-His6-IgA-tetranectin-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 Riesenberg et al. was used (Riesenberg, D., et al., J. Biotechnol. 20 (1991) 17-27): 27.6 g/l glucose*H_2O, 13.3 g/l KH_2PO_4, 4.0 g/l $(NH_4)_2HPO_4$, 1.7 g/l citrate, 1.2 g/l $MgSO_4$*7 H_2O, 60 mg/l iron(III)citrate, 2.5 mg/l $CoCl_2$*6 H_2O, 15 mg/l $MnCl_2$*4 H_2O, 1.5 mg/l $CuCl_2$*2 H_2O, 3 mg/l H_3BO_3, 2.5 mg/l Na_2MoO_4*2 H_2O, 8 mg/l $Zn(CH_3COO)_2$*2 H_2O, 8.4 mg/l Titriplex 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 $MgSO_4$*7 H_2O. The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH_3 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 NH_4Cl, 1.94 g/l L-leucine, 2.91 g/l L-proline, 0.74 g/l L-methionine, 17.3 g/l KH_2PO_4*$H2_O$, 2.02 g/l $MgSO_4$*7 H_2O, 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 (pO_2) 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 tetranectin-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 tetranectin-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 tetranectin-apolipoprotein A-I

Inclusion body preparation was carried out by resuspension of harvested bacteria cells in a potassium phosphate buffered solution or a Tris buffered solution (0.1 M, supplemented with 1 mM $MgSO_4$, 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 tetranectin-apolipoprotein A-I.

Example 4

Refolding and lipidation of tetranectin-apolipoprotein A-I

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 tetranectin-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 overnight 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 tetranectin-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 tetranectin-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 tetranectin-apolipoprotein A-I monomer.

b) POPC and cholate

Table 6: Lipid particle formation with tetranectin-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).

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

*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 tetranectin-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 (UV280 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 tetranectin-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 7: Lipid particle formation of tetranectin-apolipoprotein A-I using pure POPC. Molar ratio apolipoprotein:phospholipid has been calculated based on the tetranectin-apolipoprotein A-I monomer.

molar ratio apolipoprotein: phospholipid	protein conc. before dialysis [mg/ml]*	protein conc. after dialysis [mg/ml]*	yield [%]	observation after dialysis
1:40	3.5	2.67	76	precipitation
1:45	3.5	2.74	78	precipitation
1:50	3.5	2.94	84	precipitation
1:55	3.5	3.05	87	precipitation
1:60	3.5	3.19	91	precipitation
1:65	3.5	3.34	95	precipitation

molar ratio apolipoprotein: phospholipid	protein conc. before dialysis [mg/ml]*	protein conc. after dialysis [mg/ml]*	yield [%]	observation after dialysis
1:70	3.5	3.52	100**	
1:75	3.5	3.56	100**	
1:80	3.5	3.57	100**	

* volume before and after dialysis 2.6 ml

**within SD of the method

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 8.

Table 8: Summary of size exclusion chromatography results; percentages were calculated by integration of the area under the curve (AUC).

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

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 tetranectin-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 tetranectin-apolipoprotein A-I trimer per lipid particle. The ratio n(POPC)/n(protein monomer) gives the number of POPC molecules per tetranectin-apolipoprotein A-I monomer in the lipid particle. The number of POPC molecules per tetranectin-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 9: Summary of protein conjugate analysis of lipid particles of POPC and tetranectin-apolipoprotein A-I as shown in Figure 16.

		MW total [kDa]	MW Protein [kDa]	n (monomer)	MW lipid [kDa]	n (POPC)	n(POPC)/ n(monomer)	% protein
1:40	Main peak	238	104	3.3	135	178	54	44
	Post peak	230	148	4.6	81	107	23	65
1:45	Main peak	238	101	3.2	138	182	57	42
	Post peak	184	118	3.7	66	87	24	64
1:50	Main peak	244	100	3.1	143	188	61	41
	Post peak	187	118	3.7	70	92	25	63
1:55	Main peak	247	99	3.1	148	195	63	40
	Post peak	182	107	3.3	75	99	30	59
1:60	Main peak	248	98	3.1	150	197	64	40
	Post peak	183	106	3.3	76	100	30	58
1:65	Main peak	255	97	3.0	158	208	69	38
	Post peak	191	103	3.2	88	116	36	54
1:70	Main peak	260	97	3.0	163	214	71	37
	Post peak	196	100	3.1	95	125	40	51
1:75	Main peak	266	99	3.1	168	221	71	37
	Post peak	208	118	3.7	91	120	32	56
1:80	Main peak	275	99	3.1	176	232	75	36
	Post peak	215	112	3.5	103	136	39	52

c) DPPC and cholate

Prior to lipidation the tetranectin-apolipoprotein A-I was dialyzed against 50 mM KH_2PO_4, 250 mM arginine hydrochloride, 7 % trehalose, 10 mM methionine at pH 7.5. Tetranectin-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 10: Sample overview of lipid particles of apolipoprotein with DPPC.

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

* calculated for protein monomer

During lipid particle formation neither precipitation of protein nor turbidity through excess lipid was observed. The yield of tetranectin-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 11).

Table 11: Summary SEC-MALLS protein conjugate analysis of lipid particles of DPPC and tetranectin-apolipoprotein A-I.

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

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 12 and 13).

Table 12: Summary of SEC results; percentages were calculated by integration of the AUC.

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

Table 13: Summary protein-conjugate analysis of 75 % DPPC/25 % POPC and tetranectin-apolipoprotein A-I lipid particles.

		MW total	MW protein [kDa]	n (protein monomer)	MW lipid [kDa]	n (lipid)	n(lipid)/ n(monomer)	% protein
1:60	Main peak	257	96	3.0	161	217	72	37
	Post peak	92	75	2.3	17	23	10	82
1:65	Main peak	263	95	3.0	167	226	76	36
	Post peak	116	102	3.2	14	19	6	88
1:70	Main peak	268	95	3.0	173	234	79	35
	Post peak	93	83	2.6	10	14	5	89
1:75	Main peak	275	95	3.0	180	243	82	34
	Post peak	98	82	2.6	16	22	8	84
1:80	Main peak	279	95	3.0	184	248	84	34
	Post peak	97	86	2.7	11	15	6	89

		MW total	MW protein [kDa]	n (protein monomer)	MW lipid [kDa]	n (lipid)	n(lipid)/ n(monomer)	% protein
1:85	Pre peak	329	104	3.3	224	302	93	32
	Main peak	291	96	3.0	195	263	88	33
	Post peak	129	107	3.3	22	30	9	83
1:90	Pre peak	443	107	3.3	237	320	96	31
	Main peak	293	95	3.0	197	266	90	33
	Post peak	126	102	3.2	25	34	11	81
1:95	Pre peak	384	110	3.4	274	370	108	29
	Main peak	303	96	3.0	207	280	93	32
	Post peak	130	103	3.2	27	36	11	79
1:100	Pre peak	398	111	3.5	287	388	112	28
	Main peak	310	96	3.0	213	288	96	31
	Post peak	122	86	2.7	36	49	18	71

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:

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 14: Summary of SEC results; percentages were calculated by integration of the AUC.

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

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 15).

Table 15: Summary protein conjugate analysis of lipid particles with 50 % DPPC/50 % POPC and tetranectin-apolipoprotein A-I.

		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein
1:60	Main peak	331	124	3.9	207	277	71	38
	Post peak	131	106	3.3	24	32	10	81
1:65	Main peak	264	95	2.9	169	226	78	36
	Post peak	127	112	3.5	16	21	6	88
1:70	Main peak	273	96	3.0	178	238	79	35
	Post peak	258	213	6.7	45	60	9	82

		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein
1:75	Pre peak	319	108	3.4	211	282	83	34
	Main peak	271	93	2.9	178	238	82	34
	Post peak	126	106	3.3	20	27	8	84
1:80	Pre peak	333	108	3.4	225	301	89	32
	Main peak	278	95	2.9	184	246	85	34
	Post peak	122	100	3.1	21	28	9	83
1:85	Pre peak	359	109	3.4	250	335	98	30
	Main peak	284	94	2.9	189	253	87	33
	Post peak	132	118	3.7	14	19	5	89
1:90	Pre peak	373	109	3.4	264	353	104	29
	Main peak	286	94	2.9	192	257	89	33
	Post peak	133	110	3.4	23	31	9	83
1:95	Pre peak	390	111	3.5	278	372	106	29
	Main peak	290	94	2.9	195	261	90	33
	Post peak	162	136	4.3	26	35	8	84
1:100	Pre peak	404	113	3.5	291	390	111	28
	Main peak	293	94	2.9	199	266	92	32
	Post peak	142	107	3.3	35	47	14	75

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 straight forward and comparable to the process using pure lipids. All samples remained clear during the process and dialysis.

Table 16: Summary of SEC results; percentages were calculated by integration of the AUC.

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

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 17). 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 17: Summary protein conjugate analysis of lipid particles of 25 % DPPC/75 % POPC and tetranectin-apolipoprotein A-I.

		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein
1:60	Main peak	254	100	3.1	153	203	66	40
	Post peak	127	110	3.4	17	23	7	86
1:65	Pre peak	272	132	4.1	141	187	46	48
	Main peak	259	100	3.1	159	211	68	39
	Post peak	183	131	4.1	7	9	2	95
1:70	Pre peak	280	121	3.8	159	211	56	43
	Main peak	264	99	3.1	165	219	71	38
	Post peak	119	105	3.3	14	19	6	88

		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein
1:75	Pre peak	291	109	3.4	183	243	71	37
	Main peak	268	98	3.1	170	226	73	37
	Post peak	120	101	3.2	19	25	8	84
1:80	Pre peak	311	114	3.6	197	261	73	37
	Main peak	276	96	3.0	176	234	78	36
	Post peak	137	127	4.0	10	13	3	93
1:85	Pre peak	331	115	3.6	216	287	80	35
	Main peak	278	98	3.1	180	239	77	35
	Post peak	139	117	3.7	22	29	8	85
1:90	Pre peak	345	113	3.5	232	308	88	33
	Main peak	285	98	3.1	187	248	80	34
	Post peak	143	110	3.4	33	44	13	77
1:95	Pre peak	363	115	3.6	248	329	91	32
	Main peak	292	97	3.0	194	257	86	33
	Post peak	155	122	3.8	33	44	12	79
1:100	Pre peak	377	117	3.7	260	345	93	31
	Main peak	298	98	3.1	200	265	86	33
	Post peak	160	114	3.6	46	61	17	71

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 18: Sample overview of various approaches and observations / parameters of lipid particle formation.

sample	detergent [%]	turbidity			volume after dialysis [ml]	c after dialysis [µg/ml]	[mg] TN-Apo A-I	yield [%]
		dissolved lipid	lipidation	after dialysis				
Zwittergent 3-8								
0.1 x CMC	0.8	+++	+++	+++	2.1	2230.18	4.68	99.6
0.5 x CMC	4.2	++	++	+	2.9	1536.81	4.46	94.8
1 x CMC	8.4	+	+	+	3	1475.07	4.43	94.2
2 x CMC	16.7	-	-	-	4.3	1081.27	4.65	98.9
3 x CMC	25.1	-	-	-	5.5	839.85	4.62	98.3
Zwittergent 3-10								
0.1 x CMC	0.1	+++	+++	+++	2	2361.56	4.72	100.5
0.5 x CMC	0.6	+++	++	++	2	2221.38	4.44	94.5
1 x CMC	1.2	++	+	+	2.1	2267.16	4.76	101.3
2 x CMC	2.5	+	+	(+)	2.3	2082.18	4.79	101.9
5 x CMC	6.2	-	-	-	2.5	1941.61	4.85	103.3
10 x CMC	12.3	-	-	-	4	1073.92	4.30	91.4
Zwittergent 3-12								
0.1 x CMC	0.01	+++	+++	+++	2	2722.85	5.45	115.9
1 x CMC	0.1	+++	+++	+++	2	2158.81	4.32	91.9
2 x CMC	0.2	+++	+++	++	2	2636	5.27	112.2
20 x CMC	1.9	+	+	+	2.1	2525.69	5.30	112.8
100 x CMC	9.4	-	-	-	3.5	1567.85	5.49	116.8
300 x CMC	28.1	-	-	-	5.6	1069.04	5.99	127.4

sample	detergent [%]	turbidity			volume after dialysis [ml]	c after dialysis [µg/ml]	[mg] TN-Apo A-I	yield [%]
		dissolved lipid	lipidation	after dialysis				
Cholate								
0.1 x CMC	0.06	+++	+++	+++	2	2323.09	4.65	98.9
0.5 x CMC	0.3	+	-	-	2	2301.15	4.60	97.9
1 x CMC	0.6	-	-	-	2	2316.86	4.63	98.6
2 x CMC	1.2	-	-	-	2.5	1178.72	2.95	62.7
5 x CMC	3	-	-	-	2.5	2435.34	6.09	129.5
10 x CMC	6	-	-	-	3.5	1814.69	6.35	135.1

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

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

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

x CMC		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein	Rh (w) (QELS) [nm]
2	Pre peak Main peak	373 272	161 112	5.0 3.5	211 159	278.7 210.3	56 60	43.2 41.4	7.8 6.6
5	Pre peak Main peak	345 263	150 112	4.7 3.5	195 151	256.6 199.1	55 57	43.6 42.6	7.5 6.6
10	Pre peak Main peak	405 265	151 110	4.7 3.3	253 154	334.1 203.2	71 58	37.4 41.8	7.9 6.5

The results of lipid particle formation comprising tetranectin-apolipoprotein A-I using Zwittergent 3-12 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 tetranectin-apolipoprotein A-I molecules per particle.

Table 21: Summary of protein-conjugate analysis of lipid particles formed in the presence of Zwittergent 3-12.

x CMC		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein	Rh (w) (QELS) [nm]
100	Main peak	487	342	10.7	145	191.3	18	70.2	11.9
300	Main peak	241	208	6.5	32	43.3	7	86.4	8.5

The results of lipid particle formation comprising tetranectin-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 tetranectin-apolipoprotein A-I molecules per particle.

Table 22: Summary of protein-conjugate analysis of lipid particles formed in the presence of cholate.

CMC		MW total	MW protein	n (protein monomer)	MW lipid	n(lipid)	n(lipid)/ n (monomer)	% protein	Rh (w) (QELS) [nm]
0.5	Pre peak	1295	461	14.5	829	1091	75	35.9	12.7
	Main peak	361	153	4.8	207	273	57	42.5	7.7
	Post peak	283	115	3.6	168	221	62	40.6	6.8
1	Pre peak	1050	414	12.9	623	836	65	39.5	11.8
	Main peak	337	154	4.8	182	240	50	45.9	7.6
	Post peak	284	121	3.8	162	214	56	42.7	6.9
2	Pre peak	332	143	4.5	188	248	55	43.2	7.3
	Main peak	269	111	3.5	158	209	60	41.2	6.5
5	Pre peak	314	143	4.5	171	225	50	45.6	7.5
	Main peak	278	118	3.7	158	208	56	42.7	6.8
10	Pre peak	292	135	4.2	156	206	50	46.3	7.3
	Main peak	271	115	3.6	155	204	57	42.6	6.6

Example 5

Rapid dilution method for refolding and lipid particle formation

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 tetranectin-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 23: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with POPC.

Peak	MW total [kDa]	MW protein [kDa]	n (protein monomer)	MW lipid [kDa]	n (lipid)	n (lipid) / n (protein)	% protein
Pre Peak	347	141	4.4	207	272	62	41
Main Peak	269	111	3.5	159	209	60	41

Tetranectin-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 tetranectin-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 24: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with a POPC/DPPC/cholate mixture.

Peak	MW total [kDa]	MW Protein [kDa]	n Protein (APO-Monomer)	MW Lipid [kDa]	n Lipid	n Lipid / n Protein	% Protein
Pre Peak	419	167	5.2	251	333	64	41
Main Peak	252	101	3.2	151	200	63	41

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

Peak	MW total [kDa]	MW protein [kDa]	n (protein monomer)	MW lipid [kDa]	n (lipid)	n (lipid) / n (protein)	% protein
Main peak	263	102	3.2	161	214	67	39
Post peak	182	85	2.7	97	129	48	47

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 26: Summary protein conjugate analysis of lipid particle obtained by rapid dilution with different dilution ratios.

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

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 27: Summary of protein conjugate analysis.

Starting material		MW total	MW protein	n (protein monomer)	MW lipids	Number of lipids	Ratio lipid:protein
denatured	Mainpeak	235	71	2.2	163	216	1: 97

Example 6

Lipid particle formation starting from denatured or native protein

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, 10mM 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 tetranectin-apolipoprotein A-I (see Figure 22).

Table 28: Summary of protein conjugate analysis.

Starting material		MW total	MW protein	n (protein monomer)	MW lipids	Number of lipids	Ratio lipid:protein
native	Prepeak (60%)	321	131	4.1	190	250	61
	Mainpeak (40%)	269	107	3.3	162	213	65
denatured	Mainpeak (>90%)	269	111	3.5	159	209	60

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 then 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 NaN_3; 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 µg/ml recombinant LCAT enzyme (ROAR biochemical) in 125 µl (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaN_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 3a: Apparent kinetic parameters.

substrate [% POPC]	K_m [nM]	V_{max} [n mole ester/h/U LCAT]
100	4.6	1.6
75	0.4	1.9
50	0.5	1.8
25	1.0	1.7
0	6.9	1.8

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 29).

Table 29: Different samples.

tetranectin-apolipoprotein A-I with	**molar ratio apolipoprotein: phospholipid**	**preparation method**
100 % POPC/ 0 % DPPC	1:60	cholate
75 % POPC/ 25 % DPPC	1:60	cholate
50 % POPC/ 50 % DPPC	1:70	cholate
0 % POPC/ 100 % DPPC	1:80	cholate
-	not	

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.

tetranectin-apolipoprotein A-I with	**C_L [ml/h/kg]**	**V_{ss} [ml/kg]**	**$T_{1/2}$ [h]**	**C_{max} [mg/m]**
100 % POPC/ 0 % DPPC	0.897 ± 0.216	45.0 ± 2.5	36.9 ± 8.2	2.40 ± 0.19
0 % POPC/ 100 % DPPC	0.922 ± 0.098	37.8 ± 4.9	30.2 ± 7.7	2.29 ± 0.19
75 % POPC/ 25 % DPPC	0.815 ± 0.064	37.8 ± 5.6	34.2 ± 4.5	2.65 ± 0.28
50 % POPC/ 50 % DPPC	0.850 ± 0.135	43.1 ± 5.9	38.6 ± 10.6	2.34 ± 0.31
90 % DPPC/ 10 % SM	1.28 ± 0.62	50.7 ± 8.7	31.3 ± 8.2	1.91 ± 0.33

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 Lipoid 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 30.

Table 30: Cholesterol mobilization.

	lipids	**AUC(time dependent concentration cholesterol in blood) ---------- AUC (time dependent apolipoprotein A-I concentration in blood)**
wt-apolipoprotein A-I	soybean phospholipid mixture	0.0002 (mmol/l)/(µg/ml)).
apolipoprotein A-I Milano variant	POPC	0.0004 (mmol/l)/(µg/ml)).
tetranectin-apolipoprotein A-I as reported herein	POPC:DPPC 3:1	0.0013 (mmol/l)/(µg/ml)

Patent Claims

1. Method for producing a lipid particle comprising the following steps:

   i) providing a first solution comprising a denatured protein,
   ii) adding the first solution to a second solution comprising at least one lipid and a detergent but not the protein, and
   iii) removing the detergent from the solution obtained in step ii) and thereby producing a lipid particle.

2. Method according to claim 1, characterized in that the second solution has about 3-times to about 20-times the volume of the first solution.

3. Method according to any one of the preceding claims, characterized in that the first solution is free of lipids.

4. Method according to any one of the preceding claims, characterized in that the protein has an amino acid sequence selected from the amino acid sequences of SEQ ID NO: 01, 02, 04 to 52, 66, or 67, or comprises at least a contiguous fragment comprising at least 80 % of the amino acid sequence of SEQ ID NO: 01, 02, 04 to 52, 66, or 67.

5. Method according to claim 4, characterized in that the protein 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: 66, or SEQ ID NO: 67.

6. Method according to any one of the preceding claims, characterized in that the at least one lipid is two different phosphatidylcholines.

7. Method according to claim 6, characterized in that the first phosphatidylcholine is POPC and the second phosphatidylcholine is DPPC.

8. Method according to any one of the preceding claims, characterized in that the detergent is selected from cholic acid, Zwittergent or a salt thereof.

9. Method according to any one of the preceding claims, characterized in that the method comprises after step ii) and prior to step iii) the following step

   iia) incubating the solution obtained in step ii).

10. Method according to any one of the preceding claims, characterized in that the incubating and/or removing is at a temperature of from 4 °C to 45 °C.

11. Method according to any one of claims 9 and 10, characterized in that the incubating is for about 2 hours to about 60 hours.

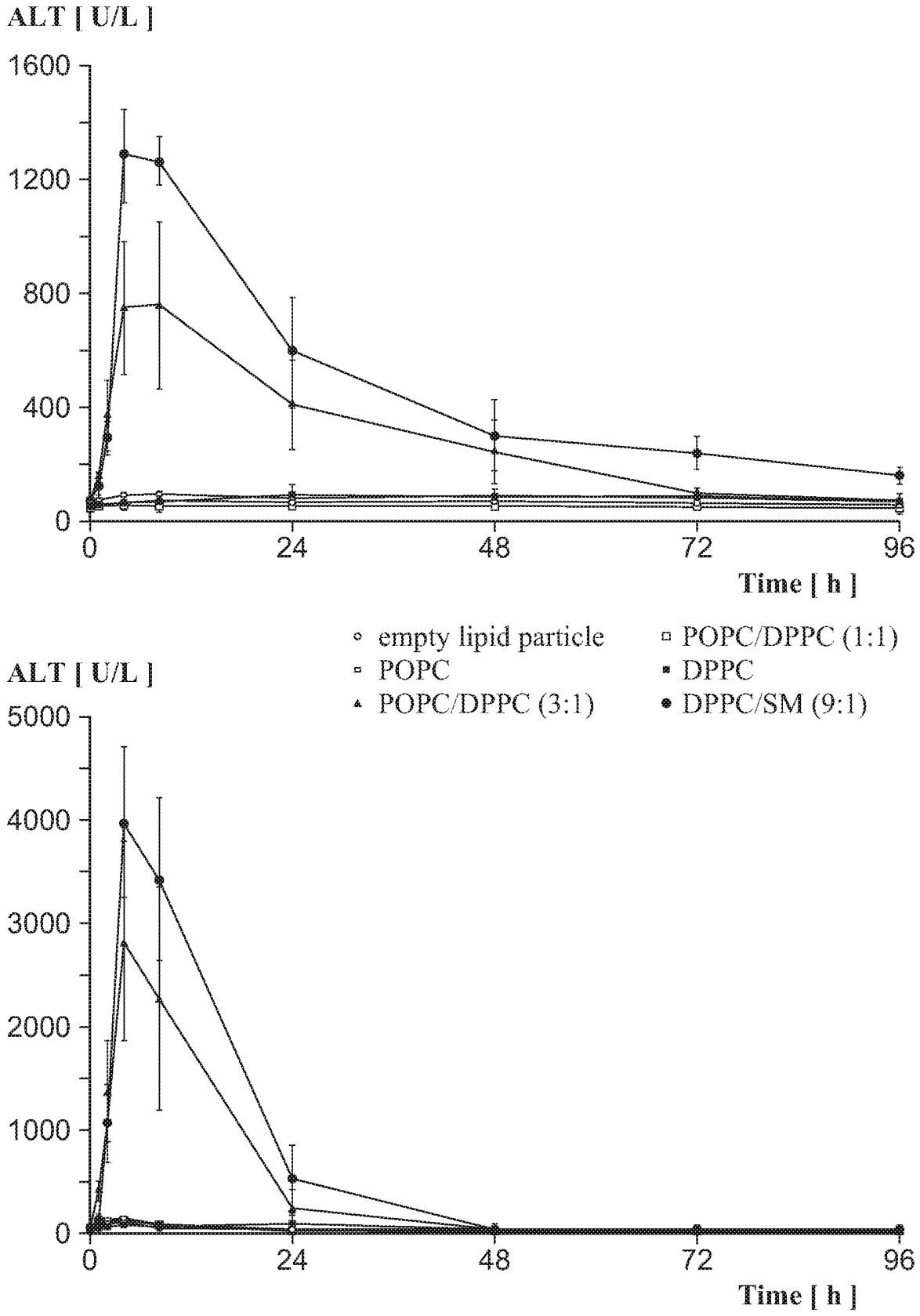
12. Method according to any one of the preceding claims, characterized in that the detergent is a detergent with a high CMC.

13. Method according to any one of the preceding claims, characterized in that the removing is by diafiltration or dialysis or adsorption.

14. A lipid particle obtained with a method according to any one of claims 1 to 13.

15. Pharmaceutical composition comprising a lipid particle according to claim 14.

Fig. 1


ALT [U/L]
1600
1200
800
400
0
0
24
48
72
96
Time [h]
empty lipid particle
POPC
POPC/DPPC (3:1)
POPC/DPPC (1:1)
DPPC
DPPC/SM (9:1)
ALT [U/L]
5000
4000
3000
2000
1000
0
0
24
48
72
96
Time [h]

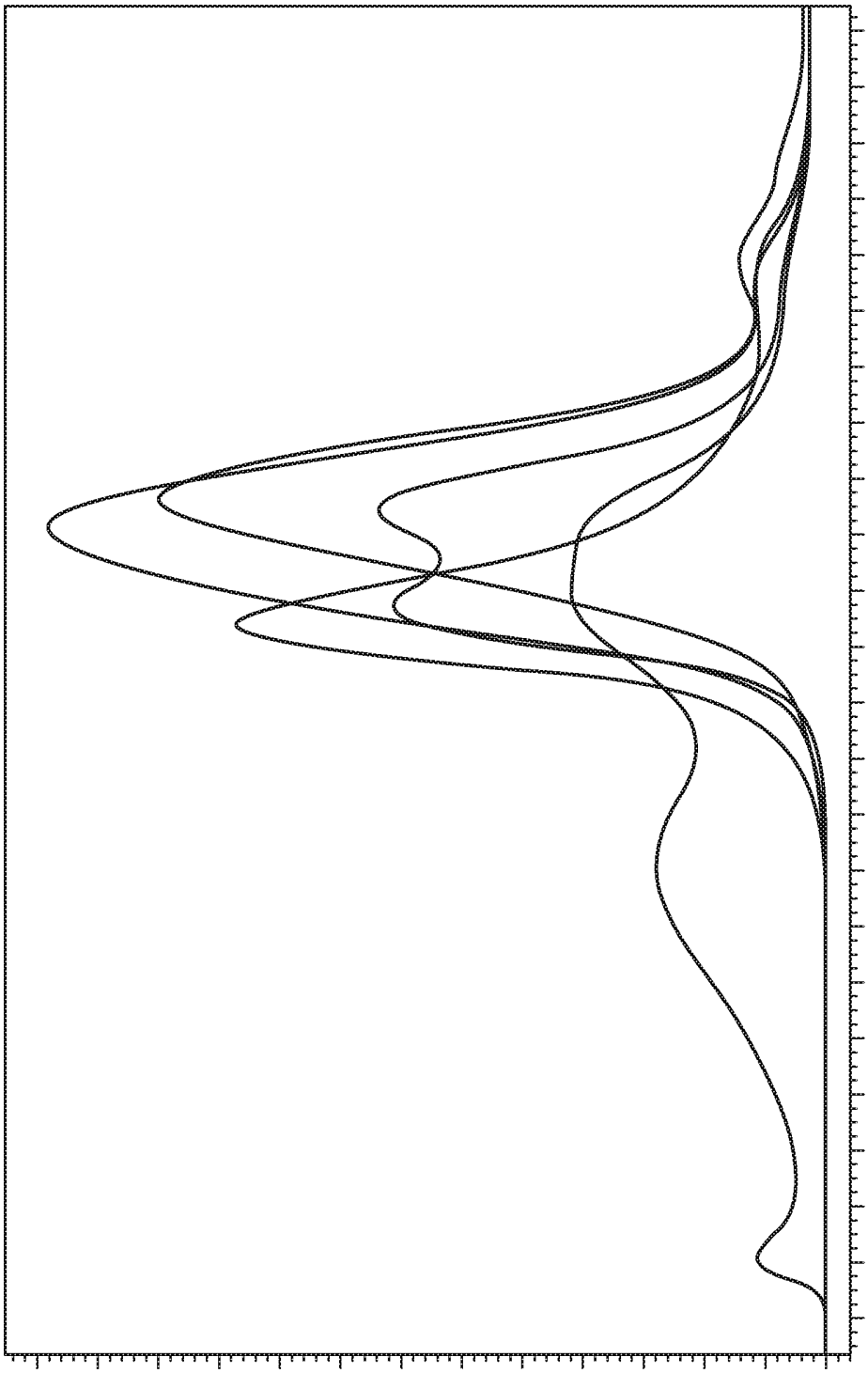


Fig. 2

Fig. 3

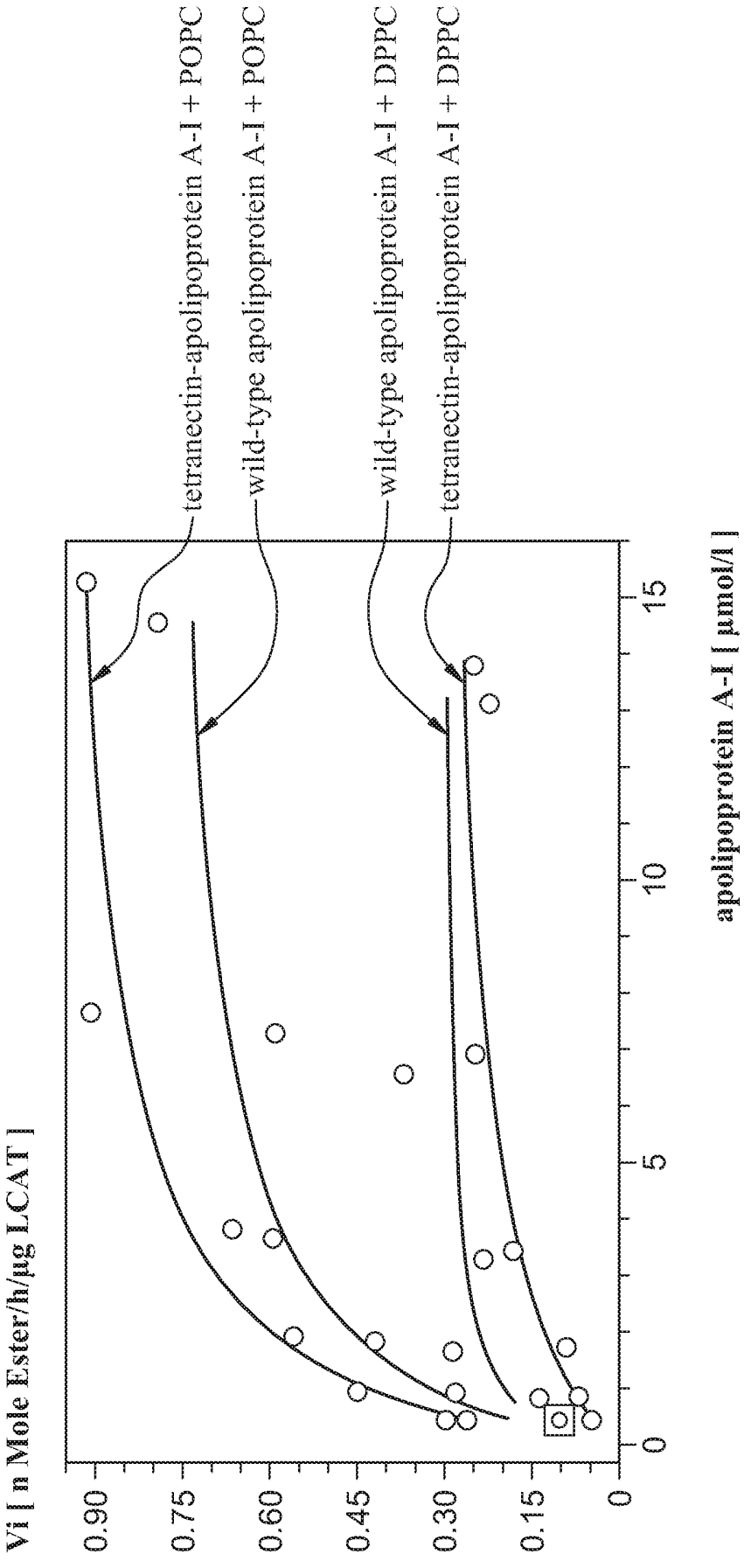

Vi [n Mole Ester/h/µg LCAT]
0.90
0.75
0.60
0.45
0.30
0.15
0
0
5
10
15
apolipoprotein A-I [µmol/l]
tetranectin-apolipoprotein A-I + POPC
wild-type apolipoprotein A-I + POPC
wild-type apolipoprotein A-I + DPPC
tetranectin-apolipoprotein A-I + DPPC

Fig. 4

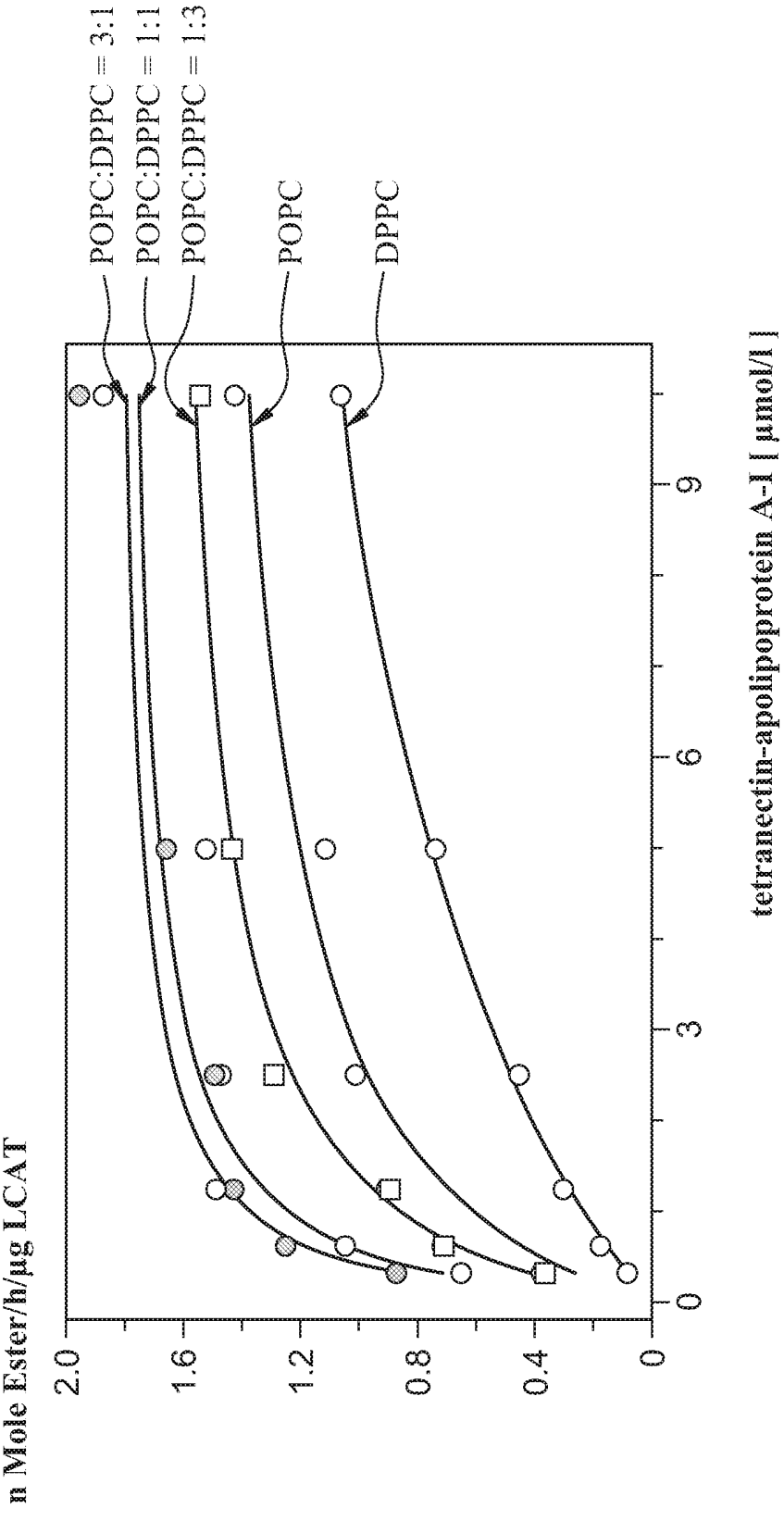

n Mole Ester/h/µg LCAT
2.0
1.6
1.2
0.8
0.4
0
0
3
6
9
tetranectin-apolipoprotein A-I [µmol/l]
POPC:DPPC = 3:1
POPC:DPPC = 1:1
POPC:DPPC = 1:3
POPC
DPPC

Fig. 5

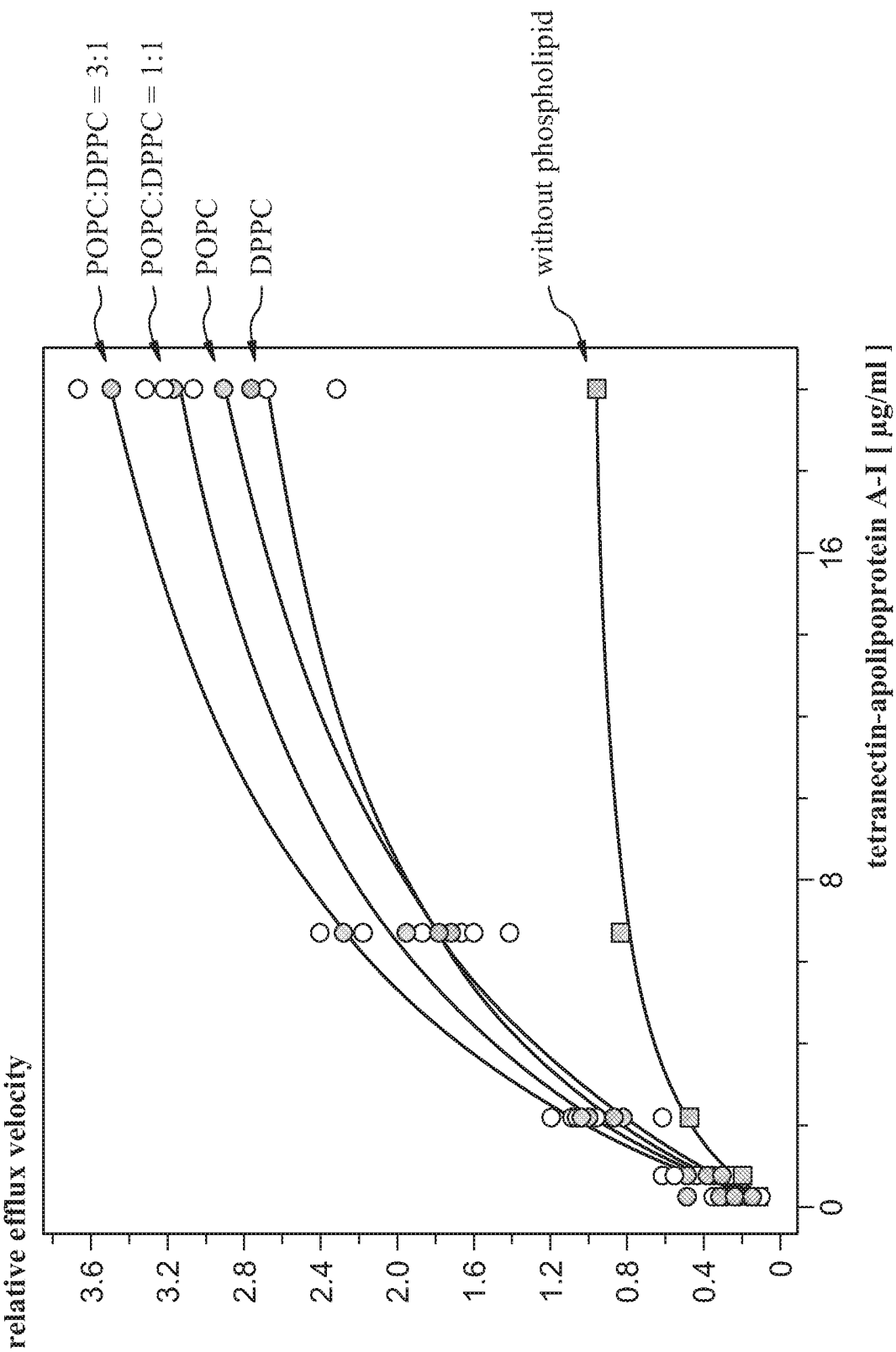

POPC:DPPC = 3:1
POPC:DPPC = 1:1
POPC
DPPC
without phospholipid
relative efflux velocity
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
0
8
16
tetranectin-apolipoprotein A-I [µg/ml]

Fig. 6

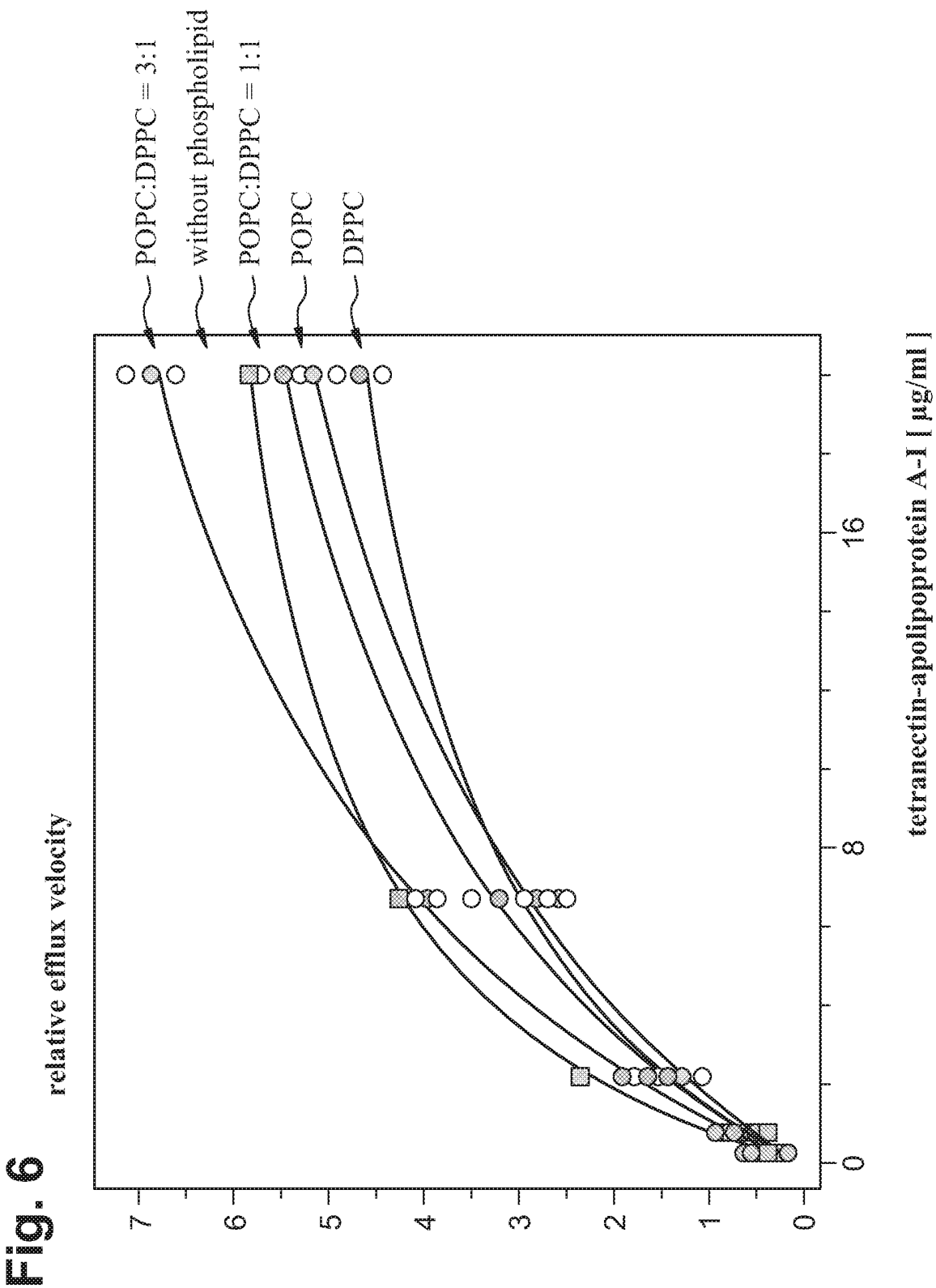

POPC:DPPC = 3:1
without phospholipid
POPC:DPPC = 1:1
POPC
DPPC
relative efflux velocity
0
1
2
3
4
5
6
7
0
8
16
tetranectin-apolipoprotein A-I [µg/ml]

Fig. 7

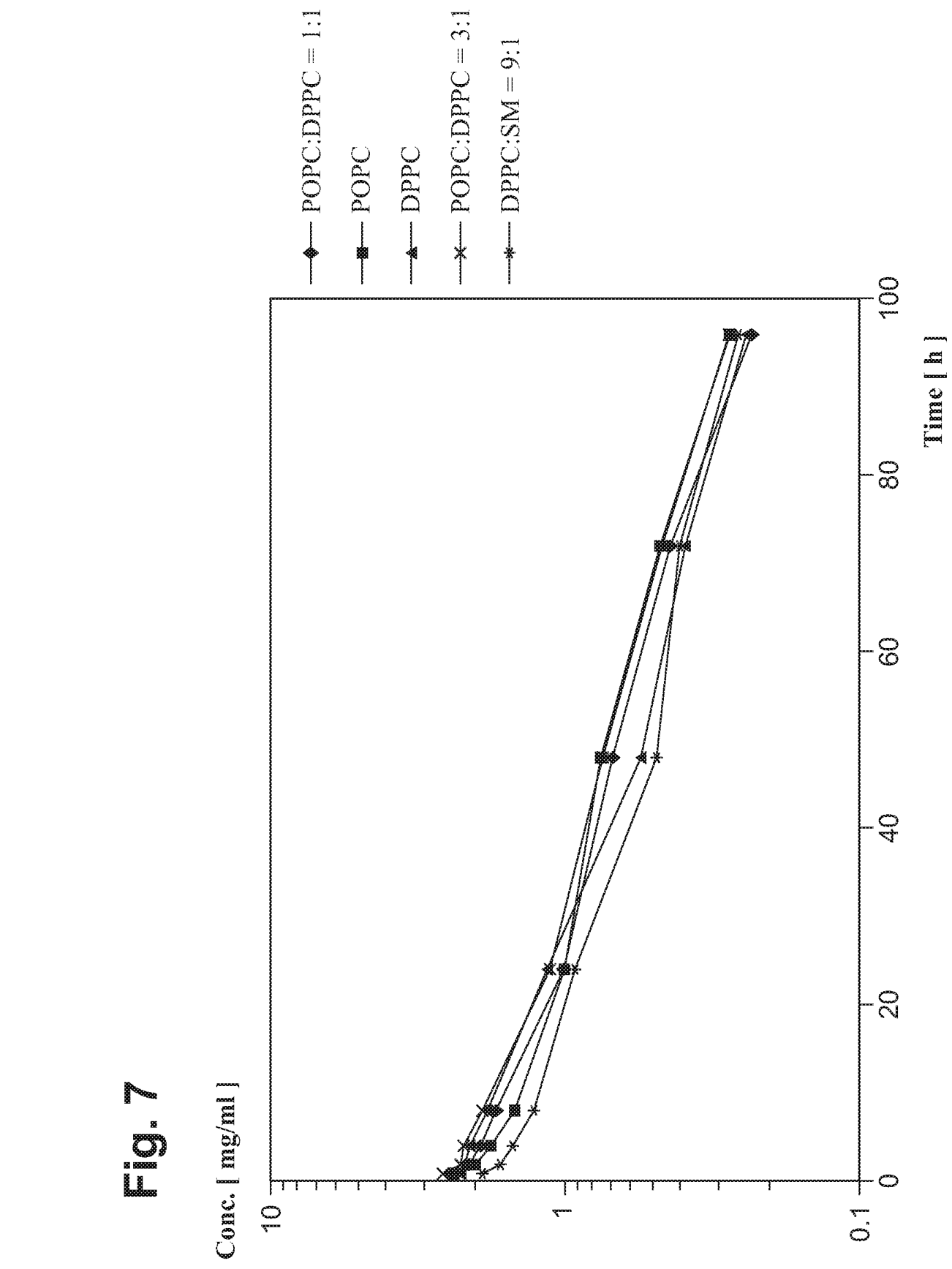

POPC:DPPC = 1:1
POPC
DPPC
POPC:DPPC = 3:1
DPPC:SM = 9:1
Conc. [mg/ml]
10
1
0.1
0
20
40
60
80
100
Time [h]

Fig. 8

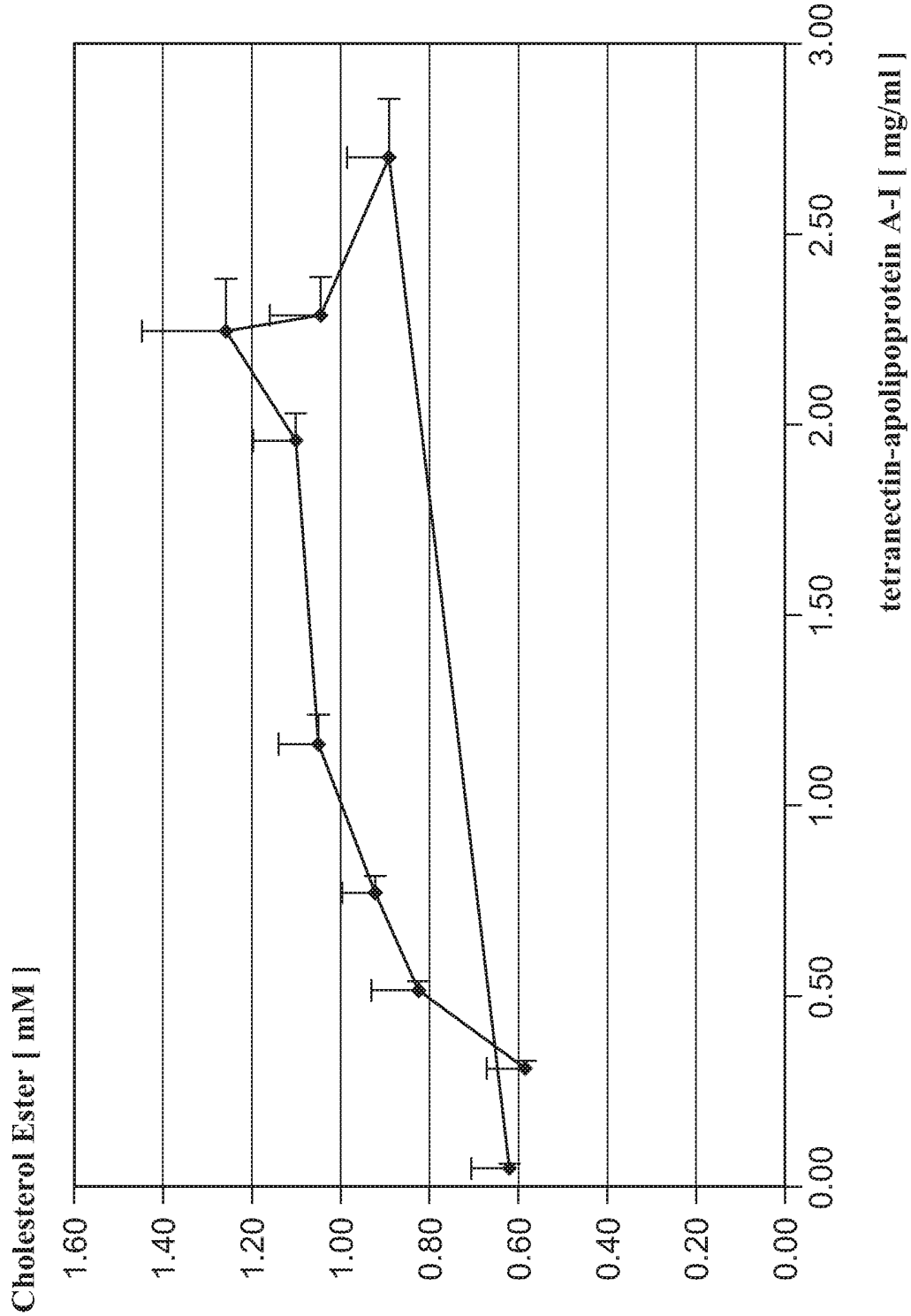
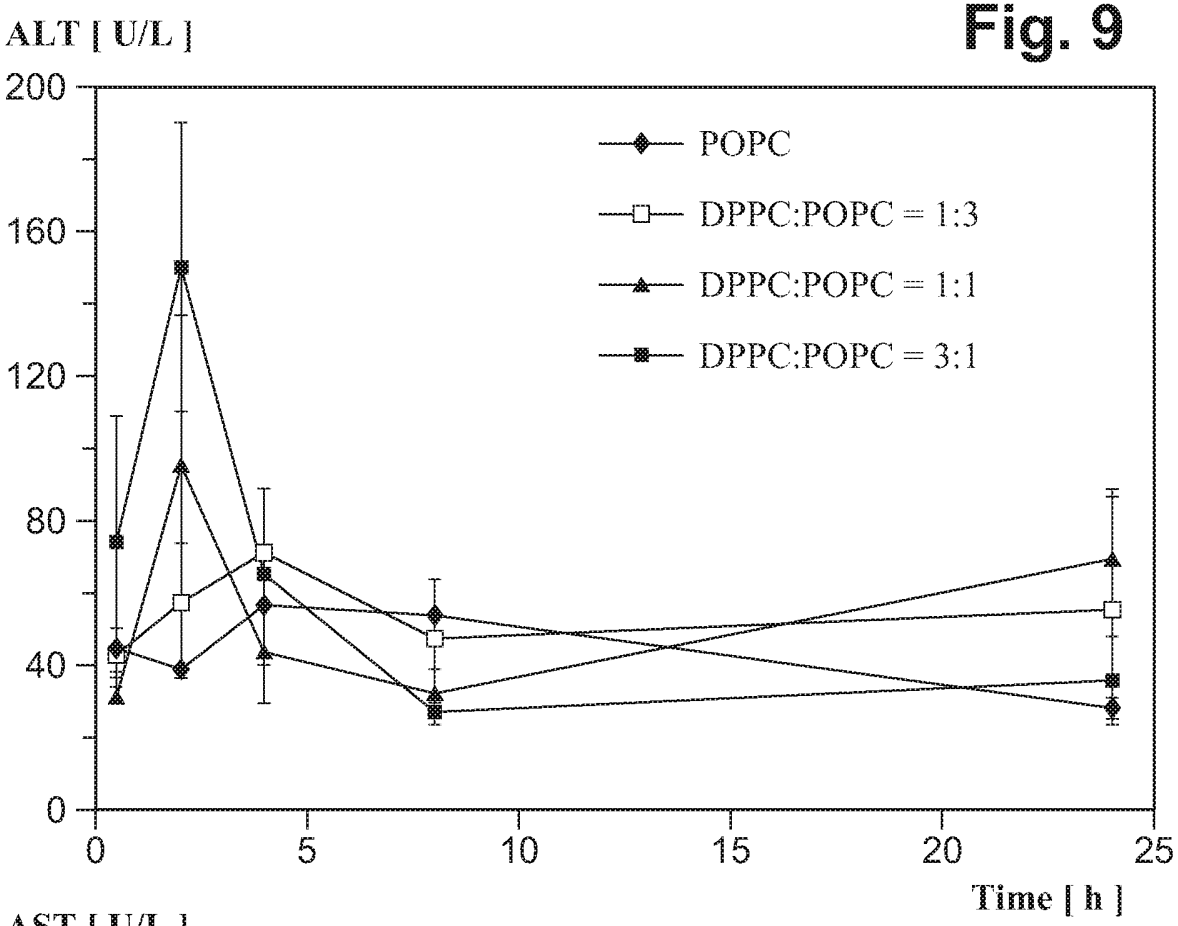

Cholesterol Ester [mM]
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
tetranectin-apolipoprotein A-I [mg/ml]

Fig. 9


ALT [U/L]
200
160
120
80
40
0
0
5
10
15
20
25
Time [h]
POPC
DPPC:POPC = 1:3
DPPC:POPC = 1:1
DPPC:POPC = 3:1

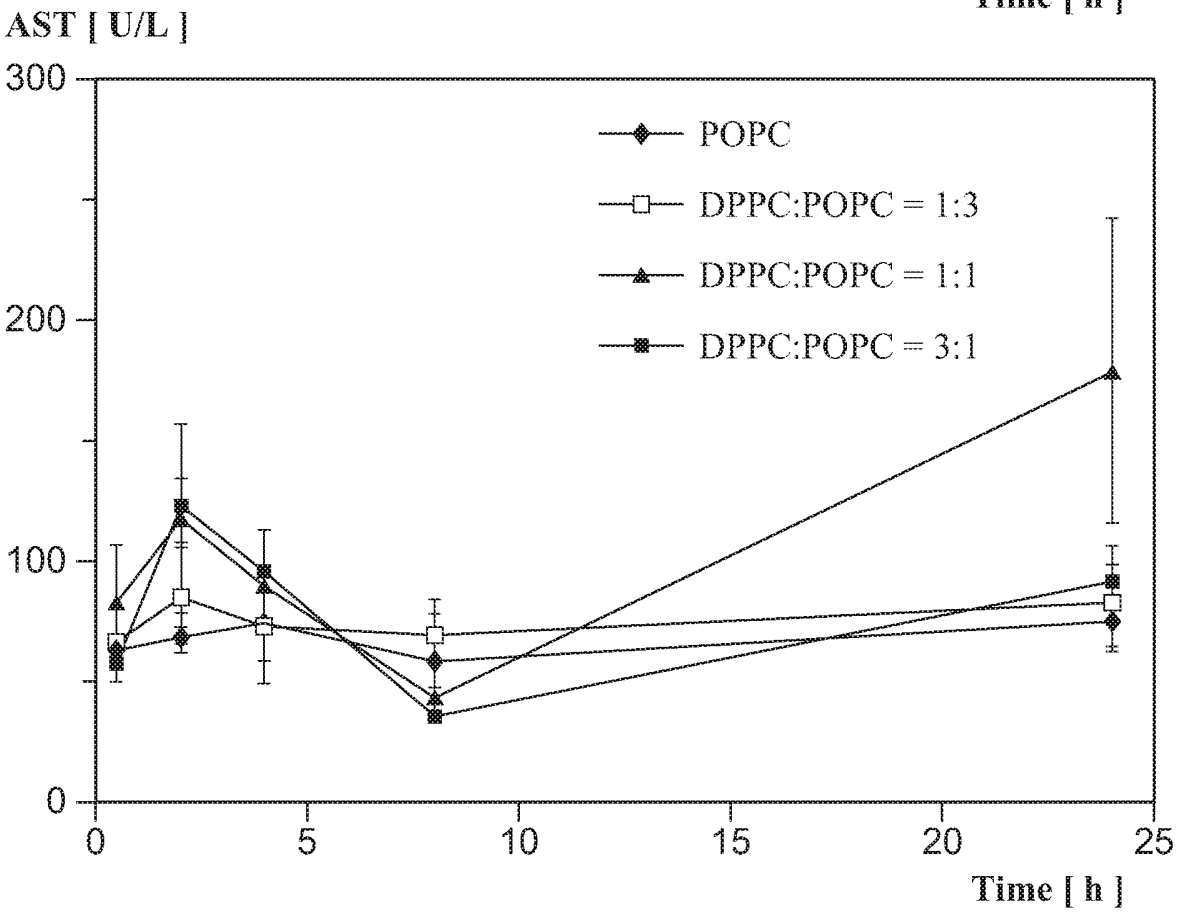
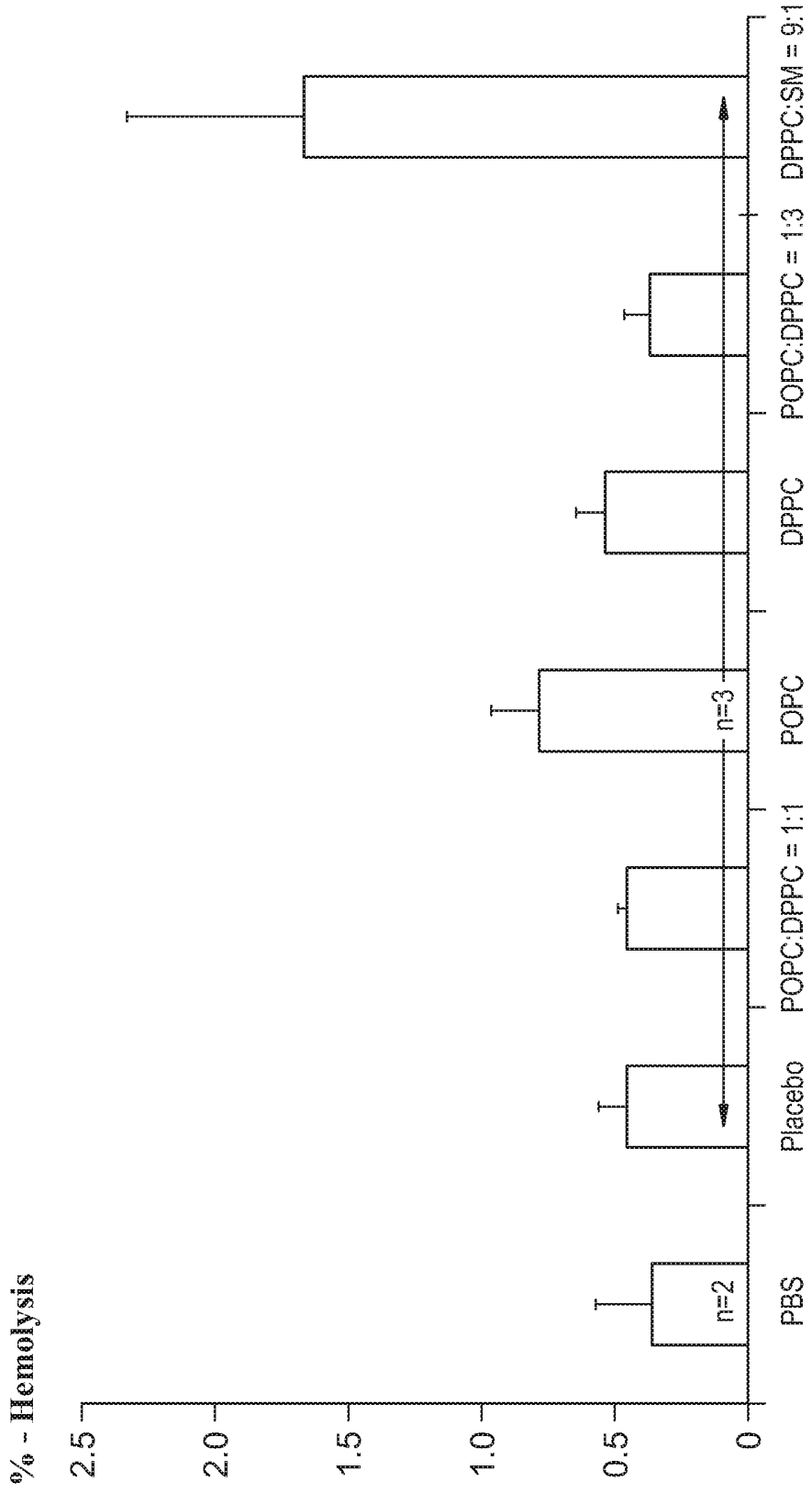

AST [U/L]
300
200
100
0
0
5
10
15
20
25
Time [h]
POPC
DPPC:POPC = 1:3
DPPC:POPC = 1:1
DPPC:POPC = 3:1

Fig. 10


% - Hemolysis
2.5
2.0
1.5
1.0
0.5
0
n=2
n=3
PBS
Placebo
POPC:DPPC = 1:1
POPC
DPPC
POPC:DPPC = 1:3
DPPC:SM = 9:1

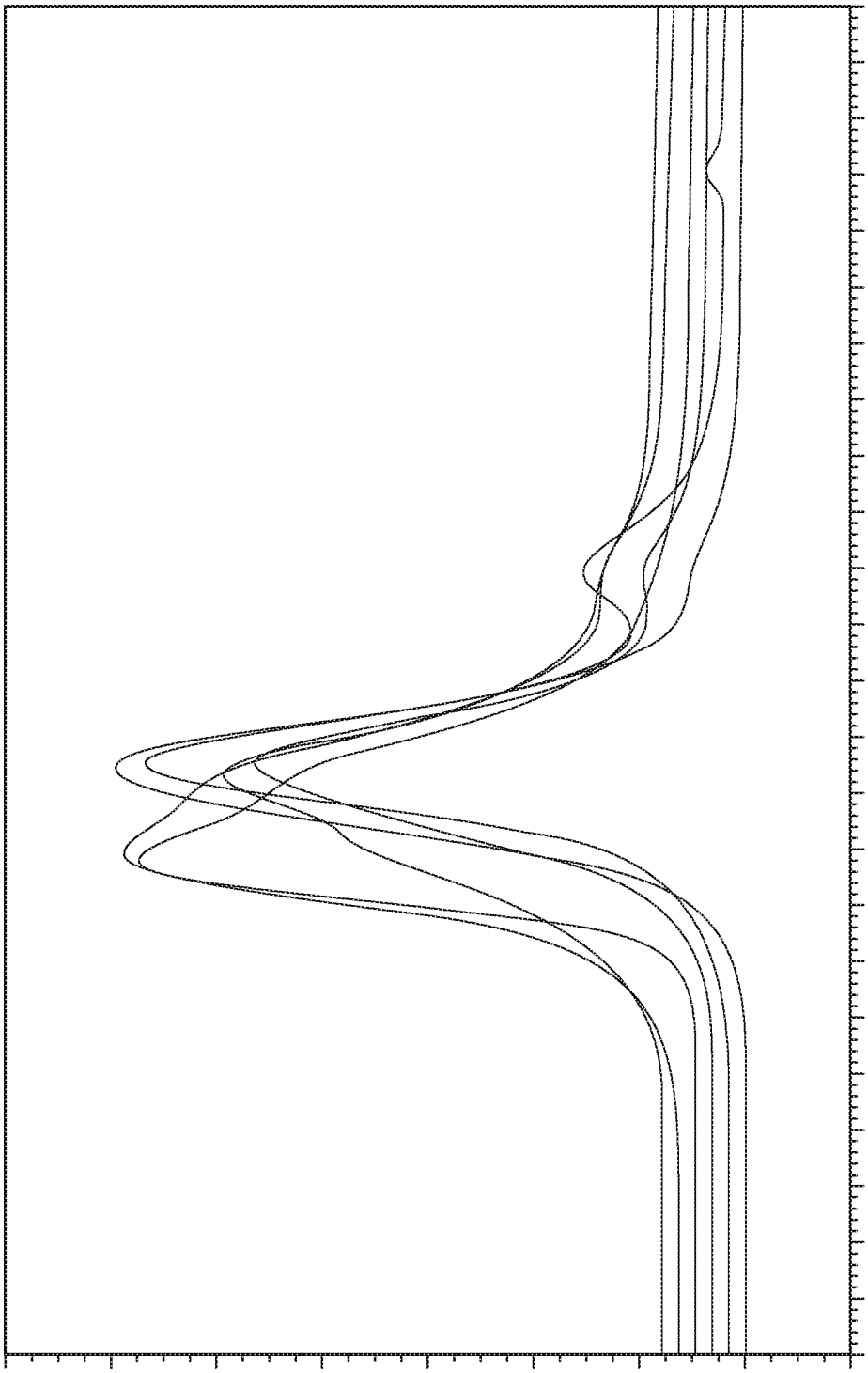


Fig. 11

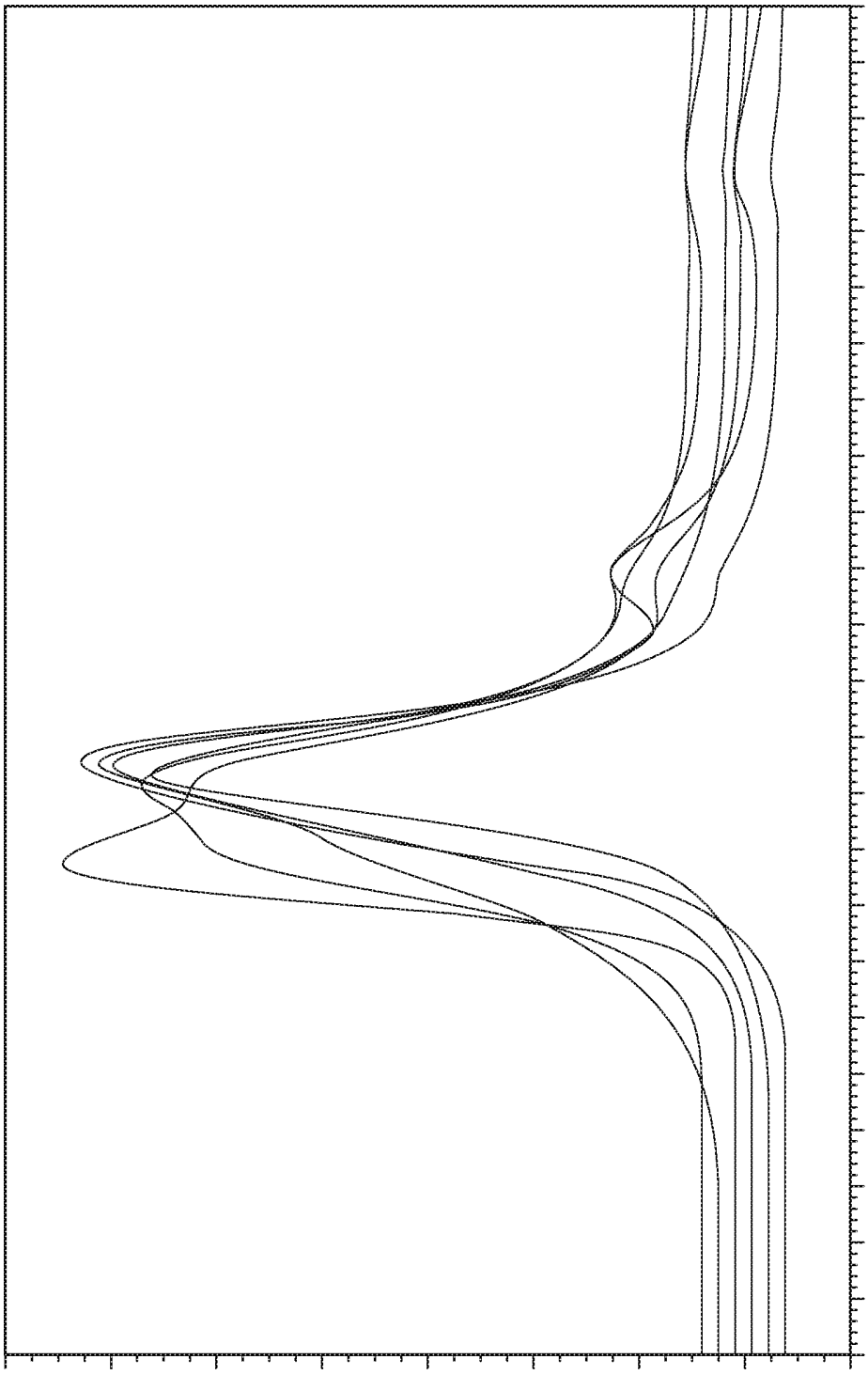
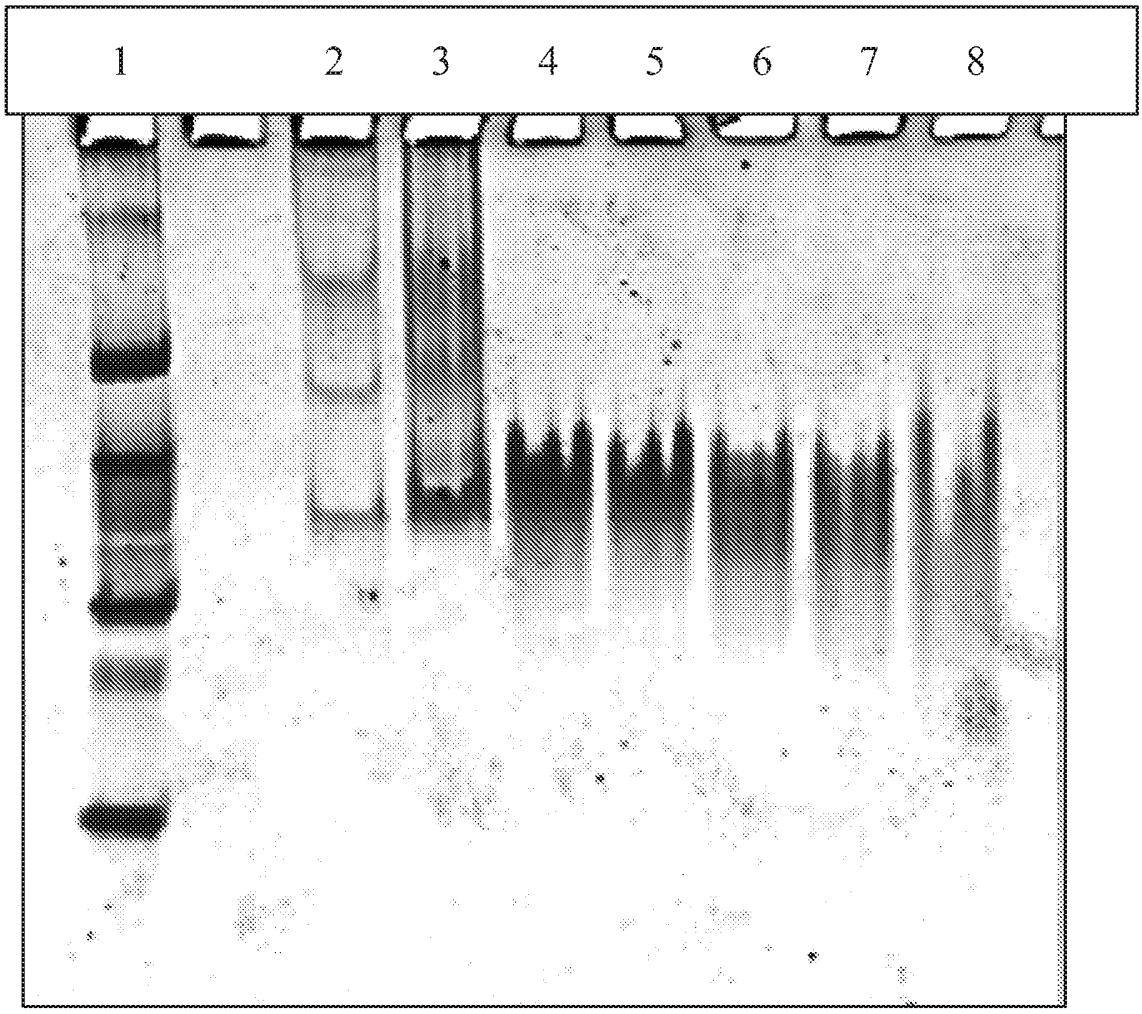


Fig. 12

Fig. 13



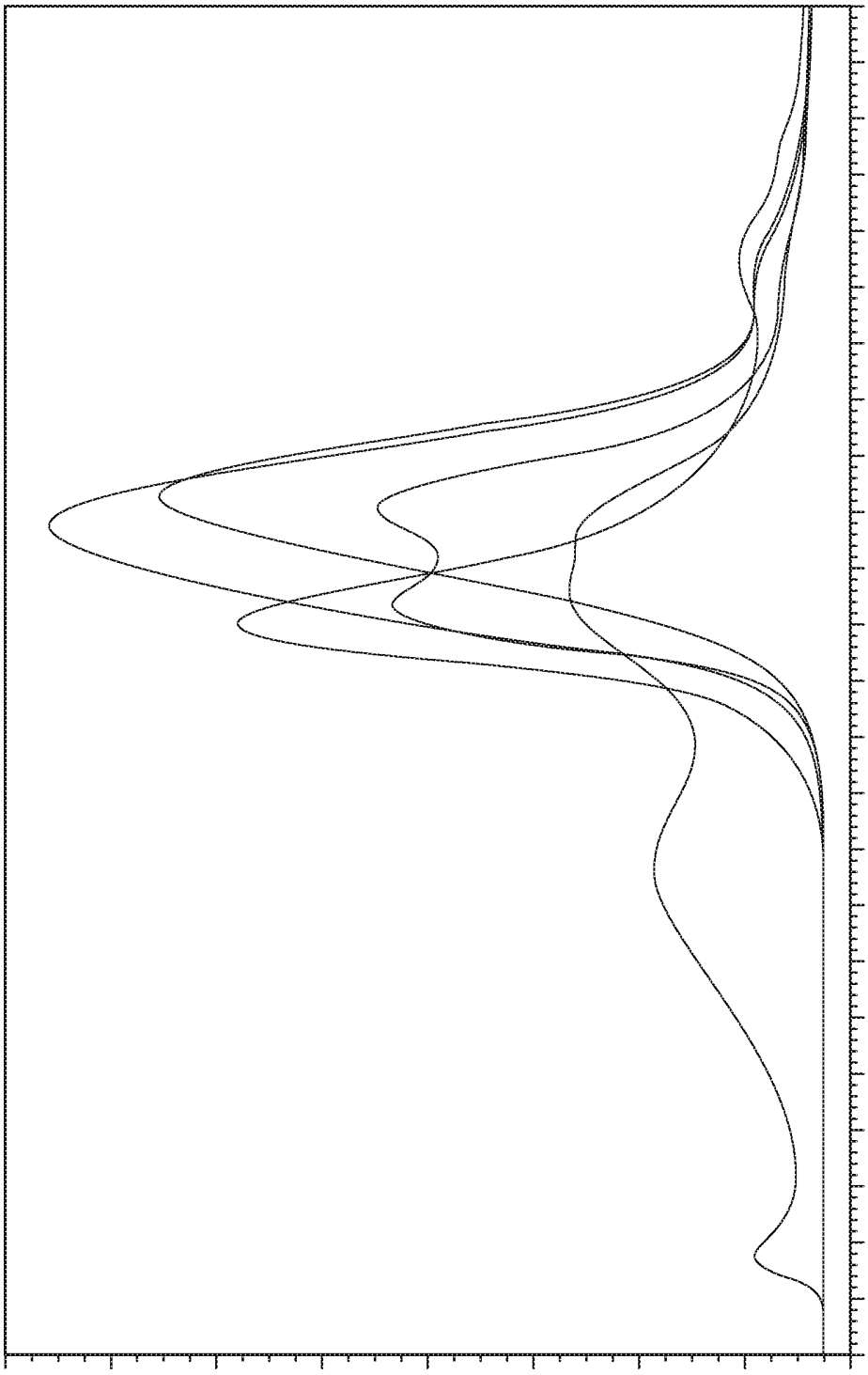


Fig. 14

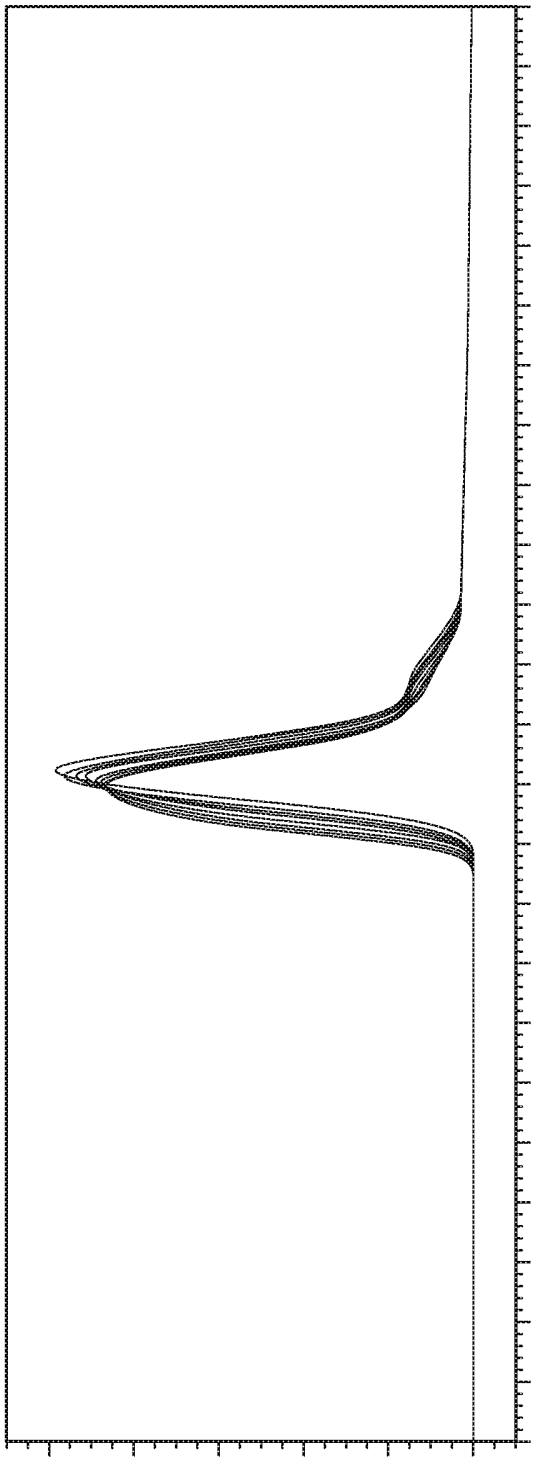


Fig. 15

Fig. 16

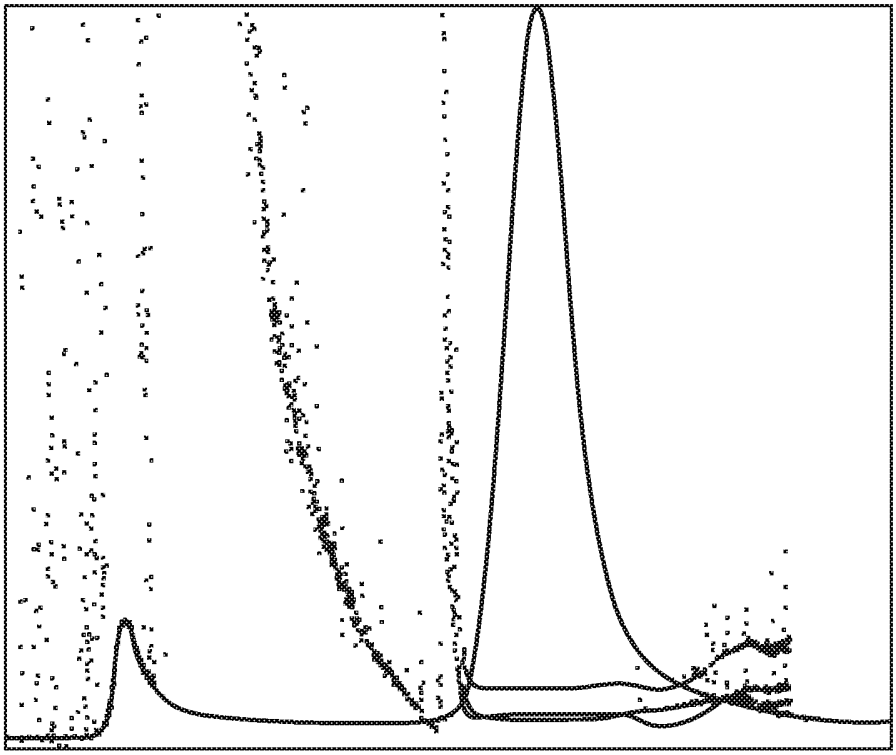
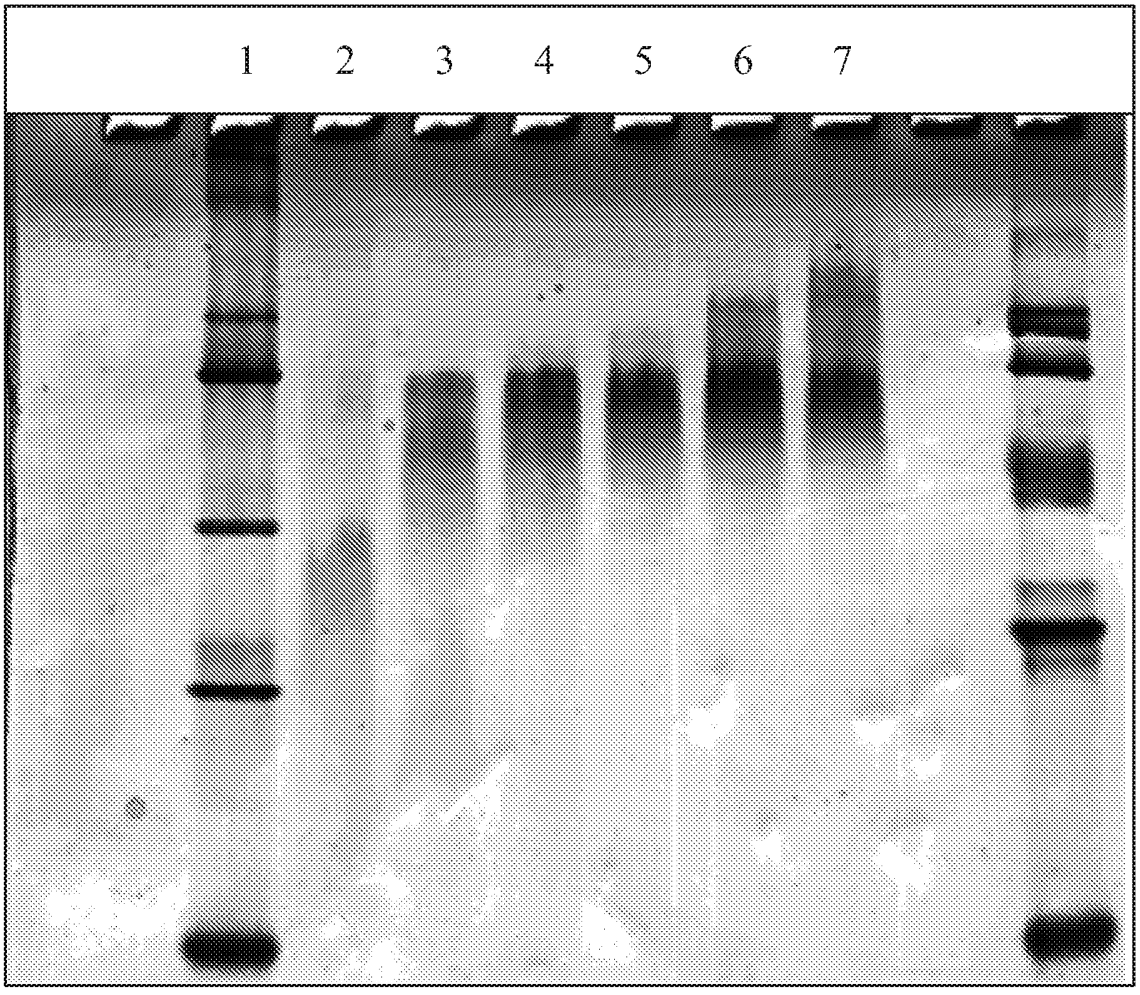


Fig. 17



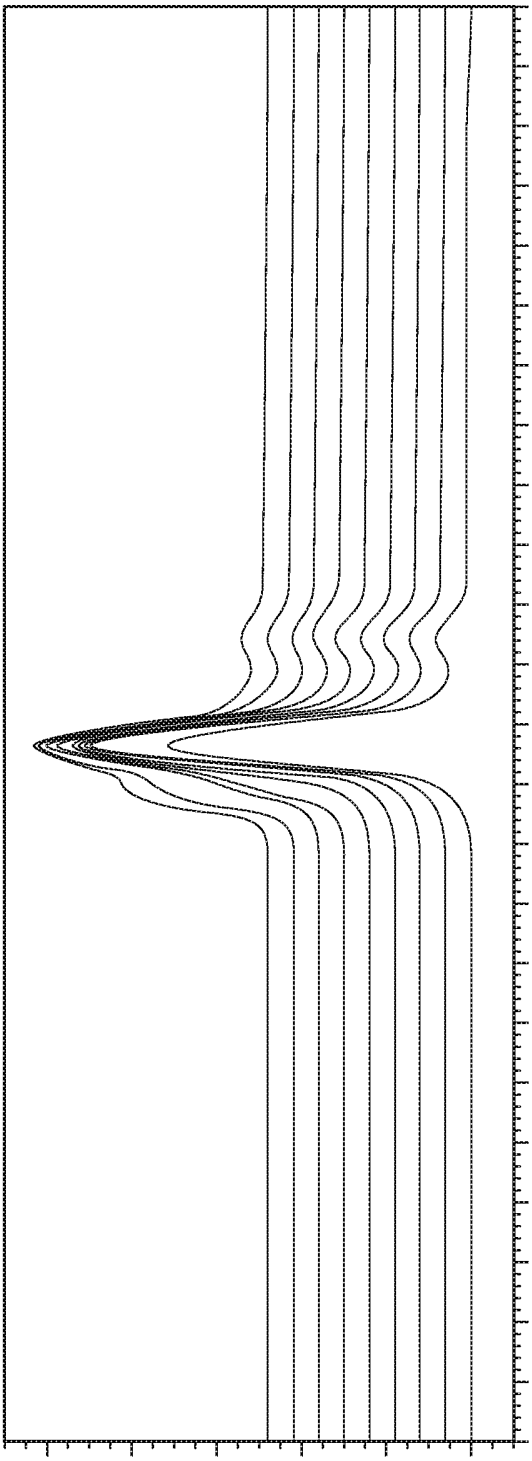


Fig. 18

Fig. 19

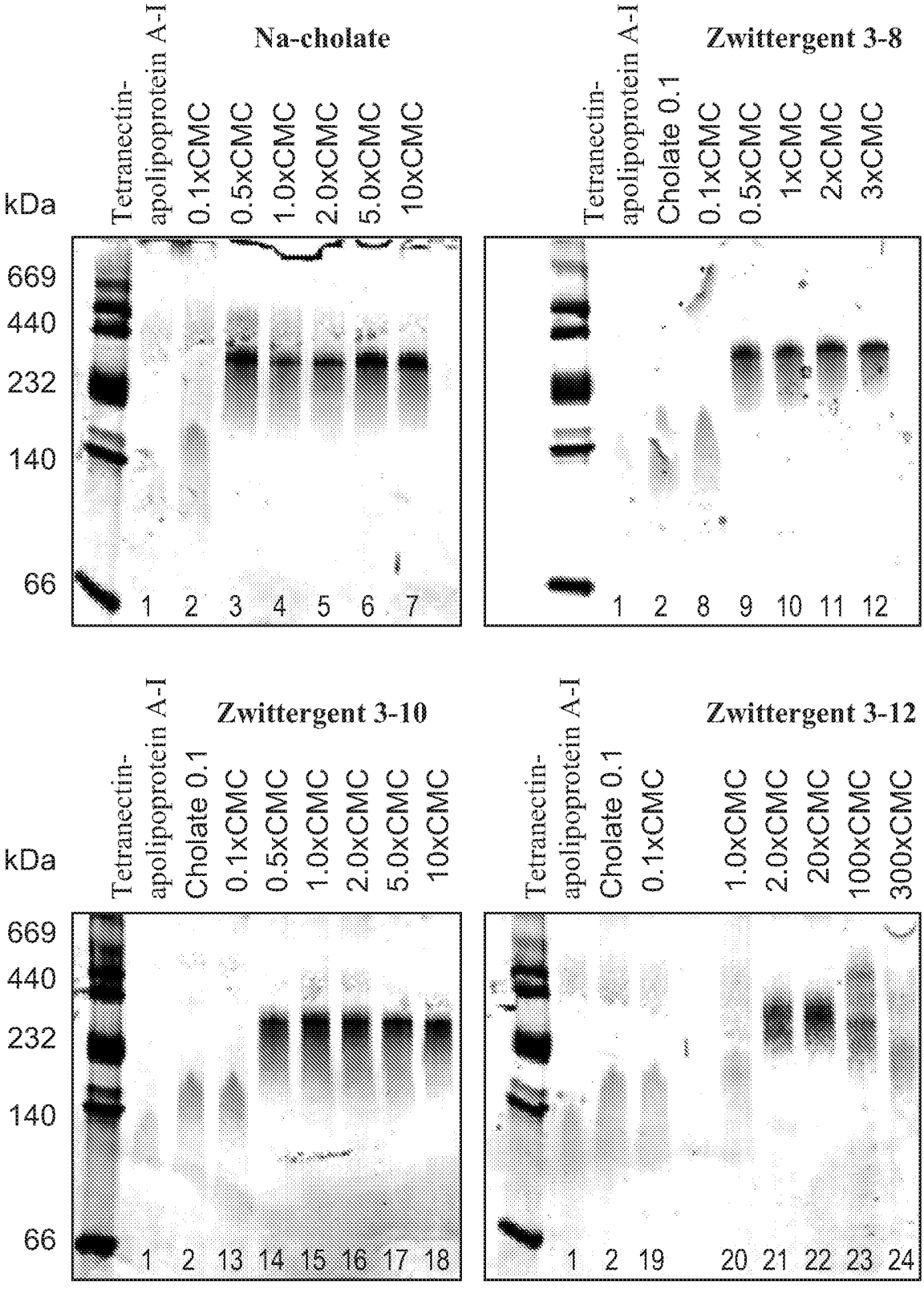

Na-cholate
Tetranectin-apolipoprotein A-I
0.1xCMC
0.5xCMC
1.0xCMC
2.0xCMC
5.0xCMC
10xCMC
kDa
669
440
232
140
66
1 2 3 4 5 6 7
Zwittergent 3-8
Tetranectin-apolipoprotein A-I
Cholate 0.1
0.1xCMC
0.5xCMC
1xCMC
2xCMC
3xCMC
1 2 8 9 10 11 12
Zwittergent 3-10
Tetranectin-apolipoprotein A-I
Cholate 0.1
0.1xCMC
0.5xCMC
1.0xCMC
2.0xCMC
5.0xCMC
10xCMC
kDa
669
440
232
140
66
1 2 13 14 15 16 17 18
Zwittergent 3-12
Tetranectin-apolipoprotein A-I
Cholate 0.1
0.1xCMC
1.0xCMC
2.0xCMC
20xCMC
100xCMC
300xCMC
1 2 19 20 21 22 23 24

Fig. 20

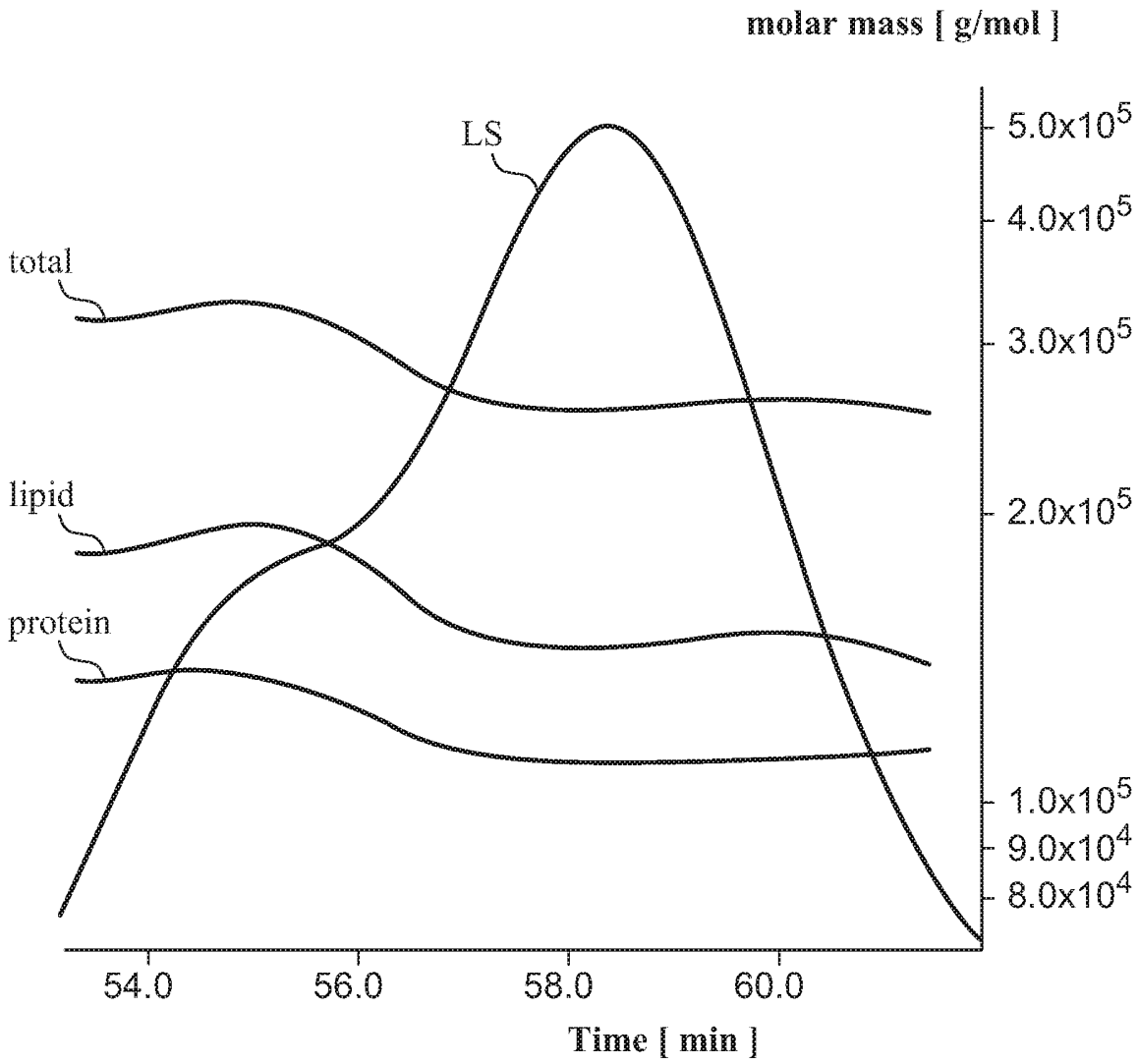

molar mass [g/mol]
LS
total
lipid
protein
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
9.0x10^4
8.0x10^4
54.0
56.0
58.0
60.0
Time [min]

Fig. 21

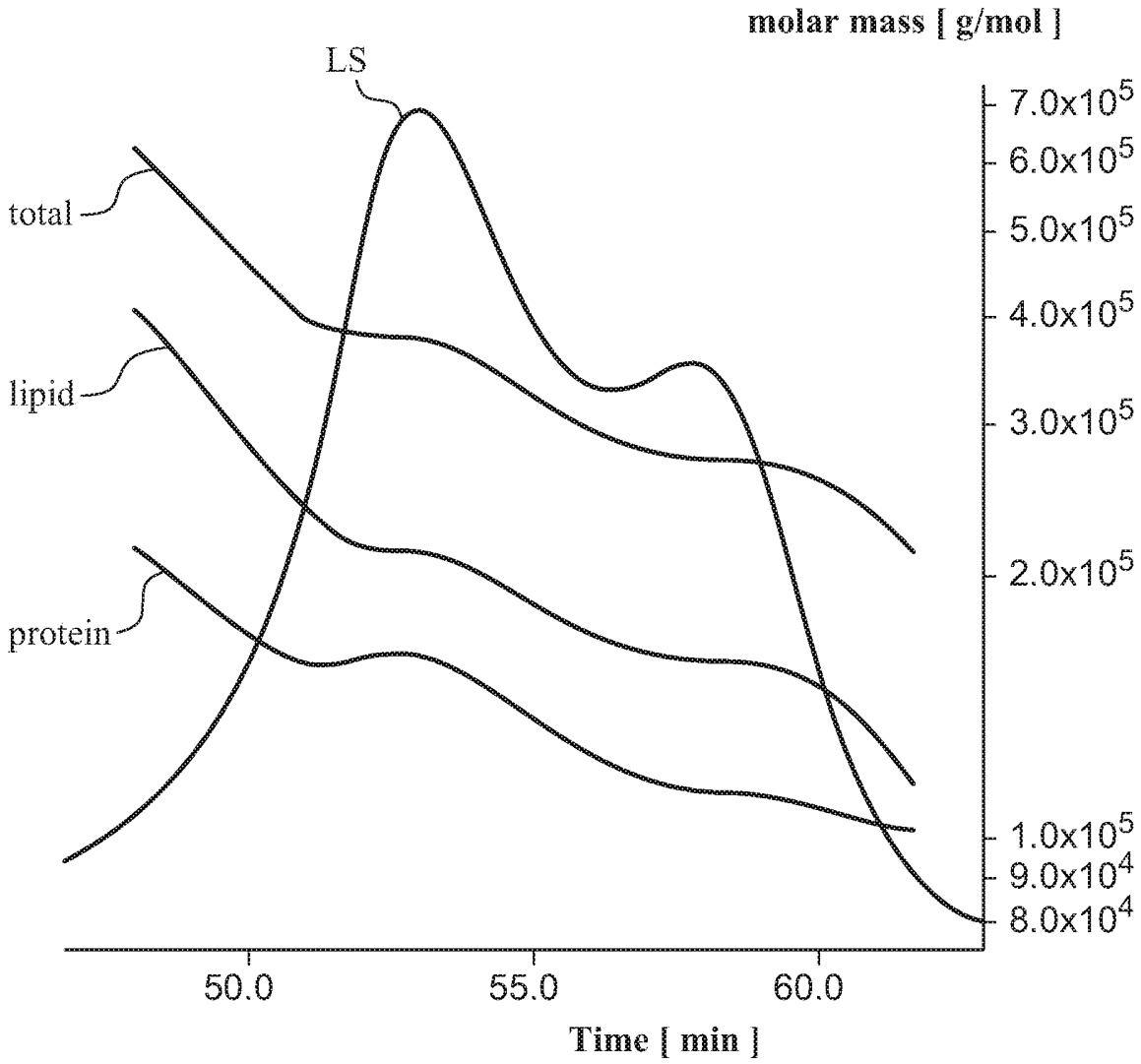
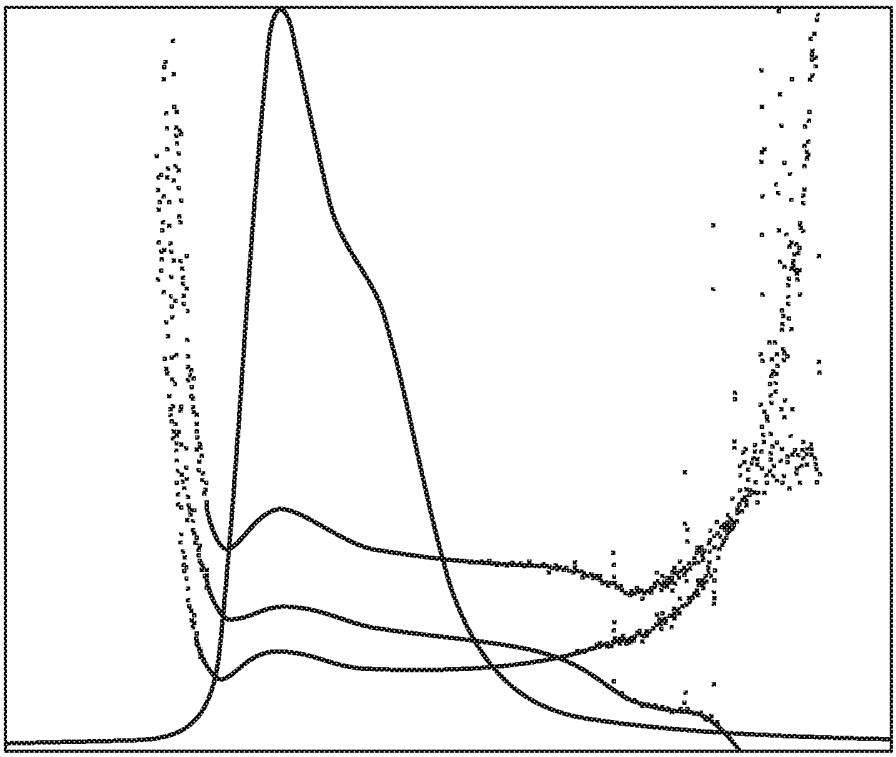

molar mass [g/mol]
LS
total
lipid
protein
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
9.0x10^4
8.0x10^4
50.0
55.0
60.0
Time [min]

Fig. 22



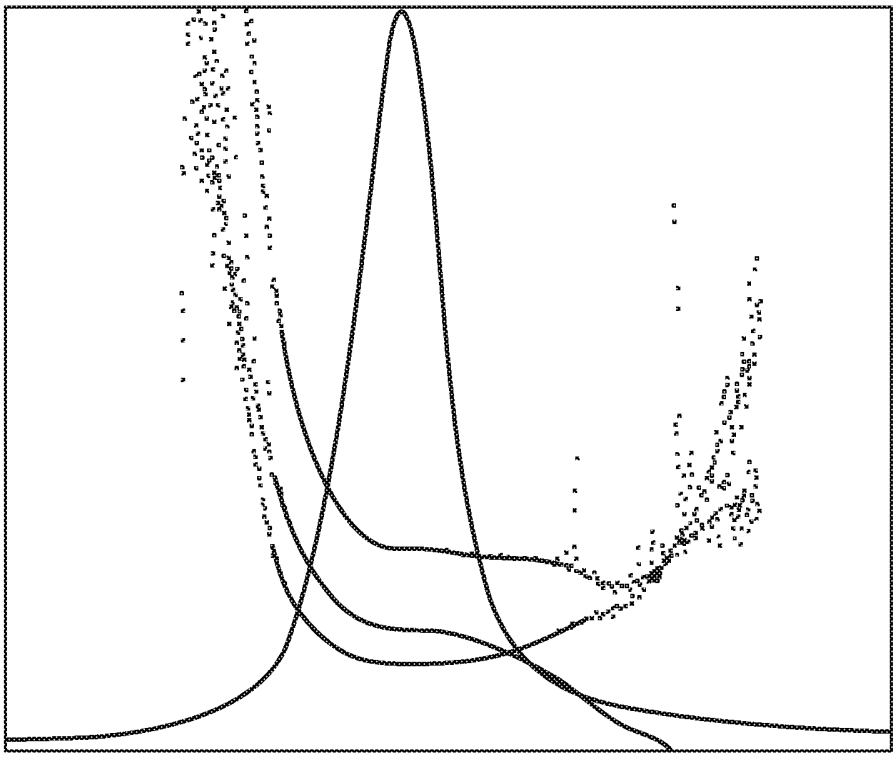
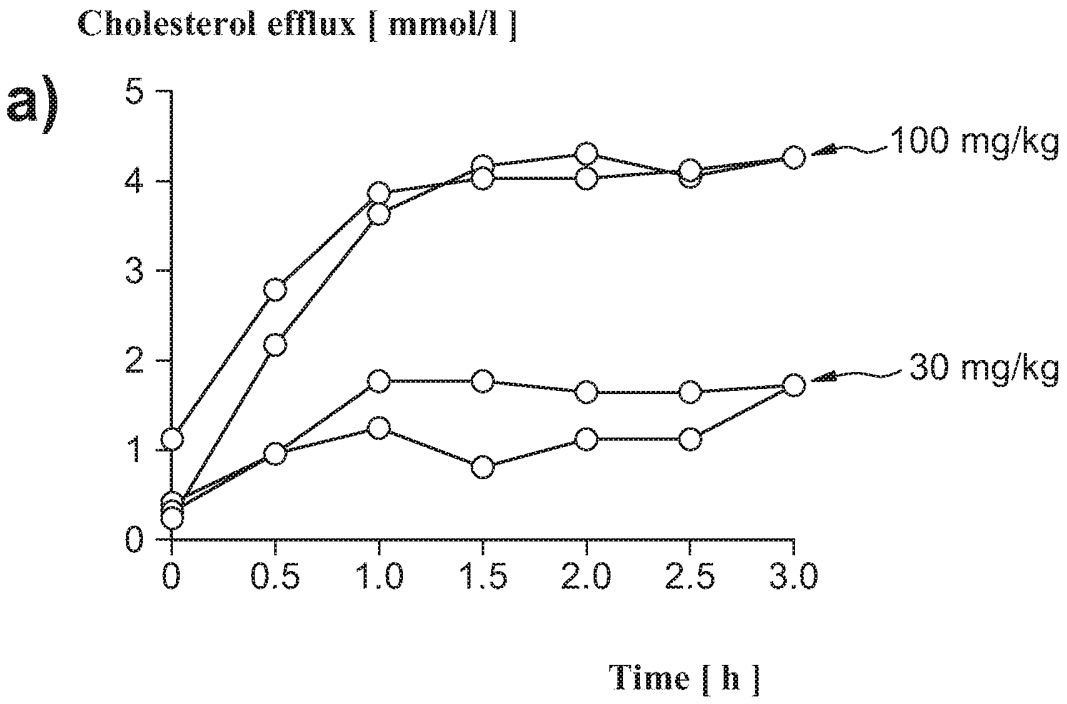


Fig. 23


Cholesterol efflux [mmol/l]
a)
5
4
3
2
1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
100 mg/kg
30 mg/kg
Time [h]

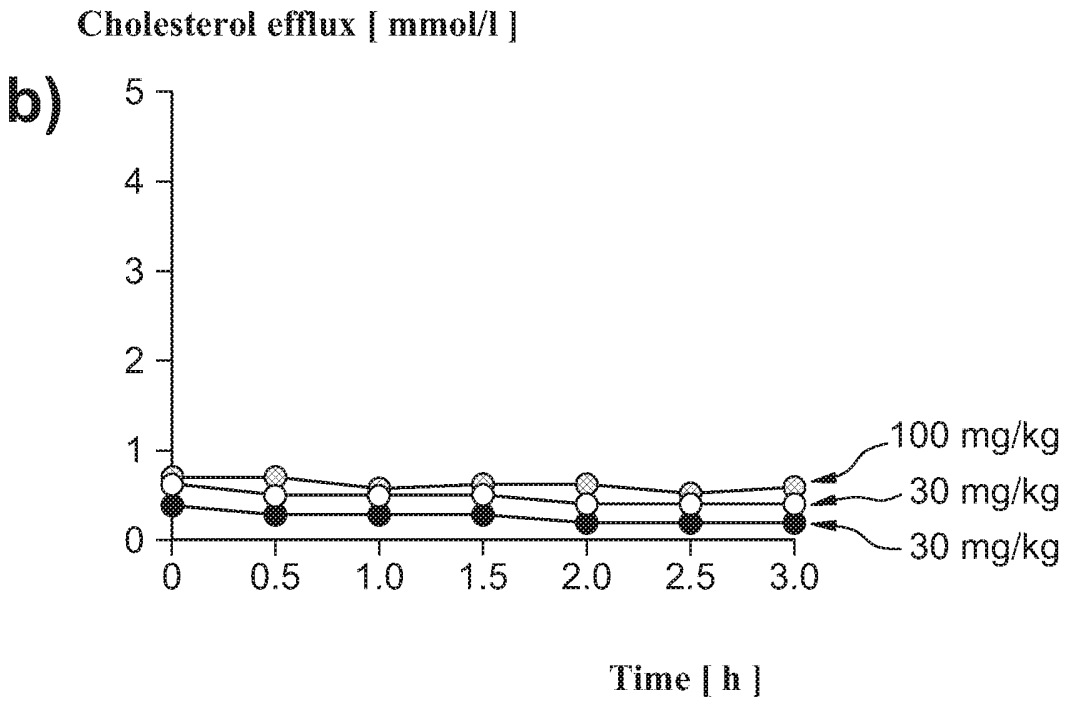
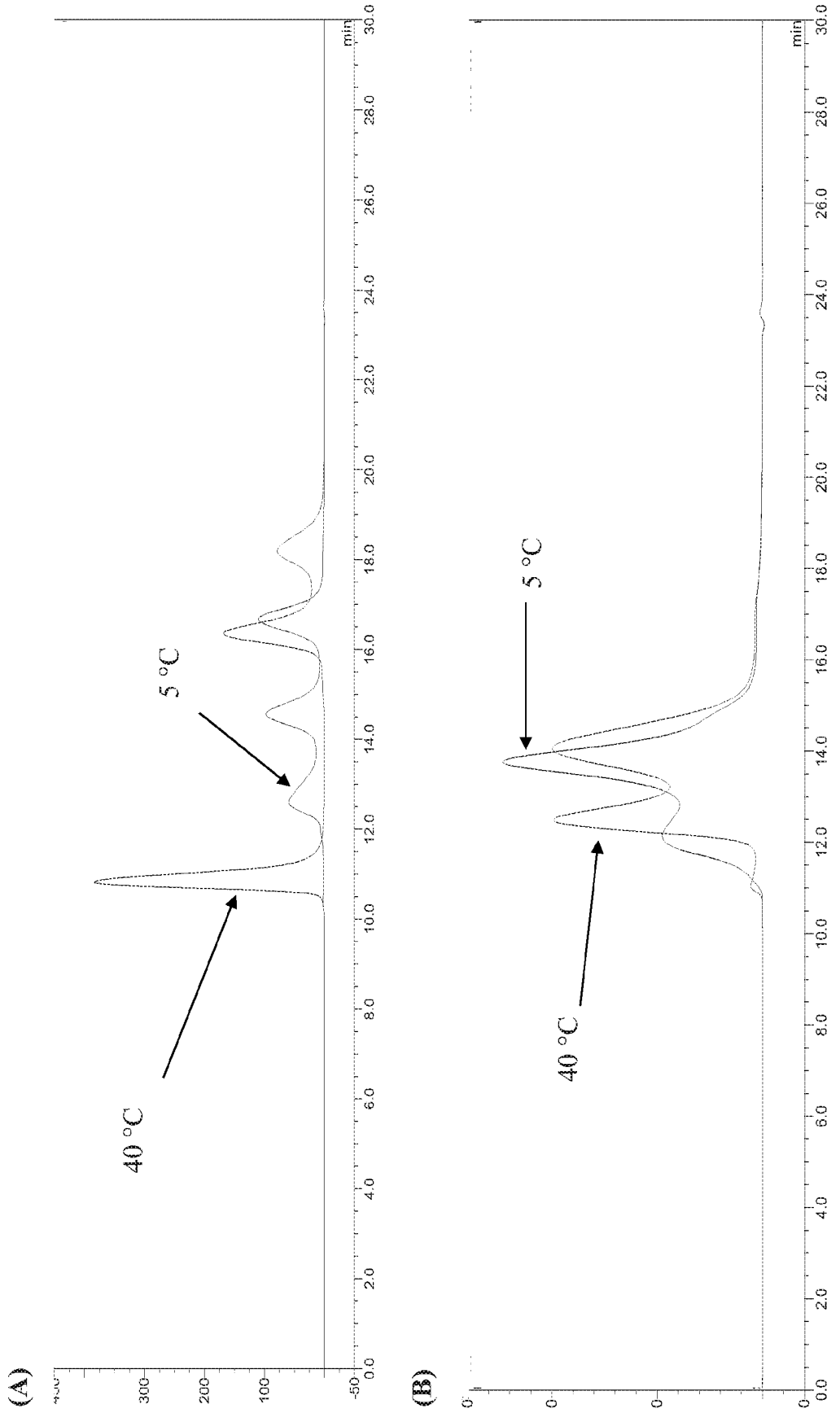

Cholesterol efflux [mmol/l]
b)
5
4
3
2
1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
100 mg/kg
30 mg/kg
30 mg/kg
Time [h]

Fig. 24



(19)中华人民共和国国家知识产权局

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(71)申请人 霍夫曼-拉罗奇有限公司
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(74)专利代理机构 中科专利商标代理有限责任公司 11021
代理人 程金山
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C12N 15/62(2006.01)
A61K 47/48(2006.01)

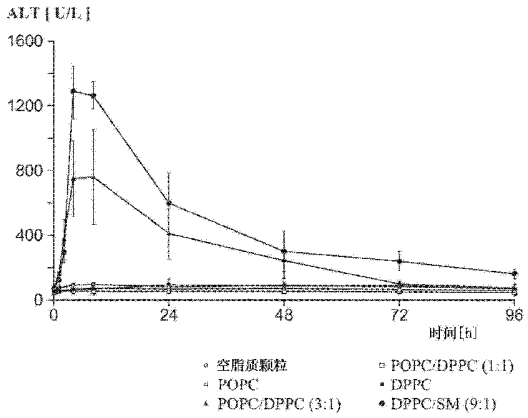
权利要求书1页 说明书51页
序列表66页 附图24页

(54)发明名称

产生四连蛋白-载脂蛋白A-I脂质颗粒的方法，脂质颗粒本身及其应用

(57)摘要

本文报道一种用于产生脂质颗粒的方法，所述方法包括下述步骤：i)提供包含变性载脂蛋白的第一溶液，ii)将所述第一溶液加入第二溶液中，所述第二溶液包含至少两种脂质和洗涤剂，但是不含载脂蛋白，和iii)从步骤ii)获得的溶液中去除所述洗涤剂并且由此产生脂质颗粒。



CN 103068368 A

1. 用于产生脂质颗粒的方法，所述方法包括下述步骤：

i) 提供包含变性蛋白的第一溶液，

ii) 将所述第一溶液加入包含至少一种脂质和洗涤剂但不含所述蛋白的第二溶液中，和

iii) 从在步骤 ii) 中获得的溶液中去除所述洗涤剂并且由此产生脂质颗粒。

2. 根据权利要求 1 所述的方法，其特征在于所述第二溶液具有所述第一溶液体积的约 3 倍到约 20 倍的体积。

3. 根据前述权利要求中任一项所述的方法，其特征在于所述第一溶液不含脂质。

4. 根据前述权利要求中任一项所述的方法，其特征在于所述蛋白具有选自 SEQ ID NO：01,02,04-52,66 或 67 的氨基酸序列的氨基酸序列，或包含至少一种包含 SEQ ID NO:01,02,04-52,66 或 67 的氨基酸序列的至少 80％的连续片段。

5. 根据权利要求 4 所述的方法，其特征在于所述蛋白是具有 SEQ IDNO:01 或 SEQ ID NO:02 或 SEQ ID NO:66 或 SEQ ID NO:67 的氨基酸序列的四连蛋白 - 载脂蛋白 A-I。

6. 根据前述权利要求任一项的方法，其特征在于所述至少一种脂质是两种不同的磷脂酰胆碱。

7. 根据权利要求 6 所述的方法，其特征在于第一磷脂酰胆碱是 POPC，并且第二磷脂酰胆碱是 DPPC。

8. 根据前述权利要求中任一项所述的方法，其特征在于所述洗涤剂选自胆酸、两性洗涤剂或它们的盐。

9. 根据前述权利要求中任一项所述的方法，其特征在于所述方法在步骤 ii) 之后和步骤 iii) 之前包括下述步骤：

iia) 温育在步骤 ii) 中获得的溶液。

10. 根据前述权利要求中任一项所述的方法，其特征在于所述温育和 / 或去除在 4℃ -45℃的温度进行。

11. 根据权利要求 9 和 10 中任一项所述的方法，其特征在于所述温育进行约 2 小时到约 60 小时。

12. 根据前述权利要求中任一项所述的方法，其特征在于所述洗涤剂是具有高 CMC 的洗涤剂。

13. 根据前述权利要求中任一项所述的方法，其特征在于所述去除通过渗滤或透析或吸附进行。

14. 通过权利要求 1-13 中任一项所述的方法获得的脂质颗粒。

15. 药物组合物，所述药物组合物包含权利要求 14 所述的脂质颗粒。

产生四连蛋白-载脂蛋白A-I脂质颗粒的方法，脂质颗粒本身及其应用

[0001] 本发明涉及脂蛋白和脂质颗粒领域。本文报道了产生包含载脂蛋白、磷脂酰胆碱和脂质的脂质颗粒的方法，以及四连蛋白-载脂蛋白A-I。

[0002] 发明背景

[0003] 血浆脂蛋白是可溶性的蛋白-脂质复合物，其在血液中进行脂质运输和代谢。基于它们的密度、尺寸、化学组成和功能，对数种主要种类的脂蛋白进行了区分。在它们之中，也被称为高密度脂质颗粒的高密度脂蛋白(HDL)颗粒由平均分子量从180kD变化到360kDa的数个亚类组成。它们的平均脂质和蛋白含量分别是50重量%。磷脂酰胆碱(PC)占总脂质的38%，其次是胆固醇酯和小量的其他极性和非极性脂质，包括游离的胆固醇。主要的蛋白成分是载脂蛋白A-I(Apo A-I)，代表人HDL中约60%的总蛋白重量。

[0004] 在人体中的胆固醇，尤其是在循环的体液如血液中的胆固醇，不以分离的分子形式存在，而是以与某些蛋白的复合物(脂蛋白)形式存在。胆固醇的主要级分与低密度脂蛋白(LDL)或与高密度脂蛋白(HDL)复合。LDL颗粒包含载脂蛋白B作为主要的蛋白质化合物，而HDL颗粒包含载脂蛋白A-I作为主要的蛋白质化合物。

[0005] 由HDL颗粒吸收的胆固醇被酶卵磷脂胆固醇酰基转移酶(LCAT)酯化。胆固醇酯具有增加的疏水性并朝向HDL颗粒的核心扩散。HDL-胆固醇-酯颗粒可以被递送到肝脏并从循环中去除。

[0006] HDL颗粒和其主要的多肽载脂蛋白A-I参与反向胆固醇运输(RCT)。其中载脂蛋白A-I增加胆固醇从细胞中的流出(例如从血管壁的细胞中流出)，脂质的结合和卵磷脂胆固醇乙酰转移酶的激活，并由此通过肝脏经血浆流动清除胆固醇。这是主动运输过程，涉及细胞膜蛋白ATP-结合-盒式-转运蛋白-A-I(ABCA-I)。

[0007] 基于载脂蛋白A-I和载脂蛋白的治疗剂，例如重构的HDL颗粒已经在上世纪的70年代晚期和80年代早期进行了鉴定。对于包含脂质颗粒的载脂蛋白A-I-Milano，可以显示临床证据(意味着在动脉硬化患者中显著的斑块减少)。载脂蛋白A-I-Milano是一种野生型载脂蛋白A-I的二聚体形式，根据载脂蛋白A-I分子天然存在的突变体进行设计。通过将氨基酸残基173(精氨酸)改变为半胱氨酸从而允许形成二硫键能够形成二聚体。

[0008] 在WO2009/131704中，报道了适合螯合胆固醇和其他分子的包含含有无机材料核心的纳米结构。在WO2009/097587中报道了用于产生纳米级别的界面双层的方法，包括在获得混合物的约1小时内从中间体混合物中消除洗涤剂。在WO2006/125304中，报道了治疗或预防冠状动脉疾病的药物组合物。在US2002/10142953中报道了涉及脂质代谢和心血管疾病的编码载脂蛋白的组合物。在WO2005/084642中，报道了脱辅基蛋白质-共螯合剂(apoprotein-cochelate)组合物。在WO2007/137400中，报道了治疗瓣膜狭窄的方法和化合物。在WO2005/041866中报道了用于治疗和预防急性冠状动脉综合征的药物制剂、方法和给药方案。

[0009] 在US6,287,590中，报道了通过共-冷冻干燥形成的肽/脂质复合物。在US6,037,323中报道了载脂蛋白A-I激动剂以及它们用于治疗血脂异常疾病

(dislipidemic disorders) 的应用。

[0010] 在 WO2009/097587 中，报道了纳米级别的界面双层、应用和产生方法。在 WO2005/065708 中报道了具有提高的耐受性的在免疫原性组合物中的疏水蛋白制剂。在 WO2006/069371 中，报道了预防、抑制和 / 或逆转动脉粥样硬化的血浆脂质化方法。在 FR2915490 中报道了包含膜蛋白的蛋白脂质体 (proteoliposomes) 的形成。

[0011] 发明概述

[0012] 本文报道了一种用于产生包含蛋白的脂质颗粒的方法。已经发现可以从包含变性蛋白的溶液通过快速稀释到包含至少一种脂质和洗涤剂的溶液中形成脂质颗粒。在该步骤中，洗涤剂的浓度被减少到 CMC 以下。使用这种方法，可以省去前面的产生天然性质 (naturation) 步骤，并且因此，使用如本文报道的方法，能够更快的产生脂质颗粒。

[0013] 在一个实施方案中，稀释是约 1 ： 3(v ： v) 到约 1 ： 20(v ： v)。

[0014] 在一个实施方案中，稀释是约 1 ： 5(v ： v) 到约 1 ： 10(v ： v)。

[0015] 在一个实施方案中，稀释是约 1 ： 5(v ： v)。

[0016] 在一个实施方案中，洗涤剂被稀释至少约 3 倍。在一个实施方案中，洗涤剂被稀释至少约 5 倍。

[0017] 本文报道的一个方面是用于产生脂质颗粒的方法，所述方法包括下述步骤 ：

[0018] i) 提供包含变性蛋白的第一溶液，

[0019] ii) 将第一溶液加入包含至少一种脂质和洗涤剂但不包含所述蛋白的第二溶液中，和

[0020] iii) 从在步骤 ii) 中获得的溶液去除洗涤剂并且由此产生脂质颗粒。

[0021] 在一个实施方案中，第一溶液不包含脂质。

[0022] 在一个实施方案中，蛋白是重组产生的蛋白。

[0023] 在一个实施方案中，蛋白是载脂蛋白。在另一个实施方案中，载脂蛋白是纯化的载脂蛋白。

[0024] 在一个实施方案中，载脂蛋白具有选自 SEQ ID NO :01,02 和 04-52 和 66-67 的氨基酸序列的氨基酸序列或包含至少一个含有 SEQ ID NO :01,02 和 04-52 和 66 和 67 的氨基酸序列的至少 80％的连续片段。

[0025] 在一个实施方案中，载脂蛋白具有氨基酸序列或是选自 SEQ ID NO :01,02 和 04-52 和 66 和 67 的氨基酸序列的至少 80％的连续片段。

[0026] 在一个实施方案中，载脂蛋白是载脂蛋白 A-I。在一个实施方案中，载脂蛋白 A-I 是人载脂蛋白 A-I。在另一个实施方案中，载脂蛋白是四连蛋白 - 载脂蛋白 A-I，其具有氨基酸序列 SEQ ID NO :01 或 SEQ ID NO :02 或 SEQ ID NO :66 或 SEQ ID NO :67。

[0027] 在一个实施方案中，载脂蛋白具有带有选自 R151C 和 R197C 的突变 SEQ ID NO :06 的氨基酸序列。

[0028] 在一个实施方案中，第二溶液具有是所述第一溶液体积的至少两倍的体积。

[0029] 在一个实施方案中，所述第二溶液具有所述第一溶液体积的约 3 倍到约 20 倍。在一个实施方案中，所述第二溶液具有所述第一溶液体积的约 5 倍到约 10 倍。

[0030] 在一个实施方案中，至少一种脂质选自磷脂、脂肪酸和类固醇脂质。

[0031] 在一个实施方案中，至少一种脂质是任选地彼此独立地选自磷脂、脂肪酸和类固

醇脂质的至少两种脂质。在另一个实施方案中，至少一种脂质来自 1-4 种脂质，即选自包括一种脂质、两种脂质、三种脂质和四种脂质的组。

[0032] 在一个实施方案中，第二溶液包含磷脂、脂质和洗涤剂。

[0033] 在一个实施方案中，第二溶液由磷脂、脂质、洗涤剂和缓冲盐组成。

[0034] 在一个实施方案中，脂质是两种不同的磷脂。在另一种实施方案中，脂质是两种不同的磷脂酰胆碱。在另一个实施方案中，第一磷脂酰胆碱和第二磷脂酰胆碱的不同之处在于一个或两个脂肪酸残基或脂肪酸残基衍生物，其被酯化为磷脂酰胆碱的甘油主链。在一个实施方案中，第一磷脂酰胆碱是 POPC，第二磷脂酰胆碱是 DPPC。

[0035] 在一个实施方案中，洗涤剂选自基于糖的洗涤剂，基于聚氧乙烯的洗涤剂，基于胆汁盐的洗涤剂，合成洗涤剂或其组合。在另一个实施方案中，洗涤剂选自胆酸，两性洗涤剂 (Zwittergent) 或它们的盐。

[0036] 在本文报道的方法的一个实施方案中，第一溶液基本不含脂质颗粒。

[0037] 在一个实施方案中，所述方法在步骤 ii) 之后和步骤 iii) 之前包括一个下述步骤 iia) ：温育在步骤 ii) 中获得的溶液。在一个实施方案中，温育和 / 或去除在从 4℃ -45℃的温度进行。

[0038] 在一个实施方案中，用洗涤剂温育多肽约 0.5 小时到约 60 小时。在一个实施方案中，用洗涤剂温育多肽约 0.5 小时到约 20 小时。在一个实施方案中，用洗涤剂温育多肽约 2 小时到约 60 小时。在一个实施方案中，用洗涤剂温育多肽约 12 小时到约 20 小时。在一个实施方案中，用洗涤剂温育多肽约 16 小时。

[0039] 在一个实施方案中，洗涤剂是具有高 CMC 的洗涤剂。在另一个实施方案中，洗涤剂是具有至少 5mM 的 CMC 的洗涤剂。在另一个实施方案中，洗涤剂是具有至少 10mM 的 CMC 的洗涤剂。

[0040] 在一个实施方案中，洗涤剂的浓度在第二溶液中是至少 0.5×CMC。

[0041] 在一个实施方案中，通过渗滤或透析或吸附来进行去除。在一个实施方案中，吸附选自亲和色谱法或疏水色谱法。在一个实施方案中，去除通过透析进行。

[0042] 在一个实施方案中，第一溶液具有第一体积，第二溶液具有第二体积，在第一溶液中的蛋白具有限定的浓度，并且在第二溶液中的脂质和洗涤剂各具有限定的浓度，并且在步骤 ii) 中，载脂蛋白的浓度，脂质的浓度和洗涤剂的浓度被改变 / 减少，允许形成脂质颗粒。

[0043] 在一个实施方案中，所述方法包括下述步骤 ：

[0044] iv) 纯化脂质颗粒并由此制备脂质颗粒。

[0045] 本文报道的一个方面是通过如本文报道的方法获得的脂质颗粒。

[0046] 本文报道的一个方面是药物组合物，所述药物组合物包含用本文报道的方法获得的含有载脂蛋白的脂质颗粒，以及将本文报道的脂质颗粒用于制备治疗动脉粥样硬化的药物的应用。

[0047] 发明详述

[0048] 定义

[0049] 术语“载脂蛋白”指分别在脂质或脂蛋白颗粒中包含的蛋白。

[0050] 术语“载脂蛋白 A-I”指具有蛋白 - 脂质和蛋白 - 蛋白相互作用性质的两亲性螺

旋多肽。载脂蛋白 A-I 作为 267 个氨基酸残基的前载脂蛋白原 (prepro-apoliprotein) 由肝脏和小肠合成，所述前载脂蛋白原作为载脂蛋白原 (pro-apolipoprotein) 被分泌，其被切割为具有 243 个氨基酸残基的成熟多肽。载脂蛋白 A-I 由被接头部分分隔的 6-8 个不同的氨基酸重复序列组成，所述氨基酸重复序列分别由 22 个氨基酸残基组成，所述接头部分通常是脯氨酸，并且在某些情形中，由数个残基组成的片段组成。示例性的人载脂蛋白 A-I 氨基酸序列在 GenPept 数据库登录号 NM-000039 或数据库登录号 X00566 ;GenBank NP-000030.1(gi4557321) 中报道。人载脂蛋白 A-I(SEQ ID NO :06) 存在天然存在的变体，如 P27H，P27R，P28R，R34L，G50R，L84R，D113E，A-A119D，D127N，缺失 K131，K131M，W132R，E133K，R151C(氨基酸残基 151 由 Arg 变化为 Cys，载脂蛋白 A-I-Paris)，E160K，E163G，P167R，L168R，E171V，P189R，R197C(氨基酸残基 173 由 Arg 改变为 Cys，载脂蛋白 A-I-Milano) 和 E222K。还包括具有保守氨基酸修饰的变体。

[0051] 在一个实施方案中，四连蛋白 - 载脂蛋白 A-I 包含免疫球蛋白 A 蛋白酶 (IgA 蛋白酶) 的切割位点的片段。从 IgA 蛋白酶已知的识别位点包含下述序列，其中“↓”表示切割键的位置 :

[0052] Pro-Ala-Pro ↓ Ser-Pro (SEQ ID NO :61)

[0053] Pro-ProSer-Pro (SEQ ID NO :62)

[0054] Pro-Pro ↓ Ala-Pro (SEQ ID NO :63)

[0055] Pro-Pro ↓ Thr-Pro (SEQ ID NO :64)

[0056] Pro-Pro ↓ Gly-Pro (SEQ ID NO :65)，

[0057] 其中更频繁地选择和切割前三个。

[0058] 术语“载脂蛋白模拟物”指模拟各个载脂蛋白的功能的合成多肽。例如，“载脂蛋白 A-I 模拟物”是显示关于去除胆固醇 (即反向胆固醇流出) 与天然载脂蛋白 A-I 相当的生物功能的合成多肽。在一个实施方案中，载脂蛋白 A-I 模拟物包含至少一个两亲性 α - 螺旋，其具有在疏水 - 亲水界面集簇的带正电荷的氨基酸残基和在亲水性面的中心集簇的带负电荷的氨基酸残基。为了模拟载脂蛋白 A-I 的功能，载脂蛋白模拟物包含从 15-29 个氨基酸残基的重复多肽，在一个实施方案中，包含 22 氨基酸残基 (PVLDEFREKLNEELEALKQKLK(SEQ ID NO :04) ;PVLDLFRELLNELLEAL KQKLK(SEQ ID NO :05)) 的重复多肽。

[0059] 术语“至少一个”指一个、两个、三个、四个、五个、六个、七个、八个、九个或更多。术语“至少两个”指两个，三个，四个，五个，六个，七个，八个，九个，十个或更多。

[0060] 术语“心血管疾病”一般指关于心脏或血管的疾病或病症，如动脉粥样硬化、冠状动脉心脏病、脑血管疾病、主动脉疾病 (aortoiliac disease)、局部缺血性心脏病或外周血管疾病。这些疾病在作为疾病结果的恶性事件发生之前可能不会被发现，所述恶性事件如心肌梗塞、卒中、心绞痛、短暂性脑缺血性发作、充血性心力衰竭、主动脉瘤，这些恶性事件大多数情况下会导致受试者死亡。

[0061] 术语 " 胆酸盐 " 指 3α，7α，12α - 三羟基 -5β - 胆 -24- 酸或其盐，尤其是其钠盐。可以通过在它们各自的转变温度与洗涤剂溶解的脂质一起温育载脂蛋白来形成脂质颗粒。

[0062] 术语“临界胶束浓度”及其缩写“CMC”，可以交互使用，指这样的表面活性剂或洗涤剂浓度，超过所述浓度，个体洗涤剂分子 (单体) 自发团聚为胶束 (胶束，圆杆，层状结构

等）。

[0063] 术语“保守氨基酸修饰”指不会影响或改变根据本发明的脂质颗粒或载脂蛋白的特性的氨基酸序列修饰。修饰可以通过本领域已知的标准技术引入，如位点定向诱变和PCR-介导的突变。保守氨基酸修饰包括其中氨基酸残基被具有类似侧链的氨基酸残基替代的修饰。本领域已经定义了具有类似侧链的氨基酸残基家族。这些家族包括具有碱性侧链的氨基酸（例如，赖氨酸、精氨酸、组氨酸），具有酸性侧链的氨基酸（例如，天冬氨酸、谷氨酸），具有不带电荷的极性侧链的氨基酸（例如，甘氨酸、天冬酰胺、谷氨酰胺、丝氨酸、苏氨酸、酪氨酸、半胱氨酸、色氨酸），具有非极性侧链的氨基酸（例如，丙氨酸、缬氨酸、亮氨酸、异亮氨酸、脯氨酸、苯丙氨酸、甲硫氨酸），具有 β-支链侧链的氨基酸（例如，苏氨酸、缬氨酸、异亮氨酸）和具有芳香族侧链的氨基酸（例如，酪氨酸、苯丙氨酸、色氨酸、组氨酸）。因此“变体”蛋白，在本文指这样的分子，其氨基酸序列与“母体”蛋白氨基酸序列的不同在于至多 10 个添加、缺失和 / 或置换，在一个实施方案中，从约 2 个到约 5 个的添加、缺失和 / 或置换。可以通过基于 Riechmann,L.，等，Nature（自然）332(1988)323-327，和 Queen,C.，等，Proc.Natl.Acad.Sci.USA86(1989)10029-10033. 所述的分子建模的诱变来进行氨基酸序列修饰。

[0064] 术语“洗涤剂”指表面活性化学物质。“洗涤剂”通常是具有非极性疏水部分和极性亲水部分的两亲性分子。术语“两性离子洗涤剂 (zwitterionicdetergent)”指总电荷为零并且同时包含至少一个带正电荷的部分和至少一个带负电荷的部分的表面活性化学化合物。在一个实施方案中，洗涤剂选自基于糖的洗涤剂，基于聚氧化烯的洗涤剂，基于胆汁盐的洗涤剂，合成洗涤剂或其组合。术语“基于糖的洗涤剂”指选自正辛基 -β-D- 吡喃葡萄糖苷、正壬基 -β-D- 吡喃葡萄糖苷、正十二烷基 -β-D- 吡喃麦芽糖苷或 5- 环已基戊基 -β-D- 吡喃麦芽糖苷及其衍生物的洗涤剂。术语“基于胆汁盐的洗涤剂”指选自胆酸钠、胆酸钾、胆酸锂、3-[(3- 氯酰氨基丙基）二甲基铵基]- 基 - 丙烷磺酸酯 (CHAPS)、3-[(3 氯酰氨基丙基）二甲基铵基]-2- 羟基丙烷磺酸酯 (CHAPSO)，和其衍生物的洗涤剂。术语“基于聚氧化烯的洗涤剂”指选自 Tween20、Triton X-100，Pluronic F68 和其衍生物的洗涤剂。术语“合成洗涤剂”指选自两性洗涤剂 3-6、两性洗涤剂 3-8、两性洗涤剂 3-10、两性洗涤剂 3-12 和其衍生物的洗涤剂。

[0065] 术语“高密度脂蛋白颗粒”或其缩写“HDL 颗粒”，可以交互使用，指包含载脂蛋白 A-I 作为主要的蛋白化合物的脂质 - 蛋白复合物。

[0066] 术语“免疫测定法”指用单克隆抗体进行的标准固相免疫测定法，包括在吸附 / 固定在固相上的抗体（捕获抗体），抗原和与酶缀合的针对抗原的另一表位的抗体（示踪抗体）之间形成复合物。因此，形成多层结构 :固相 - 捕获抗体 - 抗原 - 示踪抗体。在由多层结构催化的反应中，抗体 - 缀合的酶的活性与温育介质中的抗原浓度成比例。标准夹心式方法也被称为双抗原桥连免疫测定法，因为捕获抗体和示踪抗体结合于抗原的不同表位。其它类型的测定法是放射性免疫测定法、荧光免疫测定法和酶联免疫测定法。进行所述测定法的方法以及实际应用和程序是本领域技术人员已知的。免疫测定法可以以均相免疫测定法或异相免疫测定法形式进行。

[0067] 术语 " 增加脂质流出 " 和其语法等同成分指自细胞或斑块增加的脂质流出的水平和 / 或速率，促进脂质流出，增强脂质流出，协助脂质流出，上调脂质流出，提高脂质流

出，和 / 或加强脂质流出。在一个实施方案中，脂质流出包括磷脂、甘油三酯、胆固醇和 / 或胆固醇酯的流出。

[0068] 术语“DMPC”指磷脂二豆蔻酰磷脂酰胆碱。

[0069] 术语“DPPC”指磷脂 1,2- 二 - 棕榈酰 -sn- 甘油 -3- 磷脂酰胆碱，也被称为 1,2- 二棕榈酰 - 磷脂酰胆碱。

[0070] 术语“多聚体”指由两个以上单体组成的复合物。多聚体在单体之间通过非共价相互作用形成。每个单体包含多聚化的结构域。在一个实施方案中，多聚体包含 2 或 3 个单体。在另一个实施方案中，多聚化结构域通过在包含在每个单体中的个体多聚化结构域之间的非共价相互作用来相互作用。术语 " 多聚化结构域 " 指能够共价或非共价缔合两个以上单体分子的氨基酸序列。多聚化结构域能够与不同、类似或相同氨基酸序列的多聚化结构域相互作用。在一个实施方案中，多聚化结构域是四连蛋白三聚化结构元件或其衍生物，其具有与 SEQ ID NO :53 的共有氨基酸序列具有至少 68％同一性的氨基酸序列。在一个实施方案中，在 SEQ ID NO :53 的位置 50 的半胱氨酸残基被不同的氨基酸残基置换，在另一个实施方案中，被丝氨酸残基或苏氨酸残基或甲硫氨酸残基置换。包含多聚化结构域的多肽可以与一个或多个也包含多聚化结构域的其它多肽缔合。可以简单地通过在适合的条件下混合多肽来起始多聚体形成。在另一个实施方案中，多聚化结构域具有 SEQ ID NO :53 的氨基酸序列，其中在氨基酸序列的 N 端或 C 端缺失或添加 1-10 个残基。在另一个实施方案中，多聚化结构域具有 SEQID NO :53 的氨基酸序列，其中在氨基酸序列的 N- 端缺失 6 或 9 个氨基酸残基。在又一个实施方案中，多聚化结构域具有 SEQ ID NO :53 的氨基酸序列，其中缺失了 N 端氨基酸残基 L 或 N 端氨基酸残基 C 和 L。在一个实施方案中，多聚化结构域是四连蛋白三聚化结构元件并且具有 SEQ IDNO :54 的氨基酸序列。在一个实施方案中，多聚体是同聚体。

[0071] 多聚体可以是同聚体或异聚体，因为包含多聚化结构域的不同载脂蛋白可以组合结合到所述多聚体中。在一个实施方案中，多聚体是三聚体同聚体。

[0072] 按照一个实施方案，多聚化结构域获自四连蛋白。在一个实施方案中，多聚化结构域包含具有 SEQ ID NO :54 的氨基酸序列的四连蛋白三聚化结构元件。四连蛋白三聚化结构元件三聚化作用由卷曲的螺旋结构导致，所述卷曲的螺旋结构与两个其他四连蛋白三聚化结构元件的卷曲的螺旋结构相互作用以形成三聚体。四连蛋白三聚化结构元件可以获自人四连蛋白，获自兔四连蛋白，获自鼠四连蛋白，或获自鲨鱼软骨的 C- 型凝集素。在一个实施方案中，四连蛋白三聚化结构元件包含与 SEQ ID NO53 的共有序列具有至少 68％，或至少 75％，或至少 81％，或至少 87％，或至少 92％同一性的序列。

[0073] 术语“非共价相互作用”指非共价结合力如离子相互作用力（例如盐桥），非离子相互作用力（例如，氢键），或疏水相互作用力（例如，范德华力或 π - 堆积相互作用）。

[0074] 术语 " 相变温度 " 指诱导脂质物理状态从有序的凝胶相（，其中烃链充分延伸并且紧密包装）向无序的液晶相（其中烃链随机定向并且流动）变化所需要的温度。可以在所用的磷脂 / 磷脂混合物的相变温度或相变温度以上的温度进行脂质颗粒的形成。在下表 1 中列出数种磷脂酰胆碱和其混合物的相变温度。

[0075] 表 1 :纯的磷脂酰胆碱和磷脂酰胆碱混合物的相变温度

[0076]

磷脂摩尔比	相变温度
POPC	4 °C (-3 °C)
DPPC	41 °C
DPPC:POPC 3:1	34 °C
DPPC:POPC 1:1	27 °C
DPPC:POPC 1:3	18 °C

[0077] 术语“磷脂酰胆碱”指由一个甘油部分，两个羧酸部分和一个磷酸胆碱部分组成的分子，其中甘油部分与其他部分分别通过酯键，即两个羧酸酯键和一个磷酸酯键共价结合，其中磷酸酯键与甘油部分的 1- 羟基或 3- 羟基结合。术语“羧酸部分”指包含至少一个酰基 (R-C(O)O) 的有机部分。磷脂酰胆碱可以是任何种类或来源的。在一个实施方案中，磷脂酰胆碱选自卵磷脂酰胆碱，大豆磷脂酰胆碱，二棕榈酰磷脂酰胆碱，二豆蔻酰磷脂酰胆碱，二硬脂酰磷脂酰胆碱，二月桂酰磷脂酰胆碱，二棕榈酰磷脂酰胆碱，1- 豆蔻酰 -2- 棕榈酰磷脂酰胆碱，1- 棕榈酰 -2- 豆蔻酰磷脂酰胆碱，1- 棕榈酰 -2- 硬脂酰磷脂酰胆碱，1- 硬脂酰 -2- 棕榈酰磷脂酰胆碱，二油酰磷脂酰胆碱，1- 棕榈酰 -2- 油酰磷脂酰胆碱，1- 油酰 -2- 棕榈酰磷脂酰胆碱，和其类似物和衍生物。

[0078] 本文所用的所有磷脂可以来自任何来源，即 (如果合适)，来自大豆、乳、蛋或甚至除了人之外的动物的内部器官，它们可以来自天然来源，或半合成来源或甚至完全合成来源。

[0079] ″ 多肽″ 是由通过肽键连接的氨基酸组成的聚合物，不管其是天然产生或合成产生的。可以将少于约 20 个氨基酸残基的多肽称为“肽”而将由两个以上多肽组成或包含一个 100 个氨基酸残基以上的多肽的分子称为“蛋白”。多肽还可以包含非氨基酸成分，如碳水化合物基团，金属离子，或羧酸酯。非氨基酸成分可以由表达多肽的细胞添加，并且可以随细胞类型变化。多肽在本文中由它们的氨基酸主链结构或编码其的核酸限定。通常不指定诸如碳水化合物的添加，但是其可以存在。

[0080] 术语“POPC”指磷脂 1- 棕榈酰 -2- 油酰 -sn- 甘油 -3- 磷脂酰胆碱，也称为 1- 棕榈酰 -2- 油酰 - 磷脂酰胆碱。

[0081] 术语“快速”指在至多 10 小时内完成的过程。快速稀释是其中在至多 10 小时内将第一溶液加入第二溶液中的过程。在一个实施方案中，该过程在至多 5 小时内完成，在另一个实施方案中，在至多 2 小时内完成。

[0082] 术语“基本不含”指包含蛋白和一种或多种脂质的溶液包含少于 5％ (w/w) 脂质颗粒，少于 2.5％脂质颗粒，少于 1％脂质颗粒或少于 0.5％脂质颗粒。

[0083] 术语“变体”还包括本文报道的载脂蛋白的变体或载脂蛋白模拟物，其中在变体中，各个载脂蛋白或载脂蛋白模拟物的氨基酸序列包含一个或多个氨基酸置换、添加或缺失。修饰可以增加或减少载脂蛋白对于载脂蛋白受体或载脂蛋白转化酶的亲和性，或可以与各个载体蛋白相比增加载脂蛋白变体的稳定性，或可以与相应载脂蛋白相比增加载脂蛋

白变体在水溶液中的溶解性，或可以与各个载脂蛋白相比增加载脂蛋白变体在宿主细胞中/由宿主细胞的重组产生。

[0084] 本文报道的

[0085] 已经发现脂质颗粒可以从包含变性蛋白但不包含洗涤剂且不包含脂质的溶液开始，通过快速稀释到包含洗涤剂和至少一种脂质但不含有蛋白的溶液中来直接形成。可以省去一般需要的产生天然性质的步骤，因此提供用于产生脂质颗粒的更简单和可靠的方法。另外，形成更均一的脂质颗粒。

[0086] 用于产生脂质颗粒的方法

[0087] 本文报道了一种用于产生包含蛋白的脂质颗粒的方法，所述方法包括下述步骤：

[0088] i) 提供包含变性蛋白的第一溶液，

[0089] ii) 将第一溶液加入包含脂质和洗涤剂但无蛋白，即不含所述蛋白的第二溶液，和

[0090] iii) 从在步骤 ii) 中获得的溶液去除洗涤剂，并且由此产生脂质颗粒。

[0091] 在一个实施方案中，用于产生包含载脂蛋白的脂质颗粒的方法，包括下述步骤：

[0092] i) 提供包含变性载脂蛋白的第一溶液，

[0093] ii) 将第一溶液加入包含脂质和洗涤剂但不含载脂蛋白的第二溶液中，和

[0094] iii) 从步骤 ii) 获得的溶液中去除洗涤剂并由此产生脂质颗粒。

[0095] 在一个实施方案中，第二溶液具有是第一溶液的体积的至少两倍的体积。

[0096] 在一个实施方案中，第二溶液具有是第一溶液的体积的约 3 倍到约 20 倍的体积。在一个实施方案中，第二溶液具有是第一溶液的体积的约 5 倍到约 10 倍的体积。

[0097] 在一个实施方案中，第二溶液包含至少两种不同的脂质，所述脂质彼此独立地选自磷脂、脂肪酸和类固醇脂质。在另一个实施方案中，至少两种不同的脂质是两种不同的磷脂酰胆碱。在一个实施方案中，第一磷脂酰胆碱是 POPC，第二磷脂酰胆碱是 DPPC。

[0098] 在一个实施方案中，洗涤剂选自胆酸、两性洗涤剂或它们的盐。

[0099] 已经报道了许多从天然存在或重组产生的多肽（如例如来自人 HDL 颗粒的载脂蛋白 A-I 或脱脂质 (delipidated) 载脂蛋白 A-I）产生脂质颗粒的不同方法。其中，例如与纯化的载脂蛋白 A-I 一起温育磷脂（如棕榈酰 -2- 油酰 -sn- 甘油 -3- 磷脂酰胆碱）与洗涤剂（如胆酸钠）的水性混合物，其中以天然，即非变性形式使用载脂蛋白 A-I。形成脂质颗粒后通过透析或渗滤去除洗涤剂。

[0100] 如本文报道的方法允许在单一步骤中完全再折叠和脂质化变性蛋白。通过使用如本文报道的方法，可以获得具有提高的产品质量的脂质颗粒，可以省却预处理蛋白所需要的时间，并且用于生物药物生产的大规模加工首次成为可能。

[0101] 本文报道的方法允许在单一步骤中完全再折叠和脂质化变性的载脂蛋白 A-I。通过使用本文报道的方法，可以获得具有提高的产品质量的脂质颗粒，可以省去预处理载脂蛋白 A-I 所消耗的时间，和生物制药生产的大规模加工第一次成为可能。

[0102] 对于脂质颗粒形成工艺开发必须要考虑的要点是 i) 需要生物活性，和 ii) 涉及脂质颗粒的可制造性的技术要求。例如，对于形成包含载脂蛋白的脂质颗粒，这些要求指向相反的方向。

[0103] 从技术观点，将选择包含具有 16 个碳原子以下链的羧酸部分的饱和磷脂（例如，二棕榈酰 -sn- 甘油 -3- 磷酸胆碱，DPPC；二豆蔻酰 -sn- 甘油 -3- 磷酸胆碱，DMPC 等）。与

此相反，从生物学数据可以认为包含具有至少 16 个碳原子链的羧酸部分的不饱和磷脂（例如，棕榈酰 -2- 油酰 -sn- 甘油 -3- 磷酸胆碱，POPC ；硬脂酰 -2- 油酰 -sn- 甘油 -3- 磷酸胆碱，SOPC）是更有效的，并且对肝无毒。

[0104] 磷脂组合的选择决定包含载脂蛋白的脂质颗粒的功效和肝安全性。在使用兔进行的包含 DMPC 的脂质颗粒的体内研究中，已经发现用 30mg/kg 处理的兔显示严重的副作用，但是存活下来，而用 100mg/kg 处理的兔则死亡。结果清楚地显示对于胆固醇的动员和因此对于分子的功效需要脂质化（图 23）。

[0105] 体外功能测试证实了包含单磷脂酰胆碱如 DPPC 或 POPC 的脂质颗粒激活了 LCAT。

[0106] 还显示对于不同磷脂的组合，胆固醇流出更高。

[0107] 表 2 ：用于体内兔研究制备的磷脂组合，不同之处在于其脂质组成

[0108]

用于产生脂质颗粒的磷脂摩尔比	LCAT 底物	胆固醇流出
POPC	是	是
POPC:DPPC 3:1	是	是

[0109]

用于产生脂质颗粒的磷脂摩尔比	LCAT 底物	胆固醇流出
POPC:DPPC 1:1	是	是
POPC:DPPC 1:3	否	是
DPPC	否	是

[0110] 这些结果也由显示对于所有组合的胆固醇动员的体内数据所证实。然而，对于仅包含单磷脂酰胆碱 DPPC，或 DPPC 和鞘磷脂 (SM) 的组合的脂质颗粒而言，可以确定肝酶的增加（图 1）。

[0111] 因此，通过如本文报道的方法获得的脂质颗粒也是一个方面。

[0112] 从技术观点，与用纯的 POPC 形成比较，用纯的 DPPC 形成脂质颗粒更为方便。通过使用不同磷脂的组合减少沉淀形成的风险。此外，与具有 4℃的相变温度的纯 POPC 比较，纯 DPPC 的 41℃的相变温度也使得制备脂质颗粒更为容易。而且，获得的产物更为均一。这也可以被通过 SEC-MALLS 进行的脂质颗粒分析所证实，所述 SEC-MALLS 是允许测定蛋白 - 脂

质组合物的分析工具（蛋白 - 缀合物分析）。在图 2 中，显示了以大小排阻色谱法 (UV280 检测）解析的样品的色谱图。可以通过多个分离的或半解离的峰的出现来观察样品的不均一性。

[0113] 当使用纯的 POPC 制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的 POPC 分子数目在一个实施方案中是 40-85，在一个实施方案中，是 50-80，在一个实施方案中，是 54-75。

[0114] 当使用纯 DPPC 制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的 DPPC 分子数目，在一个实施方案中，是 50-150，在一个实施方案中，是 65-135，在一个实施方案中，是 76-123，并且在一个实施方案中，是 86-102。

[0115] 当将摩尔比为 1 ： 3 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的磷脂分子数目，在一个实施方案中，是约 50- 约 120，在一个实施方案中，是约 65- 约 105，并且在一个实施方案中，是约 72- 约 96。

[0116] 当将摩尔比为 1 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目在一个实施方案中，是 50-120，在一个实施方案中，是 60-100，在一个实施方案中，是 71 到 92，并且在一个实施方案中是 71-85。

[0117] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 50-105。

[0118] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 60-95。

[0119] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 60-90。

[0120] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目在一个实施方案中，是 60-88。

[0121] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 62-80。

[0122] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 66-86。

[0123] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是 64-70。

[0124] 当将摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物用于制备脂质颗粒时，在脂质颗粒中每个载脂蛋白单体的脂质分子数目，在一个实施方案中，是约 66。

[0125] 对于制备包含载脂蛋白和 POPC 的脂质颗粒，在一个实施方案中，应用 1 ： 40-1 ： 100 的载脂蛋白与 POPC 的摩尔比，在一个实施方案中，应用 1 ： 40-1 ： 80 的摩尔比，在一个实施方案中，应用约 1 ： 60 的摩尔比。

[0126] 对于制备包含载脂蛋白和 DPPC 的脂质颗粒，在一个实施方案中，应用 1 ： 70-1 ： 100 的载脂蛋白与 DPPC 的摩尔比，在一个实施方案中，应用 1 ： 80-1 ： 90 的摩尔比，在一个实施方案中，应用约 1 ： 80 的摩尔比。

[0127] 对于制备包含载脂蛋白、POPC 和 DPPC 的脂质颗粒，在一个实施方案中，应用 1 ： 60-1 ： 100 的载脂蛋白与 (POPC 和 DPPC)(POPC 和 DPPC 的摩尔比 1 ： 3) 的摩尔比，在

一个实施方案中，应用 1 ： 70-1 ： 90 的摩尔比，并且在一个实施方案中，应用约 1 ： 80 的摩尔比。

[0128] 对于制备包含载脂蛋白、DPPC 和 POPC 的脂质颗粒，在一个实施方案中，应用 1 ： 60-1 ： 100 的载脂蛋白与 (POPC 和 DPPC)(POPC 和 DPPC 的摩尔比 1 ： 1) 的摩尔比，在一个实施方案中，应用 1 ： 60-1 ： 80 的摩尔比，并且在一个实施方案中，应用约 1 ： 70 的摩尔比。

[0129] 对于制备包含载脂蛋白、DPPC 和 POPC 的脂质颗粒，在一个实施方案中，应用 1 ： 60-1 ： 100 的载脂蛋白与 (POPC 和 DPPC)(POPC 和 DPPC 的摩尔比 3 ： 1) 的摩尔比，在一个实施方案中，应用 1 ： 50-1 ： 70 的摩尔比，并且在一个实施方案中，应用约 1 ： 60 的摩尔比。

[0130] 在一个实施方案中，将多肽用洗涤剂温育约 0.5 小时到约 60 小时。在一个实施方案中，将多肽用洗涤剂温育约 0.5 小时到约 20 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 2 小时到约 60 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 12 小时到约 20 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 16 小时。

[0131] 在一个实施方案中，如果使用脂质的混合物来制备脂质颗粒，混合物具有 4℃ -45℃的相变温度，在一个实施方案中，具有 10℃ -38℃的相变温度，并且在一个实施方案中，具有 15℃ -35℃的相变温度。

[0132] 对于形成包含载脂蛋白的脂质颗粒，已知不同方法，如冻干、冻融、洗涤剂溶解随后透析、微射流、超声处理和匀浆化。

[0133] 脂质颗粒可以包含，在一个实施方案中，平均 1-10 个载脂蛋白分子，在一个实施方案中，平均 1-8 个载脂蛋白分子 / 脂质颗粒，在一个实施方案中，平均 1-4 个载脂蛋白分子 / 脂质颗粒。

[0134] 在一个实施方案中，脂质颗粒可以包含平均数目为至少 1，或 2，或 3，或 4，或 5，或 6，或 7，或 8，或 9，或 10 个载脂蛋白分子 / 脂质颗粒。在一个实施方案中，平均数是 1。

[0135] 在一个实施方案中，除了载脂蛋白之外，脂质颗粒包含一种或多种其他的多肽。

[0136] 不受限制地，脂质颗粒可以充当酶促辅因子和 / 或用于吸收脂质的脂质（尤其是胆固醇）的载体。

[0137] 在如本文报道的脂质颗粒中还可以存在一种或多种洗涤剂。所述洗涤剂可以是任何药用洗涤剂，如非离子型或离子型洗涤剂。非离子洗涤剂可以是包含一个或多个羟基的有机化合物的烯化氧衍生物。在一个实施方案中，非离子洗涤剂选自乙氧基化和 / 或丙氧基化醇或酯化合物或其混合物。在另一种实施方案中，酯选自山梨糖醇和脂肪酸的酯，如脱水山梨糖醇单油酸酯或、脱水山梨醇单棕榈酸酯，油性蔗糖酯、聚氧乙烯山梨糖醇脂肪酸酯、聚氧乙烯山梨糖醇脂肪酸酯、聚氧乙烯脂肪酸酯、聚氧乙烯烷基醚、聚氧乙烯固醇醚、聚氧乙烯 - 聚丙氧烷基醚、嵌段聚合物和 cethyl 醚 (cethylether)、聚氧乙烯蓖麻油或氢化蓖麻油衍生物和聚甘氨酸脂肪酸酯。在一个实施方案中，非离子型洗涤剂选自 Pluronic®, Poloxamer®, Span®, Tween®, Polysorbate®, Tyloxapol®, Emulphor®或Cremophor®。

[0138] 离子型洗涤剂可以是胆管剂。在一个实施方案中，所述离子型洗涤剂选自胆酸或脱氧胆酸，或它们的盐和衍生物，或选自游离脂肪酸，如油酸、亚油酸和其它。

[0139] 在一个实施方案中，离子型洗涤剂选自阳离子脂质如 C_{10}-C_{24} 烷基胺或烷醇胺和阳离子胆固醇酯。在一个实施方案中，洗涤剂是具有高 CMC 的洗涤剂。在又一个实施方案中，洗涤剂是具有至少 5mM CMC 的洗涤剂。

[0140] 在一个实施方案中，脂质颗粒包含少于 0.75 重量％洗涤剂。

[0141] 在一个实施方案中，脂质颗粒包含少于 0.30 重量％洗涤剂。

[0142] 在一个实施方案中，脂质颗粒包含少于 0.1 重量％洗涤剂。

[0143] 在一个实施方案中，脂质颗粒包含少于 0.05 重量％洗涤剂。

[0144] 在一个实施方案中，所述洗涤剂选自基于糖的洗涤剂，基于聚氧乙烯的洗涤剂，基于胆汁盐的洗涤剂，合成洗涤剂或其组合。在另一个实施方案中，洗涤剂是胆酸或两性洗涤剂。

[0145] 在一个实施方案中，在根据本发明的方法中，第一溶液基本不含脂质颗粒。

[0146] 在一个实施方案中，所述方法在步骤 ii) 之后在步骤 iii) 之前，包括下述步骤 iia) 温育在步骤 ii) 中获得的溶液。在一个实施方案中，将多肽用洗涤剂温育约 0.5 小时到约 60 小时。在一个实施方案中，将多肽用洗涤剂温育约 0.5 小时到约 20 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 2 小时到约 60 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 12 小时到约 20 小时。在一个实施方案中，将所述多肽用洗涤剂温育约 16 小时。

[0147] 在一个实施方案中，温育和 / 或去除在 4℃ -45℃的温度进行。

[0148] 在一个实施方案中，通过渗滤或透析进行去除。

[0149] 在一个实施方案中，第一溶液具有第一体积，第二溶液具有第二体积，在第一溶液中的蛋白，如载脂蛋白具有限定的浓度，在第二溶液中的脂质和洗涤剂分别具有限定的浓度，其中在步骤 ii) 中，载脂蛋白的浓度、脂质的浓度和洗涤剂的浓度被改变 / 减少，允许形成脂质颗粒。通过稀释载脂蛋白溶液和添加脂质和洗涤剂，调节一方面载脂蛋白与脂质的适合比率和另一方面脂质与洗涤剂的适合比率，允许形成脂质颗粒。

[0150] 在一个实施方案中，所述方法包括下述步骤 ：

[0151] iv) 纯化脂质颗粒并由此产生脂质颗粒。

[0152] 例如，为了产生包含载脂蛋白的脂质颗粒，从技术观点可以选择包含具有 16 个原子和更短的链的羧酸部分的饱和磷脂 (例如，二棕榈酰基 -sn- 甘油 -3- 磷酸胆碱，DPPC ；二肉豆蔻酰基 -sn- 甘油 -3- 磷酸胆碱，DMPC 等)。从生物学角度来看，与此相反，可以设想包含具有至少 16 个 C 原子的链的羧酸部分的不饱和磷脂 (例如棕榈酰 -2- 油酰 -sn- 甘油 -3- 磷酸胆碱，POPC ；硬脂酰 -2- 油酰 -sn- 甘油 -3- 磷酸胆碱，SOPC) 是更有效并且是非 - 肝毒性的。

[0153] 可以使用磷脂酰胆碱 DPPC 和 POPC 及其混合物用于形成包含载脂蛋白的脂质颗粒。这些示例性的磷脂酰胆碱在一个羧酸部分不同并具有一个相同的被酯化为磷酸甘油主链的羧酸部分。当使用 DPPC 时，制备脂质颗粒更为容易。相反，POPC 在体外功能测定中更为有效，特别是作为激活卵磷脂胆固醇乙酰转移酶 (LCAT) 的底物，所述酶对于将动员的胆固醇转化为胆固醇酯是必须的。已经发现，与仅包含一种磷脂酰胆碱的脂质颗粒相比，以不同摩尔比包含两种磷脂酰胆碱，例如 POPC 和 DPPC 的混合物的脂质颗粒是有利的 (见，例如图 4)。

[0154] 例如，脂质颗粒可以仅包含 POPC。当将 1 ： 40 直至 1 ： 80 摩尔比的载脂蛋白：脂质用于产生脂质颗粒时，每个载脂蛋白单体的 POPC 分子数目可以在 54-75 之间变化。在一个实施方案中，载脂蛋白与 POPC 的摩尔比是 1 ： 40-1 ： 80，在一个实施方案中，摩尔比是 1 ： 50-1 ： 70，在一个实施方案中，摩尔比是约 1 ： 60。

[0155] 因此，对于产生包含载脂蛋白和 POPC 的脂质颗粒，载脂蛋白与 POPC 的摩尔比是从 1 ： 40 到 1 ： 100，在一个实施方案中，摩尔比是 1 ： 40 到 1 ： 80，并且在一个实施方案中，摩尔比是约 1 ： 60。

[0156] 例如，脂质颗粒可以仅包含 DPPC。当将 1 ： 40 直至 1 ： 80 摩尔比的载脂蛋白：脂质用于产生脂质颗粒时，每个载脂蛋白单体的 DPPC 分子数目可以在 76-123 之间变化。在一个实施方案中，载脂蛋白与 DPPC 的摩尔比是 1 ： 70-1 ： 100，在一个实施方案中，摩尔比是 1 ： 75-1 ： 90，在一个实施方案中，摩尔比是约 1 ： 80。

[0157] 例如，可以从摩尔比为 1 ： 3 的 POPC 和 DPPC 的混合物开始制备脂质颗粒。当将 1 ： 60 直至 1 ： 100 摩尔比的载脂蛋白：脂质用于产生脂质颗粒时，每个载脂蛋白单体的磷脂分子数目可以在 72-112 之间变化。在一个实施方案中，载脂蛋白与 (POPC 和 DPPC) 的摩尔比是 1 ： 70-1 ： 90，在一个实施方案中，摩尔比是 1 ： 75-1 ： 85，在一个实施方案中，摩尔比是约 1 ： 80。

[0158] 因此，对于产生包含载脂蛋白，POPC 和 DPPC 的脂质颗粒，载脂蛋白与 (POPC 和 DPPC)(POPC 和 DPPC 摩尔比为 1 ： 3) 的摩尔比在一个实施方案中，是 1 ： 60-1 ： 100，在一个实施方案中，摩尔比是 1 ： 70-1 ： 90，并且在一个实施方案中，摩尔比是约 1 ： 80。

[0159] 例如，可以从摩尔比为 1 ： 1 的 POPC 和 DPPC 的混合物开始制备脂质颗粒。当将 1 ： 60 直至 1 ： 100 摩尔比的载脂蛋白：脂质用于制备脂质颗粒时，每个载脂蛋白单体的磷脂分子数目可以在 71-111 之间变化。在一个实施方案中，载脂蛋白与 (POPC 和 DPPC) 的摩尔比是 1 ： 60-1 ： 80，在一个实施方案中，摩尔比是 1 ： 65-1 ： 75，在一个实施方案中，摩尔比是约 1 ： 70。

[0160] 因此，对于产生包含载脂蛋白，DPPC 和 POPC 的脂质颗粒，载脂蛋白与 (POPC 和 DPPC)(POPC 和 DPPC 摩尔比为 1 ： 1) 的摩尔比在一个实施方案中，是 1 ： 60-1 ： 100，在一个实施方案中，摩尔比是 1 ： 60-1 ： 80，并且在一个实施方案中，摩尔比是约 1 ： 70。

[0161] 例如，可以从摩尔比为 3 ： 1 的 POPC 和 DPPC 的混合物开始制备脂质颗粒。当将 1 ： 60 直至 1 ： 100 摩尔比的载脂蛋白：脂质用于制备脂质颗粒时，每个载脂蛋白单体的磷脂分子数目可以在 46-93 之间变化。在一个实施方案中，载脂蛋白与 (POPC 和 DPPC) 的摩尔比是 1 ： 50-1 ： 70，在一个实施方案中，摩尔比是 1 ： 55-1 ： 65，在一个实施方案中，摩尔比是约 1 ： 60。

[0162] 因此，对于产生包含载脂蛋白，DPPC 和 POPC 的脂质颗粒，载脂蛋白与 (POPC 和 DPPC)(其中 POPC 和 DPPC 摩尔比为 3 ： 1) 的摩尔比在一个实施方案中，是 1 ： 60-1 ： 100，在一个实施方案中，摩尔比是 1 ： 50-1 ： 70，并且在一个实施方案中，摩尔比是约 1 ： 60。

[0163] 在一个实施方案中，载脂蛋白作为载脂蛋白的水溶液提供并且可以在重组生产或任何其它来源的载脂蛋白生产后获自下游加工，并且可以包含不同浓度纯度不同的载脂蛋白。

[0164] 基本上，脂质颗粒的形成通过将多肽与洗涤剂溶解的脂质在它们各自的转变温度

一起温育来实现。通过透析去除洗涤剂导致由脂双层组成的脂质颗粒的形成。

[0165] 基本上，脂质颗粒形成可以通过将四连蛋白－载脂蛋白 A-I 或其多聚体与洗涤剂溶解的脂质在它们各自的转变温度一起温育来实现。通过透析去除洗涤剂导致脂质颗粒的形成，所述脂质颗粒由被 α－螺旋载脂蛋白围绕的脂双层组成。

[0166] 脂质颗粒可以通过沉淀和 / 或色谱步骤的组合来进行纯化。例如，过量的洗涤剂，即不是脂质颗粒的一部分的洗涤剂可以在疏水吸附色谱步骤中去除。在一个实施方案中，纯化脂质颗粒的方法的步骤包括疏水吸附色谱步骤。在另一个实施方案中，用于疏水吸附步骤的色谱材料选自 ExtractiGel D（获自 Pierce Biotechnology，Rockford IL，USA），CALBIOSORB™（获自 Calbiochem，San Diego，CA，USA），SDR30HyperD™ 溶剂－洗涤剂去除层析树脂（获自 PALL Corporation，Ann Arbor，MI，USA）。脂质颗粒用不含洗涤剂的溶液从疏水吸附材料中回收。

[0167] 在一个实施方案中，使用透析来去除具有高 CMC 的洗涤剂。

[0168] 药物和诊断组合物：

[0169] 通过如本文报道的方法获得的脂质颗粒可以用于治疗和 / 或诊断疾病或病症。

[0170] 可以将如本文报道的四连蛋白－载脂蛋白 A-I 或如本文报道的脂质颗粒用于治疗和 / 或诊断由不正常的脂质水平或脂质在身体部分（如血管中的斑块）的沉积表征的疾病或病症。

[0171] 为了确定得到的蛋白质－脂质复合物支持 LCAT 催化的胆固醇酯化的能力，通过快速添加胆固醇乙醇溶液来将胆固醇结合到如本文报道的脂质颗粒中。与包含独立于它们的载脂蛋白成分（如野生型载脂蛋白 A-I 或四连蛋白－载脂蛋白 A-I）之外的 DPPC 的复合物相比，包含纯的 POPC 的脂质颗粒是更好的 LCAT 底物（图 3）。

[0172] 在包含不同的 POPC 和 DPPC 混合物的脂质颗粒中胆固醇酯化的起始速度显示与任一种纯的磷脂酰胆碱相比，混合物是更好的 LCAT 底物，如可以从胆固醇酯化的起始速度观察到的（见表 3 和图 4）。

[0173] 表 3：在包含磷脂的不同混合物的脂质颗粒中的胆固醇酯化的起始速度

[0174]

用于制备脂质颗粒的磷脂摩尔比	K_m [μm]	V_{max} [nmol 酯 /h/μg LCAT]
POPC	4.6	1.6
POPC:DPPC 3:1	0.4	1.9
POPC:DPPC 1:1	0.5	1.8
POPC:DPPC 1:3	1.0	1.7
DPPC	0.9	1.8

[0175] 将通过将 THP-1 单核细胞白血病细胞暴露于佛波醇 -12- 肉豆蔻酸酯 -13- 乙酸酯 (phorbol myristate acetate) 并负载放射性标记的胆固醇示踪物获得的巨噬细胞如人

THP1 细胞暴露于胆固醇受体测试化合物。

[0176] 可以将通过受体测试化合物诱导的流出速度计算为在上清液中的胆固醇放射性与细胞中放射性加它们上清液的放射性总和的比率并与暴露于不包含受体的介质的细胞进行比较，并通过线性拟合分析。可以使用暴露和不暴露于 RXR-LXR 激动剂的细胞进行平行实验，所述 RXR-LXR 激动剂已知主要上调 ABCA-1 并偏向向 ABCA-1 介导的运输的流出。

[0177] 在未用 RXR-LXR 脂质颗粒预处理的细胞中，与用非脂质化四连蛋白－载脂蛋白 A-I 获得的流出比较，可以观察到在胆固醇流出中的更高增加。可以在测试系列中观察到脂质混合物对流出的仅少量影响（图 5）。在用 RXR-LXR 预处理的细胞中，可以观察到在包含非脂质化的四连蛋白－载脂蛋白 A-I 的脂质颗粒的胆固醇流出的可比较的增加。与用未经预处理的细胞观察到的比较，总的增加更高。在测试系列中仅观察到脂质混合物对流出的较小影响（图 6）。

[0178] 在兔中体内测试不同的脂质颗粒。将脂质颗粒作为静脉内输注施用，并且在施用后在 96 小时内进行系列血液取样。测定肝酶、胆固醇和胆固醇酯的值。对于所有包含起始分布相的测试脂质颗粒，血浆浓度是相当的，随后血浆浓度对数线性下降（图 7）。如可以从表 4 中观察到的，对于所有测试化合物而言，药物代谢动力学参数是类似的。观察到的半衰期接近于 1.5 天。

[0179] 表 4 :测定的药物代谢动力学参数

[0180]

用于制备脂质颗粒的磷脂摩尔比	C_L [ml/h/kg]	V_{ss} [ml/kg]	$T_{1/2}$ [h]	C_{max} [mg/ml]
POPC	0.89 ± 0.22	45.0 ± 2.5	36.9 ± 8.2	2.40 ± 0.19
POPC:DPPC 3:1	0.82 ± 0.06	37.8 ± 5.6	34.2 ± 4.5	2.65 ± 0.28
POPC:DPPC 1:1	0.85 ± 0.14	43.1 ± 5.9	38.6 ± 10.6	2.34 ± 0.31
DPPC	0.96 ± 0.10	37.8 ± 4.9	30.2 ± 7.7	2.29 ± 0.19

[0181]

用于制备脂质颗粒的磷脂摩尔比	C_L [ml/h/kg]	V_{ss} [ml/kg]	$T_{1/2}$ [h]	C_{max} [mg/ml]
DPPC:SM 9:1	1.28 ± 0.62	50.7 ± 8.7	31.3 ± 8.2	1.91 ± 0.33

[0182] 如可以从图 8 中观察到的，在血浆中动员和酯化胆固醇。甚至在四连蛋白－载脂蛋白 A-I 的浓度已经开始降低后，血浆胆固醇酯水平确实继续增加。当血浆四连蛋白－载脂蛋白 A-I 水平已经减少到约 0.5mg/ml（约 50％的正常野生型载脂蛋白 A-I），仍旧可以检测到增加的胆固醇酯水平。

[0183] 包含四连蛋白－载脂蛋白 A-I 的脂质颗粒不诱导兔和小鼠中的肝酶，如可以从图 1 和 9 中观察到的。此外，在静脉内施用后两小时获得的血浆样品中可以确定没有溶血（图 10）。

[0184] 因此，本发明的方面是药物组合物和诊断组合物，其包含如本文报道的含有载脂

蛋白的脂质颗粒或包含如本文报道的四连蛋白 - 载脂蛋白 A-I。

[0185] 如在下述表 5 中显示，与非脂质化的载脂蛋白和其它脂质颗粒比较，如本文报道的脂质颗粒具有提高的体内性质。

[0186] 表 5 ：不同载脂蛋白和脂质颗粒的体内性质

[0187]

蛋白质	包含下述的颗粒	施用于	最高的施用剂量	急性肝毒性作用	参考文献
载脂蛋白 A-I 突变体	无颗粒	大鼠	口服，1 g/kg	无毒性作用多达 500 mg/kg	US 2005/0287636
A-I，四连蛋白 - 载脂蛋白 A-I	DMPC	小鼠	静脉内 1-1.2 mg/小鼠	未描述	WO 2002/38609; Graversen (2008)
载脂蛋白原 A-I	SM	未报道	未报道	注射，在 200 mg/kg 的剂量有毒	WO 2003/096983
载脂蛋白 A-I	PG/SM	兔	静脉内 15 mg/kg	未描述	WO 2006/100567

[0188]

蛋白质	包含下述的颗粒	施用于	最高的施用剂量	急性肝毒性作用	参考文献
载脂蛋白A-I	PC (大豆)	人	80 mg/kg	因为肝功能测试异常(丙氨酸氨基转移酶的10倍增加)，治疗组在早期被中断	WO 2007/137400
载脂蛋白A-I Milano变体	POPC	人	45 mg/kg	由于升高的天冬氨酸氨基转移酶水平(3x 正常值上限)的发展，一个患者撤出	Nissen, S.E., 等, JAMA 290 (2003) 2292-2300
四连蛋白-载脂蛋白A-I	DMPC	兔	100 mg/kg	在所有测试动物中3-4小时后致死	
四连蛋白-载脂蛋白A-I	POPC/DPPC	兔	100 mg/kg	未观察到增加	
四连蛋白-载脂蛋白A-I	POPC/DPPC	大鼠	静脉内500 mg/kg	未观察到增加	
四连蛋白-载脂蛋白A-I	POPC/DPPC	食蟹猴	静脉内200 mg/kg	未观察到增加	

[0189] 可以通过在体内施用载脂蛋白后比较总胆固醇的相应排出(respectiveexcursion)与载体蛋白浓度来确定胆固醇动员到血液中的效率。对于定量评估，计算在总胆固醇的浓度-时间曲线下的基线校正面积(AUC)与在载脂蛋白的浓度-时间曲线下的面积的商。

[0190] 如本文报道的脂质颗粒，尤其是包含SEQ ID NO:01的四连蛋白-载脂蛋白和摩尔比为3：1的POPC和DPPC的脂质颗粒，显示体内增加的胆固醇动员。

[0191] 四连蛋白 - 载脂蛋白 A-I

[0192] 除了上述概括的脂质颗粒之外，本文还报道四连蛋白 - 载脂蛋白 A-I。

[0193] 四连蛋白 - 载脂蛋白 A-I 是人四连蛋白三聚化结构元件和野生型人载脂蛋白 A-I 的融合蛋白。人四连蛋白部分的氨基酸序列可以缩短前面 9 个氨基酸，从位置 10 的异亮氨酸残基（天然存在的截短位点）开始。作为这种截短的后果，缺失了在位置 4 的苏氨酸残基的 O- 糖基化位点。在四连蛋白三聚化结构元件和人载脂蛋白 A-I 之间，去除了五个氨基酸残基“SLKGS”(SEQ ID NO :03)。

[0194] 为了提高的表达和纯化，产生包含 N- 端纯化标记物，例如六组氨酸 - 标记物并且包含 IgA 蛋白酶切割位点的构建体。作为特异性切割的结果，在纯化后两个氨基酸 - 丙氨酸和脯氨酸 - 保留在根据本发明的四连蛋白 - 载脂蛋白 A-I 的 N 端并且所述四连蛋白 - 载脂蛋白 A-I 具有 SEQ ID NO :01 的序列。

[0195] 四连蛋白三聚化结构元件提供允许形成三聚化的四连蛋白 - 载脂蛋白 A-I 多聚体的结构域，所述多聚体由在每个个体四连蛋白 - 载脂蛋白 A-I 单体之间的非共价相互作用构成。

[0196] 通过使用不同的生产方法，可以省去纯化 - 标记物和 IgA 蛋白酶切割位点，形成氨基酸序列为 SEQ ID NO :02 的四连蛋白 - 载脂蛋白 A-I。

[0197] 在一个实施方案中，载脂蛋白可以是包含保守氨基酸置换的变体或是载脂蛋白 A-I 模拟物。

[0198] 载脂蛋白 A-I 可以通过酶促、通过 NMR 光谱学或通过使用单克隆或多克隆抗 - 载脂蛋白 -A-I 抗体来确定。因此，本文报道的其他方面是多克隆和单克隆抗体，其特异性结合如本文报道的四连蛋白 - 载脂蛋白 A-I。可以用本领域技术人员已知的方法获得所述抗体。此外，可以用本领域技术人员已知的方法进行用于免疫测定法中的抗体的标记。

[0199] 在一个实施方案中，载脂蛋白可以是包含保守氨基酸置换的变体，或是载脂蛋白 A-I 模拟物。在一个实施方案中，四连蛋白 - 载脂蛋白 A-I 具有 SEQ ID NO :02 或 SEQ ID NO :66 或 SEQ ID NO :67 的氨基酸序列，其中 X 选自 SEQ ID NO :68-SEQ ID NO :105。

[0200] 因此，在一个实施方案中，四连蛋白 - 载脂蛋白 A-I 具有下述氨基酸序列 IVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ(SEQ ID NO :02)。

[0201] 在一个实施方案中，四连蛋白 - 载脂蛋白 A-I 具有下述氨基酸序列 (A, G, S, T)PIVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ(SEQ ID NO :66)。

[0202] 在一个实施方案中，四连蛋白 - 载脂蛋白 A-I 具有下述氨基酸序列 (M)HHHHHHXIVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHA

KATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ(SEQ IDNO:67),其中 X 可以是下述氨基酸序列的任一个:A,G, S,P,AP,GP,SP,PP,GSAP(SEQ ID NO:68),GSGP(SEQ ID NO:69),GSSP(SEQ ID NO:70),GSPP(SEQ ID NO:71),GGGS(SEQ ID NO:72),GGGGS(SEQ ID NO:73),GGGSGGGS(SEQ ID NO:74),GGGGSGGGGS(SEQ ID NO:75),GGGSGGGSGGGS(SEQ ID NO:76),GGGGSGGGGSGGGGS(SEQ ID NO:77),GGGSAP(SEQ ID NO:78),GGGSGP(SEQ IDNO:79),GGGSSP(SEQ IDNO:80),GGGSPP(SEQ ID NO:81),GGGGSAP(SEQ ID NO:82),GGGGSGP(SEQ ID NO:83),GGGGSSP(SEQ ID NO:84),GGGGSPP(SEQ ID NO:85),GGGSGGGSAP(SEQ ID NO:86),GGGSGGGSGP(SEQ ID NO:87),GGGSGGGSSP(SEQ ID NO:88),GGGSGGGSPP(SEQ ID NO:89),GGGSGGGSGGGSAP(SEQ ID NO:90),GGGSGGGSGGGSGP(SEQ IDNO:91),GGGSGGGSGGGSSP(SEQ ID NO:92),GGGSGGGSGGGSPP(SEQ ID NO:93),GGGGSAP(SEQ ID NO:94),GGGGSGP(SEQ ID NO:95),GGGGSSP(SEQ ID NO:96),GGGGSPP(SEQ ID NO:97),GGGGSGGGGSAP(SEQ IDNO:98),GGGGSGGGGSGP(SEQ ID NO:99),GGGGSGGGGSSP(SEQ ID NO:100),GGGGSGGGGSPP(SEQ ID NO:101),GGGGSGGGGSGGGGSAP(SEQ ID NO:102),GGGGSGGGGSGGGGSGP(SEQ ID NO:103),GGGGSGGGGSGGGGSSP(SEQ ID NO:104),和 GGGGSGGGGSGGGGSPP(SEQ ID NO:105)。

[0203] 如果在大肠杆菌菌株中产生异源多肽,通常用蛋白酶不能有效切割去除氨基-端甲硫氨酸残基,由此氨基端甲硫氨酸残基部分存在于产生的多肽中。

[0204] 提供下述实施例,序列表和图来辅助理解本发明,在后附权利要求中提出的真实范围。要理解,可以在不背离本发明精神的前提下,对所述方法进行修饰。

[0205] 序列表描述

[0206] SEQ ID NO:01 四连蛋白-载脂蛋白 A-I(1).

[0207] SEQ ID NO:02 四连蛋白-载脂蛋白 A-I(2).

[0208] SEQ ID NO:03 肽.

[0209] SEQ ID NO:04 载脂蛋白 A-I 模拟物 (1).

[0210] SEQ ID NO:05 载脂蛋白 A-I 模拟物 (2).

[0211] SEQ ID NO:06 人载脂蛋白 A-I.

[0212] SEQ ID NO:07 人载脂蛋白 A-II.

[0213] SEQ ID NO:08 人载脂蛋白 A-IV.

[0214] SEQ ID NO:09 人载脂蛋白 A-V.

[0215] SEQ ID NO:10 人载脂蛋白 C-I.

[0216] SEQ ID NO:11 人载脂蛋白 C-II.

[0217] SEQ ID NO:12 人载脂蛋白 C-III.

[0218] SEQ ID NO:13 人载脂蛋白 C-IV.

[0219] SEQ ID NO:14 人载脂蛋白 D.

[0220] SEQ ID NO:15 人载脂蛋白 E.

[0221] SEQ ID NO:16 人载脂蛋白 F.

[0222] SEQ ID NO:17 人载脂蛋白 H.

[0223] SEQ ID NO:18 人载脂蛋白 L-I.

[0224] SEQ ID NO:19 人载脂蛋白 L-II.

[0225] SEQ ID NO:20 人载脂蛋白 L-III.

[0226] SEQ ID NO:21 人载脂蛋白 L-IV.

[0227] SEQ ID NO:22 人载脂蛋白 L-V.

[0228] SEQ ID NO:23 人载脂蛋白 L-VI.

[0229] SEQ ID NO:24 人载脂蛋白 M.

[0230] SEQ ID NO:25 人载脂蛋白 O.

[0231] SEQ ID NO:26 人载脂蛋白 OL.

[0232] SEQ ID NO:27 人载脂蛋白 clus.

[0233] SEQ ID NO:28 载脂蛋白 .

[0234] SEQ ID NO:29 载脂蛋白 .

[0235] SEQ ID NO:30 载脂蛋白 .

[0236] SEQ ID NO:31 载脂蛋白 .

[0237] SEQ ID NO:32 载脂蛋白 .

[0238] SEQ ID NO:33 载脂蛋白 .

[0239] SEQ ID NO:34 载脂蛋白 .

[0240] SEQ ID NO:35 载脂蛋白 .

[0241] SEQ ID NO:36 载脂蛋白 .

[0242] SEQ ID NO:37 载脂蛋白 .

[0243] SEQ ID NO:38 载脂蛋白 .

[0244] SEQ ID NO:39 载脂蛋白 .

[0245] SEQ ID NO:40 载脂蛋白 .

[0246] SEQ ID NO:41 载脂蛋白 .

[0247] SEQ ID NO:42 载脂蛋白 .

[0248] SEQ ID NO:43 载脂蛋白 .

[0249] SEQ ID NO:44 载脂蛋白 .

[0250] SEQ ID NO:45 载脂蛋白 .

[0251] SEQ ID NO:46 载脂蛋白 .

[0252] SEQ ID NO:47 载脂蛋白 .

[0253] SEQ ID NO:48 载脂蛋白 .

[0254] SEQ ID NO:49 载脂蛋白 .

[0255] SEQ ID NO:50 载脂蛋白 .

[0256] SEQ ID NO:51 载脂蛋白 .

[0257] SEQ ID NO:52 载脂蛋白 .

[0258] SEQ ID NO:53 人四连蛋白三聚化结构域 .

[0259] SEQ ID NO:54 缩短的人四连蛋白三聚化结构域

[0260] SEQ ID NO:55 人干扰素片段 .

[0261] SEQ ID NO:56 六组氨酸标记物 .

[0262] SEQ ID NO:57 融合蛋白 .

[0263] SEQ ID NO:58 引物 N1.

[0264] SEQ ID NO:59 引物 N2.

[0265] SEQ ID NO:60 IgA 蛋白酶切割位点.

[0266] SEQ ID NO:61 IgA 蛋白酶切割位点.

[0267] SEQ ID NO:62 IgA 蛋白酶切割位点.

[0268] SEQ ID NO:63 IgA 蛋白酶切割位点.

[0269] SEQ ID NO:64 IgA 蛋白酶切割位点.

[0270] SEQ ID NO:65 IgA 蛋白酶切割位点.

[0271] SEQ ID NO:66 四连蛋白 - 载脂蛋白 A-I.

[0272] SEQ ID NO:67 具有 his- 标记物的四连蛋白 - 载脂蛋白 A-I.

[0273] SEQ ID NO:68-105 接头.

[0274] 附图描述

[0275] 图 1 用五种其脂质组成不同的脂质颗粒进行的体内兔研究的结果。上部 :胆固醇动员,并且因此可以对于所有制备的批次显示功效。底部 :对于通过使用 DPPC 作为单磷脂产生的脂质颗粒注意到肝酶的增加。

[0276] 图 2 根据本发明的 POPC 和载脂蛋白的脂质颗粒的 SEC-MALLS 分析 ;摩尔比为 1 ： 20-1 ： 160。

[0277] 图 3DPPC 和 POPC 对 LCAT 活性的影响。

[0278] 图 4 在包含 POPC 和 / 或 DPPC 的脂质颗粒中的胆固醇酯化的起始速度。

[0279] 图 5 在用 RXR-LXR 激动剂激发的细胞中胆固醇向 THP-1 衍生的泡沫细胞的流出。

[0280] 图 6 在使用 RXR-LXR 激动剂激活 ABCA-I 途径后胆固醇向 THP-1 衍生的泡沫细胞流出。

[0281] 图 7 不同载脂蛋白组合物的时间依赖性血浆浓度。

[0282] 图 8 胆固醇动员和酯化在血浆中的时间和浓度进程。

[0283] 图 9 在单次静脉内注射 100mg/kg. 后在小鼠中包含本发明的载脂蛋白的不同组合物的肝酶释放的比较。

[0284] 图 10 体内兔研究 - 血浆中的自发的溶血作用。

[0285] 图 11 使用 250mM Tris-HCl,140mM NaCl,pH7.5. 进行的脂质颗粒的分析 SEC。

[0286] 图 12 使用 50mM K_2HPO_4,250mM 精氨酸盐酸盐,7.5％海藻糖在 pH7.5 进行的脂质颗粒的分析 SEC。

[0287] 图 131 ： 20-1 ： 320 摩尔比的 POPC 和四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的非变性 PAGE(泳道 1 :非变性标记物 ;泳道 2 :摩尔比 1 ： 320 ;泳道 3 :摩尔比 1 ： 160 ;泳道 4 :摩尔比 1 ： 80 ;泳道 5 :摩尔比 1 ： 80(f/t) ;泳道 6 :摩尔比 1 ： 40 ;泳道 7 :摩尔比 1 ： 20 ;泳道 8 :载脂蛋白 (形成六聚体))。

[0288] 图 141 ： 20-1 ： 160 摩尔比的 POPC 和四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 分析。

[0289] 图 15POPC 和四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC 色谱图 (UV280 信号) 的叠加。

[0290] 图 16 摩尔比为 1 ： 40 的 POPC 和四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 分析。

[0291] 图 17 用 1 ： 20-1 ： 100 摩尔比获得的 DPPC 和四连蛋白 - 载脂蛋白 A-I 的脂质颗

粒的非变性 PAGE(1 :分子量标记物 ;2 :不含脂质的四连蛋白 - 载脂蛋白 A-I ;3 ： 1 ： 20 ; 4 ： 1 ： 40 ;5 ： 1 ： 60 ;6 ： 1 ： 80 ;7 ： 1 ： 100)。

[0292] 图 18 在 1 ： 60(最上的曲线)-1 ： 100(最低的曲线) 的摩尔比获得的 POPC ： DPPC ＝ 3 ： 1 和四连蛋白 - 载脂蛋白 A-I 的混合物的脂质颗粒的 SEC-MALLS 分析 (UV280 信号)

[0293] 图 19 使用胆酸盐、两性洗涤剂 3-8,3-10 和 3-12 进行的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的非变性 PAGE SDS。在每个凝胶上的泳道 1 :纯的载脂蛋白 ;在每个凝胶上的泳道 2 :0.1xCMC 胆酸盐脂质化的样品作为参考。

[0294] 图 20 使用 3x CMC 两性洗涤剂 3-8 和 POPC(载脂蛋白 ： 磷脂摩尔比 ＝ 1 ： 60) 进行的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 蛋白缀合物分析。

[0295] 图 21 使用 2x CMC 两性洗涤剂 3-10 和 POPC(载脂蛋白 ： 磷脂摩尔比 ＝ 1 ： 60) 进行的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 蛋白缀合物分析。

[0296] 图 22 使用 POPC 进行的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 蛋白质缀合分析。上面 :从天然的四连蛋白 - 载脂蛋白 A-I 形成的脂质颗粒 ;下面 :从变性的四连蛋白 - 载脂蛋白 A-I 形成的脂质颗粒。

[0297] 图 23 用 DMPC(1 ： 100)(二豆蔻酰磷脂酰胆碱) 脂质化的四连蛋白 - 载脂蛋白 A-I 进行的体内兔研究的结果 (a) 和用在 PBS 中未脂质化的四连蛋白 - 载脂蛋白 A-I 进行的体内兔研究 (b) 结果。

[0298] 图 24 在 5℃和 40℃贮存的包含野生型载脂蛋白 A-I(A) 和如本文报道的四连蛋白 - 载脂蛋白 A-I(B) 的脂质颗粒的 SE-HPLC 色谱图。

[0299] 材料和方法

[0300] 大小排阻 -HPLC ：

[0301] 用在 ASI-100HPLC 系统 (Dionex, Idstein, 德国) 上的 Tosoh Haas TSK3000SWXL 柱进行色谱法。通过 UV 二极管阵列检测器 (Dionex) 在 280nm 监测洗脱峰。在将浓缩的样品溶解到 1mg/ml 后，将柱用由 200mM 磷酸二氢钾和 250mM 氯化钾 pH7.0 的缓冲液进行洗涤直到获得稳定的基线。在 30 分钟内在室温使用 0.5ml/min 的流速在恒溶剂成分的条件下 (isocraticcondition) 进行分析轮次。将色谱图与 Chromeleon(Dionex, Idstein, Germany) 手动整合。通过比较高分子量形式的曲线下面积 (AUC) 与单体峰值的 AUC 来确定以％表示的聚合。

[0302] 动态光散射 (DLS) ：

[0303] DLS 是测量颗粒大小，典型地在亚微米大小范围内的非侵入性技术。在本发明中，将具有温度控制石英比色杯 (25℃) 的 Zetasizer Nano S 装置 (Malvern Instruments, Worcestershire, UK) 用于监测 1nm-6μm 之间的大小范围。在 173° 的角度检测反向散射激光的光的强度。强度在取决于颗粒扩散速度的速率波动，所述颗粒扩散速度又由颗粒大小控制。因此可以通过分析散射的光强度的波动来产生颗粒大小数据 (Dahneke, B. E(ed.), Measurement of Suspended particles by Quasielectric Light Scattering(通过拟电子化光散射测量悬浮的颗粒), Wiley Inc.(1983) ;Pecora, R., DynamicLight Scattering : Application of Photon Correlation Spectroscopy(光子相关光谱学的动态光散射应用), Plenum Press(1985))。使用 DTS 软件 (Malvern) 的多窄模式计算强度的大小分布。

用未稀释的样品进行实验。

[0304] SEC-MALLS：

[0305] SEC-MALLS 是大小排阻色谱法与三个检测器系统的组合：i)UV 检测，ii) 折射指数检测和 iii) 光散射检测。对于通过大小分离，使用来自 GEHealthcare 的 Superose6 柱 10/300GL 柱。所述方法用 pH7.4 的 PBS 缓冲液恒溶剂成分地进行，应用 0.4ml/min 的流速。系列地连接三种检测器系统。通过折射指数检测器监测完整的脂质颗粒（蛋白 - 脂质颗粒）信号，而在 280nm 测定的 UV 吸光度确定由蛋白部分诱导的信号。脂质级分的比例通过将蛋白 UV 信号从完整信号中简单扣除来获得。施加光散射允许检测各自种类的分子量，并且因此可以完整和详细的描述脂质颗粒。

[0306] 洗涤剂测定

[0307] 通过与蒸发光散射检测器偶联的反相色谱法 (RP-ELSD) 进行残余的洗涤剂的测定。使用来自 Phenomenex(Aschaffenburg,Germany) 的 Luna C184.6x150mm,5μm,100 Å 作为柱。在通过 10kDa 膜离心后，将 90μl 的流过物用于 HPLC 分离。在恒溶剂成分的条件下，用包含 0.1% (v/v) 三氟乙酸的 74% (v/v) 甲醇溶液在恒溶剂成分的条件下进行洗脱。将柱温设定在 30℃。通过蒸发光散射检测器进行检测，应用 30℃的喷雾温度，80℃的蒸发温度和 1.0l/min 的气流。通过在范围为 0.22μg-7.5μg 胆酸盐的胆酸盐情形中，建立标准曲线来进行残余的洗涤剂的量化。

[0308] 蛋白测定：

[0309] 通过测定在 280nm 的光密度 (OD) 来测定蛋白浓度，使用基于氨基酸序列计算的摩尔消光系数。

[0310] 重组 DNA 技术：

[0311] 使用标准方法来操作 DNA，如在 Sambrook，J.，等.，Molecular cloning：A laboratory manual（分子克隆实验指南）；Cold Spring Harbor LaboratoryPress（冷泉港实验室），冷泉港，New York，1989 中所述。根据生产商的说明书来使用分子生物学试剂。

[0312] 实施例 1

[0313] 制备和描述大肠杆菌表达质粒

[0314] 通过重组方式制备四连蛋白 - 载脂蛋白 A-I 融合多肽。N 端到 C 端方向的表达融合多肽的氨基酸序列如下：

[0315] - 氨基酸甲硫氨酸 (M)，

[0316] - 具有 CDLPQTHSL(SEQ ID NO：55) 的氨基酸序列的干扰素序列片段，

[0317] -GS 接头，

[0318] - 具有 HHHHHH(SEQ ID NO：56) 的氨基酸序列的六组氨酸标记物

[0319] -GS 接头，

[0320] - 具有 VVAPPAP(SEQ ID NO：60) 的氨基酸序列的 IgA 蛋白酶切割位点，和

[0321] - 具有 SEQ ID NO：02 氨基酸序列的四连蛋白 - 载脂蛋白 A-I。

[0322] 如上所述的四连蛋白 - 载脂蛋白 A-I 融合多肽是前体多肽，四连蛋白 - 载脂蛋白 A-I 融合多肽通过使用 IgA 蛋白酶体外酶促切割来从所述前体多肽进行释放。

[0323] 前体多肽编码融合基因通过用已知的重组方法和技术连接适合的核酸片段来装配。通过化学合成制备的核酸序列通过 DNA 测序证实。如下制备用于产生编码 SEQ ID NO：

31 的融合蛋白的 SEQ ID NO :01 的四连蛋白 - 载脂蛋白 A-I 的表达质粒。

[0324] 制备大肠杆菌表达质粒

[0325] 质粒 4980(4980-pBRori-URA3-LACI-SAC) 是用于表达大肠杆菌中的核心 - 链霉抗生物素蛋白的表达质粒。其通过连接来自质粒 1966(1966-pBRori-URA3-LACI-T- 重复序列 ;在 EP-B1422237 中报道) 的 3142bp 长的 EcoRI/CelII- 载体片段与 435bp 的编码 EcoRI/CelII- 片段的长核心 - 链霉抗生物素蛋白产生

[0326] 核心 - 链霉抗生物素蛋白大肠杆菌表达质粒包括下述元件 :

[0327] - 用于在大肠杆菌中复制的来自载体 pBR322 的复制起点 (对应于 Sutcliffe,G., 等,Quant.Biol.43(1979)77-90 所述的 2517-3160bp 位置),

[0328] - 编码乳清酸核苷 5'- 磷酸脱羧酶的酿酒酵母的 URA3 基因 (Rose,M.et al.Gene29(1984)113-124),其允许通过大肠杆菌 pyrF 突变体菌株 (尿嘧啶营养缺陷型) 的互补进行质粒选择,

[0329] - 核心 - 链霉抗生物素蛋白表达盒,包括

[0330] -T5 杂合启动子 (根据 Bujard,H., 等 .Methods.Enzymol(酶学方法).155(1987)416-433 和 Stueber,D., 等,Immunol.Methods(免疫学方法)IV(1990)121-152 所述的 T5-PN25/03/04 杂合启动子),包括根据 Stueber,D. 等所述的合成核糖体结合位点 (见前文),

[0331] - 核心 - 链霉抗生物素蛋白基因,

[0332] - 两个噬菌体 - 来源的转录终止子, λ-T0 终止子 (Schwarz,E., 等, Nature272(1978)410-414) 和 fd- 终止子 (Beck E. 和 Zink,B.Gene1-3(1981)35-58),

[0333] - 来自大肠杆菌的 lacI 阻遏基因 (Farabaugh,P.J.,Nature274(1978)765-769)。

[0334] 通过使用唯一的侧连 EcoRI 和 CelII 限制性内切酶切割位点从载体 4980 中切除核心 - 链霉抗生物素蛋白结构基因,并将 EcoRII/CelII 限制性位点侧邻的编码前体多肽的核酸插入 3142bp 长的 EcoRI/CelII-4980 载体片段来制备表达四连蛋白 - 载脂蛋白 A-I 前体多肽的最终表达质粒。

[0335] 实施例 2

[0336] 表达四连蛋白 - 载脂蛋白 A-I

[0337] 为了表达融合蛋白,使用大肠杆菌宿主 / 载体系统,其通过大肠杆菌营养缺陷型 (PyrF) 的互补能够进行无抗生素的质粒选择 (EP0972838 和 US6,291,245)。

[0338] 用表达质粒 p(IFN-His6-IgA- 四连蛋白 - 载脂蛋白 A-I) 进行电穿孔来转化大肠杆菌 K12 菌株 CSPZ-2(leuB,proC,trpE,th-1,ApyrF)。首先将转化的大肠杆菌细胞在 37℃在琼脂平板上培养。

[0339] 发酵方案 1 :

[0340] 关于预先发酵,使用根据 Sambrook 等 (Molecular Cloning :A laboratorymanual(分子克隆 :实验室手册). 冷泉港实验室出版社 ;第 2 版 (1989 年 12 月) 的 M9 培养基,其补充了约 1g/l L- 亮氨酸,约 1g/l L- 脯氨酸和约 1mg/l 硫胺 -HCl。

[0341] 关于预先发酵,用原始接种物储藏安瓿中的 2ml 接种在具有挡板的 1000ml 锥形瓶 (Erlenmeyer-flask) 中的 300ml M9- 培养基中。在 37℃,在旋转摇床上进行培养 13 小时,直到观察到 1-3 的光密度 (578nm)。

[0342] 对于发酵，使用根据 Riesenberg 等所述的分批培养基 (Riesenberg, D., 等, J.Biotechnol.20(1991)17-27) :27.6g/l 葡萄糖 *H_2O,13.3g/l KH_2PO_4,4.0g/l$(NH_4)_2HPO_4$, 1.7g/l 柠檬酸盐,1.2g/l $MgSO_4*7H_2O$,60mg/l 柠檬酸铁 (III),2.5mg/l $CoCl_2*6H_2O$, 15mg/l $MnCl_2*4H_2O$,1.5mg/l $CuCl_2*2H_2O$,3mg/l H_3BO_3,2.5mg/l $Na_2MoO_4*2H_2O$,8mg/l $Zn(CH_3COO)_2*2H_2O$,8.4mg/l Titriplex III,1.3ml/l Synperonic10％消泡剂。分别给分批培养基补充 5.4mg/l 硫胺 -HCl 和 1.2g/l L- 亮氨酸和 L- 脯氨酸。进料 1 溶液包含 700g/l 葡萄糖，补充了 19.7g/l $MgSO_4*7H_2O$。用于 pH 调节的碱溶液是分别补充了 50g/l L- 亮氨酸和 50g/l L- 脯氨酸的 12.5％ (w/v)NH_3 水溶液。所有成分溶解在去离子水中。

[0343] 发酵在 10l Biostat C DCU3 发酵器 (Sartorius, Melsungen, Germany) 中进行。用 6.4l 灭菌的发酵分批培养基加 300ml 来自预发酵的接种物开始，在 37℃，pH6.9±0.2，500mbar 和 10l/min 的通气速率进行分批发酵。在开始补充的葡萄糖消耗完之后，将温度调节到 28℃，发酵进入补料 - 分批模式。此时，通过加入进料 1 组合恒定增加的搅拌器速度 (在 10 小时内 550rpm-1000rpm 和在 16 小时内从 1000rpm-1400rpm) 和通气速率 (在 10 小时内从 10l/min-16l/min 和在 5 小时内从 16l/min-20l/min)，将溶氧 (pO2) 的相对值保持在 50％ (DO-stat，见例如 Shay, L.K., 等, J.Indus.Microbiol.Biotechnol.2(1987)79-85)。当培养约 8 小时后 pH 达到调节下限 (6.70) 时加入碱溶液，导致供应另外的氨基酸。通过在光密度 70 加入 1mM IPTG 来诱导重组治疗蛋白的表达。

[0344] 在发酵结束时，在收集之前，使用加热步骤 (其中在发酵器中的整个培养基被加热到 50℃达 1 或 2 小时)，胞质和可溶性表达的四连蛋白 - 载脂蛋白 A-I 被转到不可溶的蛋白聚集体，所谓的包含体 (见，例如 EP-B1486571)。随后，将发酵器的内容物用无逆流离心机 (13,000rpm,13l/h) 离心并将收获的生物量贮存在 -20℃直到进一步加工。只在不可溶的细胞碎片级分中发现不可溶的蛋白聚集体形式 (所谓的包含体 (IBs)) 的合成的四连蛋白 - 载脂蛋白 A-I 前体蛋白。

[0345] 只在不可溶的细胞碎片级分中发现不可溶的蛋白聚集体形式 (所谓的包含体 (IBs)) 的合成的融合蛋白。

[0346] 用 SDS- 聚丙烯酰胺凝胶电泳分析从发酵器中回收的样品，一个是诱导前的样品，其它的是在诱导蛋白表达后指定的时间点的样品。从每个样品，将相同量的细胞 ($OD_{靶标}$ = 5) 重新悬浮在 5mL PBS 缓冲液中并通过在冰上的超声处理来破坏。接着，将 100μL 每种悬浮液离心 (15,000rpm,5 分钟)，并回收每个上清液，将其转移到单独的瓶中。这是为了区分可溶性和不可溶的表达靶蛋白。向每个上清液 (= 可溶性) 级分加入 300μL 和每个沉淀 (= 不可溶) 级分加入 400μL 的 SDS 样品缓冲液 (Laemmli, U.K., Nature227(1970)680-685)。在摇动情况下，将样品在 95℃加热 15 分钟以溶解和还原在样品中的所有蛋白。在冷却到室温后，将 5μL 每种样品转到 4-20％ TGX Criterion Stain Free 聚丙烯酰胺凝胶 (Bio-Rad) 中。另外，将 5μl 分子量标准 (Precision Plus Protein Standard,Bio-Rad) 和具有已知产物蛋白浓度 (0.1μg/μl) 的 3 种量 (0.3μl,0.6μl 和 0.9μl) 的量化标准设置在凝胶上。

[0347] 在 200V 运行电泳 60 分钟，并随后将凝胶转到 GelDOC EZ 成像器 (Bio-Rad) 并用 UV 照射处理 5 分钟。使用 Image Lab 分析软件 (Bio-Rad) 分析凝胶图像。使用三个标准，计算线性回归曲线，系数 > 0.99，并且使用其计算在原始样品中的靶蛋白的浓度。

[0348] 发酵方案 2：

[0349] 关于预先发酵，使用根据 Sambrook 等 (Molecular Cloning:A laboratorymanual(分子克隆：实验室手册). 冷泉港实验室出版社；第 2 版 (1989 年 12 月) 的 M9 培养基，其补充了约 1g/l L- 亮氨酸，约 1g/l L- 脯氨酸和约 1mg/l 硫胺 -HCl。

[0350] 关于预先发酵，从琼脂平板或用原始接种物储藏安瓿中的 1-2ml 接种在具有挡板的 1000ml 锥形瓶中的 300ml 改良 M9- 培养基中。在 37℃，在旋转摇床上进行培养 13 小时，直到观察到 1-3 的光密度 (578nm)。

[0351] 对于发酵和高产率表达四连蛋白 - 载脂蛋白 A-I，使用下述批次培养基和进料：

[0352] 8.85g/l 葡萄糖，63.5g/l 酵母提取物，2.2g/l NH_4Cl，1.94g/l L- 亮氨酸，2.91g/l L- 脯氨酸，0.74g/l L- 甲硫氨酸，17.3g/l KH_2PO_4*$H2_0$，2.02g/l$MgSO_4$*$7H_2O$，25.8mg/l 硫胺 -HCl，1.0ml/l Synperonic 10％消泡剂。进料 1 溶液包含 333g/l 酵母提取物和 333g/l 85％ - 甘油，分别补充了 1.67g/l L- 甲硫氨酸和 5g/l L- 亮氨酸和 L- 脯氨酸。进料 2 是 600g/l L- 脯氨酸的溶液。用于 pH 调节的碱性溶液是 10％ (w/v)KOH 溶液，作为酸使用 75％葡萄糖溶液。所有成分溶解在去离子水中。

[0353] 发酵在 10l Biostat C DCU3 发酵器 (Sartorius，Melsungen，Germany) 中进行。用 5.15l 灭菌的发酵分批培养基加 300ml 来自预发酵的接种物开始，在 25℃，pH6.7±0.2，300mbar 和 10 l/min 的通气速率进行分批发酵。在开始补充的葡萄糖消耗完之前，培养物达到 15 的光密度 (578nm)，并且发酵进入进料 - 批次模式，此时进料 1 以 70g/h 开始。在培养物中监测葡萄糖浓度，将进料 1 增加到最大 150g/h，同时避免葡萄糖累积并保持 pH 接近调节上限 6.9。在 50 的光密度 (578nm)，进料 2 以 10ml/h 的恒定进料速率开始。通过平行增加搅拌器速度 (500rpm-1500rpm)，通气速率 (10l/min-20l/min) 和压力 (300mbar-500mbar) 保持溶氧 (pO_2) 的相对值在 50％以上。通过在 90 的光密度加入 1mM IPTG 来诱导重组治疗蛋白的表达。

[0354] 用 SDS- 聚丙烯酰胺凝胶电泳分析从发酵器中回收的七个样品，一个是诱导前的样品，其它的是在诱导蛋白表达后指定的时间点的样品。从每个样品，将相同量的细胞 ($OD_{靶标}$ = 5) 重新悬浮在 5mL PBS 缓冲液中并通过在冰上的超声处理来破坏。接着，将 100μL 每种悬浮液离心 (15,000rpm，5 分钟)，并回收每个上清液，将其转移到单独的瓶中。这是为了区分可溶性和不可溶的表达靶蛋白。向每个上清液 (= 可溶性) 级分加入 300μL 和每个沉淀 (= 不可溶) 级分加入 200μL 的 SDS 样品缓冲液 (Laemmli，U.K.，Nature227(1970)680-685)。在摇动情况下，将样品在 95℃加热 15 分钟以溶解和还原在样品中的所有蛋白。在冷却到室温后，将 5μL 每种样品转到 10％ Bis-Tris 聚丙烯酰胺凝胶 (Novagen) 中。另外，将 5μl 分子量标准 (Precision Plus Protein Standard，Bio-Rad) 和具有已知产物蛋白浓度 (0.1μg/μl) 的 3 种量 (0.3μl，0.6μl 和 0.9μl) 的量化标准设置在凝胶上。

[0355] 将电泳在 200V 运行 35 分钟，接着用考马斯亮蓝 R 染料对凝胶进行染色，用加热的水退色并将其转到光密度计上进行数字化 (GS710，Bio-Rad)。使用 Quantity One1-D 分析软件 (Bio-Rad) 分析凝胶图像。使用三个标准，计算线性回归曲线，系数 > 0.98，并且使用其计算在原始样品中的靶蛋白的浓度。

[0356] 在发酵结束时，在收集之前，使用加热步骤 (其中在发酵器中的整个培养基被加

热到 50℃达 1 或 2 小时），胞质和可溶性表达的四连蛋白 - 载脂蛋白 A-I 被转到不可溶的蛋白聚集体，所谓的包含体（见，例如 EP-B1486571）。加热步骤后，只在不可溶的细胞碎片级分中发现 IBs 形式的合成的四连蛋白 - 载脂蛋白 A-I 前体蛋白。

[0357] 将发酵器的内容物冷却到 4-8℃，用无逆流离心机（13,000rpm，131/h）离心，并将收获的生物量贮存在 -20℃直到进一步加工。取决于表达的构建体，总的收获的生物量产率范围在 39g/l 和 90g/l 干物质。

[0358] 实施例 3

[0359] 制备四连蛋白 - 载脂蛋白 A-I

[0360] 通过在磷酸钾缓冲溶液或 Tris 缓冲溶液（0.1M，补充 1mM $MgSO_4$，pH6.5）中再悬浮收集的细菌细胞进行包含体制备。在加入 DNAse 后，通过在 900bar 的压力匀浆来破坏细胞。将包含 1.5M NaCl 和 60mM EDTA 的缓冲溶液加入匀浆的细胞混悬液。在用 25％（w/v）HCl 将 pH 值调节到 5.0 后，在进一步离心步骤后获得最终的包含体浆液。将浆液贮存在 -20℃，单次使用的灭菌的塑料袋中直到进一步加工。

[0361] 将包含体浆液（约 15kg）溶解在盐酸胍溶液（150 1，6.7M）中。在通过深度过滤澄清加溶物后，将溶液加入 Zn- 螯合的亲和性色谱材料中。通过 Zn- 螯合色谱材料纯化融合多肽并通过 IgA 蛋白酶切割。随后，用阴离子交换色谱法和阳离子交换色谱法步骤进一步纯化多肽。这些步骤在包含尿素的溶液（7M），即在变性条件下进行。将这些步骤用于去除多肽片段、内毒素和其他的杂质。进行包含 6.7M 盐酸胍的溶液中的渗滤。获得的最终溶液包含变性的四连蛋白 - 载脂蛋白 A-I。

[0362] 实施例 4

[0363] 四连蛋白 - 载脂蛋白 A-I 的再折叠和脂质化

[0364] a）一般方法

[0365] 将纯的结晶 POPC 或 DPPC（Lipoid，Switzerland）溶解在水性缓冲液（脂质化缓冲液）中，所述缓冲液以 1 ： 1.35 摩尔比磷脂：胆酸盐包含胆酸盐。将所述混合物在氮气气氛下温育并且在室温（POPC）或在 55℃（DPPC）避光，直到获得澄清溶液。将澄清的脂质 - 胆酸盐溶液冷却到 4℃（POPC）或贮存在 41℃（DPPC）。在限定的载脂蛋白：磷脂比率，在 4℃（POPC）或 41℃（DPPC）加入纯化的四连蛋白 - 载脂蛋白 A-I。对于脂质颗粒形成，在氮气气氛下在 4℃（POPC）或 41℃（DPPC）并且避光将反应混合物温育过夜。最终，通过针对脂质化缓冲液的大量透析（4℃ /41℃）来去除胆酸盐。最终，离心样品来去除沉淀的材料。

[0366] 如上制备包含纯的 POPC 或纯的 DPPC 的胆酸盐溶解的脂质溶液。通过在需要的比率组合脂质溶液来制备脂质混合物随后在各自 T_m（T_m ＝相变温度）贮存。如对于纯的脂质溶液所述进行，但是在选择的脂质混合物的各自 T_m 进行四连蛋白 - 载脂蛋白 A-I 的脂质颗粒形成。

[0367] 已经测试了下述脂质化缓冲液：

[0368] 1. 在 pH7.5，用 250mM 盐酸精氨酸，7.5％蔗糖补充的 50mM 磷酸钾缓冲液

[0369] 2. 在 pH7.5，用 250mM 盐酸精氨酸，7.5％蔗糖，10mM 甲硫氨酸补充的 50mM 磷酸氢二钾缓冲液

[0370] 3. 在 pH7.5，用 140mM NaCl，10mM 甲硫氨酸补充的 250mM tris- 羟基氨基甲烷

(TRIS)

[0371] 4. 在 pH7.5，用 250mM 盐酸精氨酸、7％海藻糖，10mM 甲硫氨酸补充的 50mM 磷酸氢二钾缓冲液。

[0372] 通过分析 SEC 评估从四连蛋白－载脂蛋白 A-I 样品形成的脂质颗粒的均匀性（图 11 和 12）。总之，与磷脂的选择相比，脂质缓冲液的选择仅具有微小的作用。DPPC- 脂质颗粒作为一个主峰洗脱，而且 POPC- 脂质颗粒显示两个峰值模式。脂质化缓冲液的选择受到载脂蛋白的纯化过程以及稳定的无脂质的载脂蛋白的供应的影响。显示能够与脂质化缓冲液无关地形成脂质颗粒。在各种测试的缓冲液中，最适合的脂质化缓冲液鉴定为 250mM Tris、140mM NaCl，10mM 甲硫氨酸，pH7.5。

[0373] 脂质化混合物分别包含限定量的载脂蛋白，并且相应计算磷脂，例如 POPC 的量。脂质的摩尔量的计算都基于四连蛋白－载脂蛋白 A-I 单体。

[0374] b)POPC 和胆酸盐

[0375] 表 6：使用纯的 POPC 用四连蛋白－载脂蛋白 A-I 作为实例形成脂质颗粒。计算蛋白单体的载脂蛋白：磷脂的摩尔比。对照：不加入脂质（纯的 Apo）温育载脂蛋白和不加入载脂蛋白（无 Apo）温育脂质。

[0376]

载脂蛋白:磷脂摩尔比	过夜温育后观察到的	透析前的蛋白浓度 [mg/ml]	透析后的蛋白浓度 [mg/ml]	透析后观察到的
1:320	澄清	0.67	n.d.	浑浊
1:160	澄清	1.34	1.47	澄清
1:80	澄清	2.68	2.6	澄清
1:40	澄清	5.36	4.87	澄清
1:20	浑浊	10.73	5.02	浑浊*
仅 Apo	浑浊	2.68	0.51	浑浊*
无 Apo	澄清	-	-	澄清

[0377] *在离心后澄清

[0378] 在整个过程中，从 1 ： 40-1 ： 160 的摩尔比保持澄清。没有观察到通过过量磷脂的浑浊也没有观察到蛋白沉淀。

[0379] 通过非变性 PAGE 分析脂质颗粒样品（见图 13）。用样品 1 ： 80（泳道 4）发现最均一的条带模式。此外，1x 冻 / 融（-80℃）也没有改变样品的外观（泳道 5）。样品 1 ： 320 和 1 ： 160 的条带模式显示不均匀的产物，导致多个条带（泳道 2 和 3）。样品 1 ： 40 以及此外 1 ： 20 具有在主产物条带以下的另外的条带（泳道 6 和 7）。纯的四连蛋白－载脂蛋白 A-I 的迁移模式在图 13 的泳道 8 中显不。

[0380] SEC-MALLS 分析用于获得关于脂质颗粒和它们的载脂蛋白 - 磷脂组成的均一性的更详细的信息（蛋白 - 缀合物分析）。图 14 显示 SEC 分辨的样品的色谱图 (UV280 检测）。在这里，将 1 ： 160 样品分为三个单独的峰。1 ： 80 样品显示包含至少两个不同大小的种类，如作为双峰所显示的。从样品 1 ： 20 获得的峰值显示最均匀的产物。

[0381] 在 5 的步骤中，使用四连蛋白 - 载脂蛋白 A-I(3.84mg/ml ;10mg/ 样品）进行实验，并且载脂蛋白：磷脂摩尔比从 1 ： 40 增加到 1 ： 80。在低于 1 ： 40 的摩尔比，脂质颗粒形成是不完全的。通过实验排除了在大于 1 ： 80 的摩尔比，在通过透析去除胆酸盐之后，样品变得浑浊。而且，在更高的脂质比率，脂质颗粒变得更加的不均匀。

[0382] 表 7 ：使用纯的 POPC 的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒形成。基于四连蛋白 - 载脂蛋白 A-I 单体计算载脂蛋白：磷脂的摩尔比。

[0383]

载脂蛋白:磷脂摩尔比	透析前的蛋白浓度 [mg/ml]*	透析后的蛋白浓度 [mg/ml]*	产率[%]	透析后观察到的
1:40	3.5	2.67	76	沉淀
1:45	3.5	2.74	78	沉淀
1:50	3.5	2.94	84	沉淀
1:55	3.5	3.05	87	沉淀
1:60	3.5	3.19	91	沉淀
1:65	3.5	3.34	95	沉淀
1:70	3.5	3.52	100**	
1:75	3.5	3.56	100**	
1:80	3.5	3.57	100**	

[0384] * 在透析前和透析后的体积 2.6ml

[0385] ** 在所述方法的 SD 内

[0386] 在 -3℃的转变温度温育过程中，所有的样品保持视觉上的澄清。在通过透析去除胆酸盐后，观察到样品 1 ： 40-1 ： 65 的增加的浊度。可以通过离心去除沉淀并且随后样品保持澄清。

[0387] SEC-MALLS 分析用于获得关于形成的脂质颗粒和它们的载脂蛋白 - 磷脂组成的均一性的详细信息（蛋白 - 缀合物分析）。在分析大小排阻色谱法上所有的脂质颗粒是可比地均一 (SEC ;图 15)，显示小的后峰值，这在更低的摩尔比是更为显著的。此外，在峰值模式上存在更高的摩尔比向更高分子量的显著的移动。在表 8 中提供各自保留时间。

[0388] 表 8 ：大小排阻色谱法结果的总结 ：通过曲线下面积 (AUC) 的积分计算百分比

[0389]

UV280	保留时间 主峰 **[min.]**	主峰 **[%]**	后峰 **[%]**	总面积 **[mAU*min]**
POPC 1:40	56.2	89.3	10.7	322.3
POPC 1:45	55.9	89.7	10.4	331.3
POPC 1:50	55.8	90.0	10.0	333.2
POPC 1:55	55.7	91.0	9.1	342.5
POPC 1:60	55.6	90.8	9.2	331.7
POPC 1:65	55.3	90.9	9.2	337.2
POPC 1:70	55.2	91.1	8.9	326.5
POPC 1:75	55.1	91.3	8.7	347.1
POPC 1:80	54.8	92.0	8.0	347.8

[0390] 蛋白-缀合物分析(在表8中总结)能够计算从SEC柱上洗脱的每种脂质颗粒的蛋白(MW蛋白)以及脂质成分(MW脂质)的总分子量。基于四连蛋白-载脂蛋白A-I单体(32.7kDa)和POPC(760Da)的分子量,可以计算脂质颗粒的组成(n蛋白和n POPC)。对应于每个脂质颗粒的四连蛋白-载脂蛋白A-I三聚体,在所有摩尔比的脂质颗粒主峰中发现的载脂蛋白成分的分子量是约100kDa。n(POPC)/n(蛋白单体)的比率提供在脂质颗粒中每个四连蛋白-载脂蛋白A-I单体的POPC分子的数目。尽管应用了从1:40直至1:80的摩尔比,每个四连蛋白-载脂蛋白A-I单体的POPC分子的数目在54和75之间变化。%蛋白值是脂质化程度的参数。蛋白在脂质颗粒中的百分比越低,脂质化程度越高。

[0391] 表9:如在图16中显示的,POPC和四连蛋白-载脂蛋白A-I的脂质颗粒的蛋白缀合物分析总结。

[0392]

		MW 总计 [kDa]	MW 蛋白 [kDa]	n (单体)	MW 脂质 [kDa]	n (POPC)	n(POPC)/ n(单体)	% 蛋白
1:40	主峰	238	104	3.3	135	178	54	44
	后峰	230	148	4.6	81	107	23	65
1:45	主峰	238	101	3.2	138	182	57	42
	后峰	184	118	3.7	66	87	24	64
1:50	主峰	244	100	3.1	143	188	61	41
	后峰	187	118	3.7	70	92	25	63
1:55	主峰	247	99	3.1	148	195	63	40
	后峰	182	107	3.3	75	99	30	59
1:60	主峰	248	98	3.1	150	197	64	40
	后峰	183	106	3.3	76	100	30	58
1:65	主峰	255	97	3.0	158	208	69	38
	后峰	191	103	3.2	88	116	36	54
1:70	主峰	260	97	3.0	163	214	71	37
	后峰	196	100	3.1	95	125	40	51
1:75	主峰	266	99	3.1	168	221	71	37
	后峰	208	118	3.7	91	120	32	56
1:80	主峰	275	99	3.1	176	232	75	36
	后峰	215	112	3.5	103	136	39	52

[0393] c)DPPC 和胆酸盐

[0394] 在脂质化之前，用 pH7.5 的 50mM KH_2PO_4，250mM 精氨酸盐酸盐，7％海藻糖，10mM 甲硫氨酸透析四连蛋白－载脂蛋白 A-I。在 5 的步骤中，使用 1 ： 60-1 ： 100 的摩尔比（脂质浓度增加）对四连蛋白－载脂蛋白 A-I(3.84mg/ml，3mg/ 样品）进行脂质化。脂质化缓冲液是 250mM Tris-HCl，140mMNaCl，10mM 甲硫氨酸，pH7.5.

[0395] 表 10 ：载脂蛋白与 DPPC 的脂质颗粒的样品综述

[0396]

摩尔比载脂蛋白:磷脂*	在 o/n 温育后观察到的	基于蛋白的产率 [%]
1:20	澄清	85
1:40	澄清	88
1:60	澄清	89
1:80	澄清	91
1:100	澄清	94
仅 Apo	澄清	86
无 Apo	澄清	沉淀的 DPPC

[0397] *对于蛋白单体计算的

[0398] 在脂质颗粒形成过程中,没有观察到蛋白沉淀或通过过量脂质的浑浊。在最终产物中的四连蛋白-载脂蛋白 A-I 的产率越高,用于脂质化的 DPPC 越多。

[0399] 在非变性 PAGE 上在 1 : 20 样品中发现残余的无脂质的载脂蛋白(泳道 3,图 17)。在非变性 PAGE 上,1 : 40 和 1 : 60 样品看上去最均匀(泳道 4 和 5),而 1 : 80 和 1 : 100 样品在主要的脂质颗粒条带上还包含另外的更高的分子条带(泳道 6 和 7)。

[0400] SEC-MALLS 蛋白缀合物分析用于表征在 DPPC 脂质颗粒形成之后获得的脂质颗粒的组成 (MW DPPC :734Da)。在 1 : 80 和以下的摩尔比获得均匀的 SEC 峰。在更高的脂质比率,出现前-峰(见,例如在表 11 中的 1 : 90 的样品)。

[0401] 表 11 :DPPC 和四连蛋白-载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 蛋白缀合物分析总结

[0402]

摩尔比 载脂蛋白:磷脂	峰	MW 总[kDa]	MW 蛋白 kDa]	n (蛋白)	MW 脂质[kDa]	n (DPPC)/ n (蛋白)	% 蛋白
1:60	1	724	298	9.0	425	193	41.2
1:65	1	281	109	3.3	171	77	38.9
1:70	1	273	103	3.1	169	76	37.9
1:75	1	286	103	3.1	183	83	36.0
1:80	1	295	100	3.0	194	88	34.1
1:85	1	307	99	3.0	207	94	32.6
1:90	1	361	117	3.5	244	110	32.6
	2	319	101	3.0	217	98	31.8
1:95	1	397	134	4.0	262	118	33.8
	2	327	100	3.0	226	102	30.8
1:100	1	405	132	4.0	273	123	32.6
	2	344	101	3.0	243	110	29.3

[0403] 用 1 ： 80-1 ： 90 摩尔比发现脂质化的最高程度（蛋白的最低百分比）。此外，DLS 揭示在 1 ： 80-1 ： 90（＞ 98％）的比率的最为均匀的颗粒形成，颗粒大小为 14-17nm。

[0404] d) 75％ DPPC/25％ POPC

[0405] 如在本实施例 a)-c) 项中报道的相应地进行脂质颗粒形成，使用下述参数 ：

[0406] 蛋白 ：四连蛋白 - 载脂蛋白 A-I 在 3.84mg/ml，3mg/ 样品

[0407] 脂质化缓冲液 ：250mM Tris-HCl，140mM NaCl，10mM 甲硫氨酸 pH7.5

[0408] 脂质化 ：在 34℃

[0409] 透析 ：在 4℃

[0410] 测试的摩尔比 ： 在 5 的步骤中，1 ： 60-1 ： 100，具有增加的脂质

[0411] 脂质颗粒形成简单易行，并且与用纯的脂质进行的方法相当。在所述方法和透析过程中，所有的样品保持澄清。对于所有测试的比率（～ 85％），脂质颗粒的产率是类似的。SEC-MALLS 分析显示 1 ： 80 的摩尔比导致具有 90.9％主峰，无前峰和 9.1％后峰的最均匀的脂质颗粒。蛋白缀合物分析显示在所有样品的主要种类中，1 个四连蛋白 - 载脂蛋白 A-I 三聚体 / 脂质颗粒的存在（见图 18 和表 12 和 13）。

[0412] 表 12 ：SEC 结果的总结 ；通过 AUC 积分计算百分比

[0413]

UV280	保留时间 主峰	前峰 [%]	主峰 [%]	后峰 [%]	总 [mAU*min]
75/25 DPPC/POPC 1:60	58.3	-	89.7	10.3	360.5
75/25 DPPC/POPC 1:65	58.3	-	89.2	10.8	383.7
75/25 DPPC/POPC 1:70	58.3	-	89.5	10.5	376.8
75/25 DPPC/POPC 1:75	58.4	-	90.3	9.7	367.0
75/25 DPPC/POPC 1:80	58.3	-	90.9	9.1	383.5
75/25 DPPC/POPC 1:85	58.2	10.4	79.5	10.1	356.4
75/25 DPPC/POPC 1:90	58.3	10.2	81.5	8.3	344.6
75/25 DPPC/POPC 1:95	58.0	16.9	74.9	8.2	377.4
75/25 DPPC/POPC 1:100	58.0	21.0	70.4	7.7	365.0

[0414] 表 13 :75% DPPC/25% POPC 和四连蛋白 - 载脂蛋白 A-I 脂质颗粒的蛋白 - 缀合物分析总结 .

[0415]

		MW 总计	MW 蛋白 [kDa]	n (蛋白单体)	MW 脂质 [kDa]	n (脂质)	n(脂质)/ n(单体)	% 蛋白
1:60	主峰	257	96	3.0	161	217	72	37
	后峰	92	75	2.3	17	23	10	82
1:65	主峰	263	95	3.0	167	226	76	36
	后峰	116	102	3.2	14	19	6	88
1:70	主峰	268	95	3.0	173	234	79	35
	后峰	93	83	2.6	10	14	5	89
1:75	主峰	275	95	3.0	180	243	82	34
	后峰	98	82	2.6	16	22	8	84
1:80	主峰	279	95	3.0	184	248	84	34
	后峰	97	86	2.7	11	15	6	89
	前峰	329	104	3.3	224	302	93	32
1:85	主峰	291	96	3.0	195	263	88	33
	后峰	129	107	3.3	22	30	9	83
	前峰	443	107	3.3	237	320	96	31
1:90	主峰	293	95	3.0	197	266	90	33
	后峰	126	102	3.2	25	34	11	81

[0416]

		MW 总计	MW 蛋白 [kDa]	n (蛋白单体)	MW 脂质 [kDa]	n (脂质)	n(脂质)/n(单体)	% 蛋白
	前峰	384	110	3.4	274	370	108	29
1:95	主峰	303	96	3.0	207	280	93	32
	后峰	130	103	3.2	27	36	11	79
	前锋	398	111	3.5	287	388	112	28
1:100	主峰	310	96	3.0	213	288	96	31
	后峰	122	86	2.7	36	49	18	71

[0417] e)50％ DPPC/50％ POPC

[0418] 如在本实施例 a)-c) 项中报道的相应地进行脂质颗粒形成，使用下述参数：

[0419] 蛋白：四连蛋白 - 载脂蛋白 A-I 在 3.84mg/ml，3mg/ 样品

[0420] 脂质化缓冲液：250mM Tris-HCl，140mM NaCl，10mM 甲硫氨酸 pH7.5

[0421] 脂质化：在 27℃

[0422] 透析：在室温

[0423] 测试的摩尔比：在 5 的步骤中，1 ： 60-1 ： 100，具有增加的脂质

[0424] 所有的样品在所述方法和透析过程中保持澄清。对于所有测试的比率，脂质颗粒的产率是类似的。

[0425] 表 14：SEC 结果的总结；通过 AUC 的积分计算百分比。

[0426]

UV280	保留时间 主峰 [min]	前峰 [%]	主峰 [%]	后峰 [%]	总计 [mAU*min]
50/50 DPPC/POPC 1:60	58.2	-	88.9	11.1	341.3
50/50 DPPC/POPC 1:65	58.3	-	89.3	10.7	349.6
50/50 DPPC/POPC 1:70	58.3	-	89.9	10.1	336.9
50/50 DPPC/POPC 1:75	58.2	6.1	84.3	9.6	347.4
50/50 DPPC/POPC 1:80	58.1	8.5	82.2	9.3	356.9
50/50 DPPC/POPC 1:85	58.0	11.3	79.8	8.9	352.7
50/50 DPPC/POPC 1:90	58.0	14.4	77.1	8.5	356.5

[0427]

UV280	保留时间 主峰 [min]	前峰 [%]	主峰 [%]	后峰 [%]	总计 [mAU*min]
50/50 DPPC/POPC 1:95	58.0	19.3	72.6	8.1	367.0
50/50 DPPC/POPC 1:100	57.9	36.6	65.8	7.6	365.3

[0428] 使用 50％ DPPC 和 50％ POPC 的脂质混合物来用于四连蛋白－载脂蛋白 A-I 的脂质颗粒形成，在 1 ： 70 的摩尔比获得最均匀的产物（见表 14）。产物关于主峰是 89.9％纯的，并且包含一个单一的四连蛋白－载脂蛋白 A-I 三聚体（见表 15）。

[0429] 表 15：具有 50％ DPPC/50％ POPC 和四连蛋白－载脂蛋白 A-I 的脂质颗粒的蛋白缀合物分析总结

[0430]

		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	%蛋白
1:60	主峰	331	124	3.9	207	277	71	38
	后峰	131	106	3.3	24	32	10	81
1:65	主峰	264	95	2.9	169	226	78	36
	后峰	127	112	3.5	16	21	6	88
1:70	主峰	273	96	3.0	178	238	79	35
	后峰	258	213	6.7	45	60	9	82
	前峰	319	108	3.4	211	282	83	34
1:75	主峰	271	93	2.9	178	238	82	34
	后峰	126	106	3.3	20	27	8	84
	前峰	333	108	3.4	225	301	89	32
1:80	主峰	278	95	2.9	184	246	85	34
	后峰	122	100	3.1	21	28	9	83
	前峰	359	109	3.4	250	335	98	30
1:85	主峰	284	94	2.9	189	253	87	33
	后峰	132	118	3.7	14	19	5	89
	前峰	373	109	3.4	264	353	104	29

[0431]

		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/n (单体)	%蛋白
1:90	主峰	286	94	2.9	192	257	89	33
	后峰	133	110	3.4	23	31	9	83
	前峰	390	111	3.5	278	372	106	29
1:95	主峰	290	94	2.9	195	261	90	33
	后峰	162	136	4.3	26	35	8	84
	前峰	404	113	3.5	291	390	111	28
1:100	主峰	293	94	2.9	199	266	92	32
	后峰	142	107	3.3	35	47	14	75

[0432] f)25% DPPC/75% POPC

[0433] 如在本实施例 a)-c) 项中报道的相应地进行脂质颗粒形成,使用下述参数 :

[0434] 蛋白 :四连蛋白 - 载脂蛋白 A-I 在 3.84mg/ml,3mg/ 样品

[0435] 脂质化缓冲液 :250mM Tris-HCl,140mM NaCl,10mM 甲硫氨酸 pH7.5

[0436] 脂质化 :在 18℃

[0437] 透析 :在室温

[0438] 测试的摩尔比 :在 5 的步骤中,1 ： 60-1 ： 100,具有增加的脂质

[0439] 脂质颗粒形成简单易行,并且与使用纯的脂质的方法相当。在所述方法和透析过程中,所有样品保持澄清。

[0440] 表 16 :SEC 结果的总结 ;通过 AUC 的积分计算百分比。

[0441]

UV280	保留时间 主峰 [min]	前峰 %	主峰 %	后峰 %	总计 [mAU*min]
25/75 DPPC/POPC 1:60	58.2	-	90.2	9.8	342.6
25/75 DPPC/POPC 1:65	58.2	4.6	85.9	9.4	345.6
25/75 DPPC/POPC 1:70	58.1	8.8	82.3	8.9	353.2
25/75 DPPC/POPC 1:75	58.0	9.0	82.4	8.6	357.5
25/75 DPPC/POPC 1:80	57.9	10.8	81.2	8.0	356.7
25/75 DPPC/POPC 1:85	57.9	21.2	71.0	7.8	366.3
25/75 DPPC/POPC 1:90	57.8	26.1	66.4	7.5	357.8
25/75 DPPC/POPC 1:95	57.7	32.7	60.5	6.8	365.9
25/75 DPPC/POPC 1:100	57.6	36.1	57.5	6.4	373.4

[0442] 使用 25％ DPPC 和 75％ POPC 的脂质混合物来形成四连蛋白－载脂蛋白 A-I，在 1 ： 60 的摩尔比获得最均匀的产物（见表 17）。产物关于主峰是 90.2％纯的，并且包含一个单一的四连蛋白－载脂蛋白 A-I 三聚体（见表 15）。

[0443] 表 17 :25％ DPPC/75％ POPC 和四连蛋白－载脂蛋白 A-I 的脂质颗粒的蛋白缀合物总结。

[0444]

		MW 总计	MW 蛋白质	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	%蛋白
1:60	主峰	254	100	3.1	153	203	66	40
	后峰	127	110	3.4	17	23	7	86
	前峰	272	132	4.1	141	187	46	48

[0445]

		MW 总计	MW 蛋白质	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/n (单体)	%蛋白
1:65	主峰	259	100	3.1	159	211	68	39
	后峰	183	131	4.1	7	9	2	95
	前峰	280	121	3.8	159	211	56	43
1:70	主峰	264	99	3.1	165	219	71	38
	后峰	119	105	3.3	14	19	6	88
	前峰	291	109	3.4	183	243	71	37
1:75	主峰	268	98	3.1	170	226	73	37
	后峰	120	101	3.2	19	25	8	84
	前峰	311	114	3.6	197	261	73	37
1:80	主峰	276	96	3.0	176	234	78	36
	后峰	137	127	4.0	10	13	3	93
	前峰	331	115	3.6	216	287	80	35
1:85	主峰	278	98	3.1	180	239	77	35
	后峰	139	117	3.7	22	29	8	85
	前峰	345	113	3.5	232	308	88	33
1:90	主峰	285	98	3.1	187	248	80	34
	后峰	143	110	3.4	33	44	13	77
	前峰	363	115	3.6	248	329	91	32
1:95	主峰	292	97	3.0	194	257	86	33
	后峰	155	122	3.8	33	44	12	79
	前峰	377	117	3.7	260	345	93	31
1:100	主峰	298	98	3.1	200	265	86	33
	后峰	160	114	3.6	46	61	17	71

[0446] g) 使用两性洗涤剂形成脂质颗粒

[0447] 如在本实施例的 a)-c) 项报道（使用具有下述参数）相应地进行脂质颗粒的形成，除了用合成洗涤剂两性洗涤剂取代胆酸盐：

[0448] 蛋白：四连蛋白 - 载脂蛋白 A-I，在 23.5mg/ml

[0449] 缓冲液：50mM Tris-HCl，7.2M 盐酸胍，10mM 甲硫氨酸，pH8

[0450] 脂质化缓冲液：250mM Tris-HCl，140mM NaCl，pH7.5

[0451] 100％ POPC，载脂蛋白：磷脂摩尔比＝ 1 ： 60

[0452] 表 18：脂质颗粒形成的各种方法和观察 / 参数的样品综述

[0453]

样品	洗涤剂[%]	浊度			透析后的体积[ml]	c 透析后 [μg/ml]	[mg] TN-Apo A-I	产率 [%]
		溶解的脂质	脂质化	透析后				
两性洗涤剂 3-8								
0.1 x CMC	0.8	+++	+++	+++	2.1	2230.18	4.68	99.6
0.5 x CMC	4.2	++	++	+	2.9	1536.81	4.46	94.8
1 x CMC	8.4	+	+	+	3	1475.07	4.43	94.2
2 x CMC	16.7	-	-	-	4.3	1081.27	4.65	98.9
3 x CMC	25.1	-	-	-	5.5	839.85	4.62	98.3
两性洗涤剂 3-10								
0.1 x CMC	0.1	+++	+++	+++	2	2361.56	4.72	100.5
0.5 x CMC	0.6	+++	++	++	2	2221.38	4.44	94.5
1 x CMC	1.2	++	+	+	2.1	2267.16	4.76	101.3
2 x CMC	2.5	+	+	(+)	2.3	2082.18	4.79	101.9
5 x CMC	6.2	-	-	-	2.5	1941.61	4.85	103.3
10 x CMC	12.3	-	-	-	4	1073.92	4.30	91.4
两性洗涤剂 3-12								
0.1 x CMC	0.01	+++	+++	+++	2	2722.85	5.45	115.9
1 x CMC	0.1	+++	+++	+++	2	2158.81	4.32	91.9
2 x CMC	0.2	+++	+++	++	2	2636	5.27	112.2
20 x CMC	1.9	+	+	+	2.1	2525.69	5.30	112.8
100 x CMC	9.4	-	-	-	3.5	1567.85	5.49	116.8
300 x CMC	28.1	-	-	-	5.6	1069.04	5.99	127.4
胆酸盐								
0.1 x CMC	0.06	+++	+++	+++	2	2323.09	4.65	98.9

[0454]

样品	洗涤剂[%]	浊度			透析后的体积[ml]	c 透析后 [μg/ml]	[mg] TN-Apo A-I	产率 [%]
		溶解的脂质	脂质化	透析后				
0.5 x CMC	0.3	+	-	-	2	2301.15	4.60	97.9
1 x CMC	0.6	-	-	-	2	2316.86	4.63	98.6
2 x CMC	1.2	-	-	-	2.5	1178.72	2.95	62.7
5 x CMC	3	-	-	-	2.5	2435.34	6.09	129.5
10 x CMC	6	-	-	-	3.5	1814.69	6.35	135.1

[0455] 在非变性 PAGE 上分析包含四连蛋白 - 载脂蛋白 A-I 的脂质颗粒。无脂质的四连蛋白 - 载脂蛋白 A-I 在 140kDa 迁移（在图 19 中泳道 1），而脂质颗粒显示向 232kDa 和 440kDa 之间的更高分子量的特征性移动。

[0456] 在所有用各自洗涤剂的仅 0.1x CMC 制备的样品中检测无脂质的四连蛋白 - 载脂蛋白 A-I，但是没有检测到脂质颗粒（图 19，泳道 2，8，13，和 19）。然而，0.5x CMC 浓度的洗涤剂足以使两性洗涤剂 3-8 和 3-10 能够使四连蛋白 - 载脂蛋白 A-I 形成脂质颗粒（泳道 3，9，和 14）。使用两性洗涤剂 3-12，没有发生脂质颗粒形成，直到达到 2.0x CMC 的浓度（泳道 21）。

[0457] 图 20 显示使用 3x CMC 两性洗涤剂 3-8 和 POPC（载脂蛋白：磷脂摩尔比＝ 1 ： 60）的包含四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的 SEC-MALLS 色谱图。蛋白 - 缀合物分析的结果总结在表 18 中。脂质颗粒级分由两种不同的种类组成，如在 SEC 色谱图中在两个重叠的峰中显示的。然而，这两个种类非常相似，不同之处主要在每个颗粒的四连蛋白 - 载脂蛋白 A-I 分子的数目（对于峰 1 为 4.2 和对于峰 2 为 3.5）。

[0458] 表 19 ：在两性洗涤剂 3-8 存在时形成的脂质颗粒的蛋白 - 缀合物分析的总结。

[0459]

x CMC		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	% 蛋白	Rh (w) (QELS) [nm]
2	前峰	345	147	4.6	198	261.5	57	42.5	7.7
	主峰	268	113	3.6	154	203.2	56	42.4	6.5
3	前峰	323	134	4.2	188	249.9	60	41.6	7.4
	主峰	257	110	3.5	146	192.9	55	43.0	6.5

[0460] 图 21 显示 SEC-MALLS 分析的色谱图和表 19 使用 2x CMC 两性洗涤剂 3-10 和 POPC（载脂蛋白：磷脂摩尔比＝ 1 ： 60）的包含四连蛋白 - 载脂蛋白 A-I 的脂质颗粒的蛋

白－缀合物分析的总结。两个峰包含分别含有 3.5 和 5 个四连蛋白－载脂蛋白 A-I 分子的脂质颗粒。

[0461] 表 20：在存在两性洗涤剂 3-10 时形成的脂质颗粒的蛋白－缀合物分析总结。

[0462]

x CMC		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	% 蛋白	Rh (w) (QELS) [nm]
2	前峰	373	161	5.0	211	278.7	56	43.2	7.8
	主峰	272	112	3.5	159	210.3	60	41.4	6.6
5	前峰	345	150	4.7	195	256.6	55	43.6	7.5
	主峰	263	112	3.5	151	199.1	57	42.6	6.6
10	前峰	405	151	4.7	253	334.1	71	37.4	7.9
	主峰	265	110	3.3	154	203.2	58	41.8	6.5

[0463] 使用两性洗涤剂 3-12 和 POPC（载脂蛋白：磷脂摩尔比＝ 1 ： 60）的包含四连蛋白－载脂蛋白 A-I 的脂质颗粒形成的结果总结在表 21 中。脂质颗粒级分由两种不同的种类组成，如在 SEC 色谱图中在两个重叠的峰中显示的。然而，这两个种类非常相似，不同之处主要在每个颗粒的四连蛋白－载脂蛋白 A-I 分子的数目。

[0464] 表 21：在存在两性洗涤剂 3-12 时形成的脂质颗粒的蛋白－缀合物分析总结。

[0465]

x CMC		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	% 蛋白	Rh (w) (QELS) [nm]
100	主峰	487	342	10.7	145	191.3	18	70.2	11.9
300	主峰	241	208	6.5	32	43.3	7	86.4	8.5

[0466] 使用胆酸盐和 POPC（载脂蛋白：磷脂摩尔比＝ 1 ： 60）的包含四连蛋白－载脂蛋白 A-I 的脂质颗粒形成的结果总结在表 21 中。脂质颗粒级分由两种不同的种类组成，如在 SEC 色谱图中在两个重叠的峰中显示的。然而，这两个种类非常相似，不同之处主要在每个颗粒的四连蛋白－载脂蛋白 A-I 分子的数目。

[0467] 表 22：在存在胆酸盐时形成的脂质颗粒的蛋白－缀合物分析的总结

[0468]

CMC		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	n(脂质)	n(脂质)/ n (单体)	% 蛋白	Rh (w) (QELS) [nm]
0.5	前峰	1295	461	14.5	829	1091	75	35.9	12.7
	主峰	361	153	4.8	207	273	57	42.5	7.7
	后峰	283	115	3.6	168	221	62	40.6	6.8
1	前峰	1050	414	12.9	623	836	65	39.5	11.8
	主峰	337	154	4.8	182	240	50	45.9	7.6
	后峰	284	121	3.8	162	214	56	42.7	6.9
2	前峰	332	143	4.5	188	248	55	43.2	7.3
	主峰	269	111	3.5	158	209	60	41.2	6.5
5	前峰	314	143	4.5	171	225	50	45.6	7.5
	主峰	278	118	3.7	158	208	56	42.7	6.8
10	前峰	292	135	4.2	156	206	50	46.3	7.3
	主峰	271	115	3.6	155	204	57	42.6	6.6

[0469] 实施例 5

[0470] 用于再折叠和脂质颗粒形成的迅速稀释方法

[0471] a)POPC 和胆酸钠

[0472] 将四连蛋白 - 载脂蛋白 A-I 在大肠杆菌中表达并且根据实施例 1-3 纯化（方法 1)。纯化后，将缓冲液通过渗滤改变为 pH7.4 的包含 250mM Tris，140mM NaCl，6.7M 盐酸胍的溶液。将蛋白浓度调节到 28mg/ml。

[0473] 通过在室温将 100 摩尔 /l POPC 溶解在包含 250mM Tris-HCl，140mMNaCl，135mM 胆酸钠的 pH7.4 的缓冲液中来制备脂质贮存溶液。将脂质贮存溶液在室温温育 2 小时。通过将 77ml 脂质贮存混合物稀释到 1478ml 的 250mM Tris-HCl，140mM NaCl，pH7.4 中来制备再折叠缓冲液。将该缓冲液在室温，搅拌另外 7 小时。

[0474] 再折叠和脂质颗粒形成通过将在 250mM Tris，140mM NaCl，6.7M 盐酸胍，pH7.4 中的 162ml 四连蛋白 - 载脂蛋白 A-I 加入再折叠缓冲液来起始。这导致盐酸胍的 1 ： 10 稀释液。将溶液在室温温育 16 小时，同时持续搅拌。通过渗滤进行洗涤剂的去除。

[0475] 表 23 :通过用 POPC 快速稀释获得的脂质颗粒的蛋白缀合物分析的总结

[0476]

峰	MW 总计 [kDa]	MW 蛋白 [kDa]	n (蛋白单体)	MW 脂质 [kDa]	n (脂质)	n (脂质) / n (蛋白)	% 蛋白
前峰	347	141	4.4	207	272	62	41
主峰	269	111	3.5	159	209	60	41

[0477] 将四连蛋白 - 载脂蛋白 A-I 在大肠杆菌中表达并且根据实施例 1-3 纯化（方法 2)。纯化后，将缓冲液通过渗滤改变为 pH7.4 的包含 50mM Tris，10mM L- 甲硫氨酸，6.7M 盐酸胍的溶液。将蛋白浓度调节到 20.4mg/ml。

[0478] 通过在室温将 100 摩尔 /l 磷脂 (POPC ： DPPC 比率为 3 ： 1) 溶解在包含 250mM Tris-HCl，140mM NaCl，10mM L- 甲硫氨酸，135mM 胆酸钠的 pH7.4 的缓冲液中来制备脂质贮存溶液。通过将 3.7ml 脂质贮存溶液稀释到 35.6ml 的 250mM Tris-HCl，140mM NaCl，pH7.4 中来制备再折叠缓冲液。将该缓冲液在室温，搅拌另外 2 小时。

[0479] 再折叠和脂质颗粒形成通过将在 50mM Tris，10mM L- 甲硫氨酸，6.7M 盐酸胍，pH8.0 中的 9.8ml 四连蛋白 - 载脂蛋白 A-I 加入再折叠缓冲液来起始。这导致盐酸胍的 1 ： 5 稀释液。将溶液在室温温育过夜，同时持续搅拌。通过渗滤进行洗涤剂的去除。

[0480] 表 24 :通过用 POPC/DPPC/ 胆酸盐混合物快速稀释获得的脂质颗粒的蛋白缀合物分析总结。

[0481]

峰	MW 总计[kDa]	MW 蛋白质 [kDa]	n 蛋白质 (APO-单体)	MW 脂质 [kDa]	n 脂质	n 脂质 / n 蛋白	% 蛋白
前峰	419	167	5.2	251	333	64	41
主峰	252	101	3.2	151	200	63	41

[0482] b)POPC 和 DPPC 和胆酸钠

[0483] 将四连蛋白 - 载脂蛋白 A-I 在大肠杆菌中表达并且根据实施例 1-3 纯化。纯化后，将缓冲液通过渗滤改变为 pH7.4 的包含 250mM Tris，140mMNaCl，6.7M 盐酸胍的溶液。将蛋白浓度调节到 30mg/ml。

[0484] 制备两种单独的脂质贮存溶液。通过在室温将 100 摩尔 /l POPC 溶解在包含 250mM Tris-HCl，140mM NaCl，135mM 胆酸钠的 pH7.4 的缓冲液中来制备溶液 A。在 41℃，通过将 100moles/l DPPC 溶解在 250mM Tris-HCl，140mM NaCl，135mM 胆酸钠 pH7.4 中来制备溶液 B。将脂质贮存溶液 A 和 B 以 3 ： 1 的比率混合并且在室温温育 2 小时。通过将 384ml 脂质贮存溶液稀释到 6365ml 的 250mM Tris-HCl，140mM NaCl，pH7.4 中来制备再折叠缓冲液。将该缓冲液在室温，搅拌另外 24 小时。

[0485] 再折叠和脂质颗粒形成通过将在 250mM Tris,140mM NaCl,6.7M 盐酸胍,pH7.4 中的 750ml 四连蛋白 - 载脂蛋白 A-I 加入再折叠缓冲液来起始。这导致盐酸胍的 1 ： 10 稀释液。将溶液在室温温育至少 12 小时,同时持续搅拌。通过渗滤进行洗涤剂的去除。

[0486] 表 25 :通过用 POPC ： DPPC ＝ 1 ： 1 快速稀释获得的脂质颗粒的蛋白缀合物分析总结

[0487]

峰	MW 总计[kDa]	MW 蛋白质 [kDa]	n (蛋白质单体)	MW 脂质 [kDa]	n (脂质)	n (脂质) / n (蛋白质)	% 蛋白质
主峰	263	102	3.2	161	214	67	39
后峰	182	85	2.7	97	129	48	47

[0488] c) 不同的盐酸胍浓度

[0489] 将根据本发明的四连蛋白 - 载脂蛋白 A-I 在大肠杆菌中表达并且从包含体通过金属螯合物亲和性色谱法进行纯化 (见实施例 1-3)。纯化后,将缓冲液通过渗滤改变为 pH7.4 的包含 250mM Tris,140mM NaCl,6.7M 盐酸胍的溶液。将蛋白浓度调节到 28mg/ml。

[0490] 通过在室温将 100 摩尔 /l POPC 溶解在包含 250mM Tris-HCl,140mMNaCl,135mM 胆酸钠的 pH7.4 的缓冲液中来制备脂质贮存溶液。将脂质贮存溶液在室温温育 2 小时。通过将脂质贮存溶液稀释到 250mM Tris-HCl,140mM NaCl,pH7.4 中来制备再折叠缓冲液。将该缓冲液在室温,搅拌另外 12 小时。将不同量的四连蛋白 - 载脂蛋白 A-I 稀释到再折叠缓冲液中 :1 ： 5,1 ： 7.5,1 ： 10,1 ： 12.5。这导致盐酸胍在再折叠缓冲液中的不同残余浓度。所述溶液允许在室温搅拌 o/n 以起始再折叠和脂质颗粒形成。通过透析去除洗涤剂。

[0491] 表 26 :通过用不同稀释比率快速稀释获得的脂质颗粒的蛋白缀合物分析总结。

[0492]

稀释	峰	MW 总计[kDa]	MW 蛋白 [kDa]	n (蛋白 单体)	MW 脂质[kDa]	n (脂质)	n (脂质) / n (蛋白)	%蛋白
1:5	主	273	103	3,2	170	226	70	38
1:7.5	主	272	100	3,1	173	230	73	37
1:10	主	266	106	3,3	160	212	64	40
1:12.5	主	281	101	3,2	180	239	76	36

[0493] d) 存在尿素时的 POPC 和胆酸钠

[0494] 将四连蛋白 - 载脂蛋白 A-I 在大肠杆菌中表达并且根据实施例 1-3 纯化。纯化后,将缓冲液通过渗滤改变为 pH7.4 的包含 250mM Tris,140mMNaCl,6.7M 尿素的溶液。将蛋白浓度调节到 28mg/ml。

[0495] 通过在室温将 100 摩尔 /l POPC 溶解在包含 250mM Tris-HCl，140mMNaCl，135mM 胆酸钠的 pH7.4 的缓冲液中来制备脂质贮存溶液。将脂质贮存溶液在室温温育 2 小时。通过将 77ml 脂质贮存混合物稀释到 1478ml 的 250mM Tris-HCl，140mM NaCl，pH7.4 中来制备再折叠缓冲液。将该缓冲液在室温，搅拌另外 7 小时。

[0496] 再折叠和脂质颗粒形成通过将在 250mM Tris，140mM NaCl，6.7M 尿素，pH7.4 中的 162ml 四连蛋白 - 载脂蛋白 A-I 加入再折叠缓冲液来起始。这导致尿素的 1 ： 10 稀释液。将溶液在室温温育 16 小时，同时持续搅拌。通过渗滤进行洗涤剂的去除。

[0497] e)POPC 和胆酸钠和野生型载脂蛋白 A-I

[0498] 在另一种示例性第二方法中，将在 6.7M 盐酸胍，50mM Tris，10mM 甲硫氨酸，pH8.0 中的人载脂蛋白 A-I（野生型载脂蛋白 A-I）稀释 1 ： 5(v/v) 到脂质化缓冲液中，得到 0.6mg/ml 的蛋白浓度。所述脂质化缓冲液由 7mM 胆酸盐，4mM POPC 和 1.3mM DPPC 组成，对应的脂质：蛋白比率为 240 ： 1。将 SEC-MALLS 用于分析复合物形成。在由约 200 脂质分子组成的复合物中发现约两个载脂蛋白分子。

[0499] 表 27 ：蛋白缀合物分析总结

[0500]

原材料		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	脂质的数目	脂质:蛋白的比率
变性的	主峰	235	71	2.2	163	216	1:97

[0501] 实施例 6

[0502] 从变性或天然蛋白开始的脂质颗粒形成

[0503] 如在实施例 4 中报道的方法（第一方法）需要天然载脂蛋白用于脂质颗粒形成，而在实施例 5 中报道的方法（第二方法）用充分变性的载脂蛋白开始进行脂质颗粒形成。

[0504] 在示例性的第一方法中，将在 6.7M 盐酸胍，50mM Tris，10mM 甲硫氨酸，pH8.0 中的变性四连蛋白 - 载脂蛋白 A-I 使用由 250mM Tris，140mMNaCl，10mM 甲硫氨酸组成的 pH7.5 的缓冲液进行广泛透析，蛋白浓度为 3.46mg/ml。接着，加入 POPC 和胆酸盐的混合物以在溶液中产生 6mMPOPC 和 8mM 胆酸盐的最终浓度。这对应于 60 分子 POPC/ 四连蛋白 - 载脂蛋白 A-I 单体的比率 (60 ： 1)。随后，通过渗滤去除洗涤剂。通过 SEC-MALLS 分析形成的蛋白 - 脂质复合物。使用这种方法，形成不均匀的产物，其中形成的种类中有约 60％包括超过三种的四连蛋白 - 载脂蛋白 A-I 单体。

[0505] 在示例性的第二方法中，将在 6.7M 盐酸胍，50mM Tris，10mM 甲硫氨酸，pH8.0 中的变性的四连蛋白 - 载脂蛋白 A-I 直接稀释 1 ： 10(v/v) 到脂质化缓冲液中，得到 2.5mg/ml 的蛋白浓度。所述脂质化缓冲液由 6mM 胆酸盐和 4.5mM POPC 组成，对应的脂质与蛋白比率是 60 ： 1。使用这种方法，形成均匀的产物，包括超过 90％的单一形成种类，其中每四连蛋白 - 载脂蛋白 A-I 分子结合 60 分子的脂质（见图 22)。

[0506] 表 28 ：蛋白缀合物分析总结

[0507]

原材料		MW 总计	MW 蛋白	n (蛋白单体)	MW 脂质	脂质数目	脂质:蛋白的比率
天然的	前峰(60%)	321	131	4.1	190	250	61
	主峰 (40%)	269	107	3.3	162	213	65
变性的	主峰 (>90%)	269	111	3.5	159	209	60

[0508] 实施例 7

[0509] 用胆酸盐 - 和两性洗涤剂 - 溶解的 POPC/DPPC 对胰岛素 -F 进行的脂质化

[0510] 选择用于脂质颗粒形成的蛋白是商购的胰岛素 (Humalog®, InsulinLispro, Lilly)。该蛋白的分子量是 5808Da。为了增加胰岛素在脂质颗粒中的检测极限,将蛋白用 NHS- 荧光素 (6-[荧光素 -5(6)- 羧基氨基] 己酸 N- 羟基琥珀酰亚胺酯, Sigma Aldrich#46940-5MG-F) 标记。

[0511] 使用 POPC 和 DPPC 的 1 ： 1 混合物如在实施例 4 中报道进行两性洗涤剂 - 和胆酸盐介导的 NHS- 荧光素 - 标记的胰岛素 (胰岛素 -F) 的脂质化。将 0.5mM 脂质混合物溶解于在 PBS pH7.4 中的 1x CMC 胆酸盐,2x CMC 两性洗涤剂 3-8 或 5x CMC 两性洗涤剂 3-10 中。脂质的溶解在超声波浴中在 45℃ 1 小时来实现。将胰岛素 -F 加入溶解的脂质中,蛋白 : 脂质的摩尔比为 1 ： 2(两性洗涤剂 3-8) 或 1 ： 1.2(两性洗涤剂 3-10 和胆酸盐)。在室温,将脂质化混合物温育 1 小时,随后使用 PBS pH7.4 进行广泛透析以去除洗涤剂。

[0512] 使用荧光检测 (494nm ext.,521nm em.) 和 UV280 吸收将形成的脂质颗粒和对照样品在 SE-HPLC 上进行分析。在 SE-HPLC 上对每种脂质化方法分析三种不同的样品 : 溶解在 PBS 中的胰岛素 -F,在 PBS 中不含胰岛素 F 的脂质体和包含胰岛素 -F 的脂质颗粒。非脂质化的胰岛素 -F 在约 40min 的洗脱时间从柱上洗脱,并且通过荧光和 UV280 检测来检测峰。脂质化的胰岛素 F- 样品以两个单独的峰从柱上洗脱,所述峰通过荧光和 UV280 检测。迟峰 (峰的最大值在约 40min) 与胰岛素 -F 对照样品共同迁移。在 15min 洗脱时间的早峰比纯的胰岛素 -F 具有更高的分子量,并且由脂质化的胰岛素 -F 组成。不含脂质颗粒的蛋白在 15min 的洗脱时间洗脱。

[0513] 实施例 8

[0514] 载脂蛋白的应用

[0515] a)DPPC 和 POPC 对 LCAT 活性的影响

[0516] 检查包含棕榈酰油酰磷脂酰胆碱 (POPC) 或二棕榈酰磷脂酰胆碱 (DPPC) 和重组野生型载脂蛋白 A-I 或四连蛋白 - 载脂蛋白 A-I 的脂质颗粒支持通过 LCAT 进行胆固醇酯化的能力。

[0517] 氚化的胆固醇 (4% ; 相对于基于摩尔的磷脂酰胆碱含量) 通过加入胆固醇乙醇溶液来结合在脂质颗粒中。在存在在 125μl(10mM Tris,150mM NaCl,1mM EDTA,1mM NaN_3 ; pH7.4 ;2mg/ml HuFAF 白蛋白 ;4mMβ- 巯基乙醇) 中的 0.2μg/ml 重组 LCAT 酶 (ROAR 生物化学) 时,在 37℃进行 1 小时测试得到的蛋白 - 脂质复合物支持 LCAT 催化的胆固醇酯化的能力。通过加入氯仿 : 甲醇 (2 ： 1) 来终止反应并提取脂质。在通过 TLC 分离胆固醇 - 胆

固醇酯并闪烁计数后计算“百分比”酯化。因为少于 20％的示踪物被结合到形成的酯中，在反应条件下反应速率能够认为是恒定的。使用 XLfit 软件 (IDBS)，数据拟合于米－曼方程 (Michaelis Mentenequation)。对于这些结果的显示，见图 3。

[0518] b)DPPC/POPC 混合物对 LCAT 活性的影响

[0519] 通过将重组野生型载脂蛋白 A-I 与 3H 胆固醇、DPPC/POPC 混合物和胆酸盐以 1 ： 4 ： 80 ： 113 摩尔比混合后，使用胆酸盐作为洗涤剂来制备脂质颗粒。DPPC/POPC 混合物包含任一 100％ POPC ;75％ POPC ;50％ POPC ;25％ POPC。

[0520] 在通过透析去除胆酸盐后，测试得到的蛋白－脂质复合物支持 LCAT 催化的胆固醇酯化的能力。通过加入胆固醇乙醇溶液将 ^{3}H 胆固醇 (4％ ;相对于基于摩尔的磷脂酰胆碱含量）结合在脂质颗粒中。在存在在 125μl(10mM Tris，150mM NaCl，1mM EDTA，1mM NaN_3 ; pH7.4 ;2mg/ml HuFAF 白蛋白 ;4mMβ－巯基乙醇）中的 0.2μg/ml 重组 LCAT 酶 (ROAR 生物化学）时，在 37℃进行 1 小时测试得到的蛋白－脂质复合物支持 LCAT 催化的胆固醇酯化的能力。通过加入氯仿：甲醇 (2 ： 1) 来终止反应并提取脂质。在通过 TLC 分离胆固醇－胆固醇酯并闪烁计数后计算“百分比”酯化。因为少于 20％的示踪物被结合到酯中，在反应条件下反应速率能够认为是恒定的。使用 XLfit 软件 (IDBS)，数据拟合于米－曼方程并显示在图 4 中。

[0521] 表 3a :表观动力学参数

[0522]

底物 [% POPC]	K_m [nM]	V_{max} [n 摩尔酯/h/U LCAT]
100	4.6	1.6
75	0.4	1.9
50	0.5	1.8
25	1.0	1.7
0	6.9	1.8

[0523] c) 胆固醇向 THP-1 衍生的泡沫细胞的流出

[0524] 通过将 THP-1 单核细胞白血病细胞暴露于佛波醇 -12- 肉豆蔻酸酯 -13- 乙酸酯 (phorbol myristate acetate) 获得巨噬细胞如人 THP-1 细胞。随后，通过将细胞在存在包含 ^{3}H 胆固醇示踪物的乙酰化的 LDL 时进一步培养来负载。接着，将这些模型泡沫细胞暴露于胆固醇受体 4h-8h 测试化合物（见下）。

[0525] 收获细胞培养物上清液并将细胞在 5％ NP40 中切割。将流出分数计算为在上清液中的胆固醇放射性相对于在细胞加上清液中的放射性的总和的比率。扣除暴露于不包含受体的介质的细胞的流出并且通过线性拟合计算流出速度。使用从细胞中的流出，相对于作为参考的 10μg/ml 野生型载脂蛋白 A-I 来对流出速度进行标准化（相对流出速度）。将在两个单独的实验中获得的相对流出速度作图为胆固醇受体浓度的函数并将数据拟合于米－曼方程。

[0526] 使用暴露于 RXR-LXR 激动剂的细胞进行平行实验，所述 RXR-LXR 激动剂已知上调 ABCA-1 转运蛋白，并使胆固醇运输偏向 ABCA-1 介导的流出。

[0527] 在测试系列中仅观察到脂质混合物的微小影响（图 5 和表 29)。

[0528] 表 29 :不同的样品 .

[0529]

具有下述的四连蛋白-载脂蛋白 A-I	摩尔比 载脂蛋白：磷脂	制备方法
100 % POPC/ 0 % DPPC	1:60	胆酸盐
75 % POPC/ 25 % DPPC	1:60	胆酸盐
50 % POPC/ 50 % DPPC	1:70	胆酸盐
0 % POPC/ 100 % DPPC	1:80	胆酸盐
-	无	

[0530] RXR-LXR 预处理泡沫细胞强烈增加向非脂质化物质的流出，与未处理的细胞相比，最大速度增加 6 倍。对脂质颗粒的影响则小得多，具有 2 倍增加，反映了 ABCA-1 转运蛋白向胆固醇流出的较小贡献（图 6）。

[0531] d) 体内研究

[0532] 研究了 5 种脂质颗粒变体：

[0533] i) 仅 POPC

[0534] ii) 仅 DPPC

[0535] iii)POPC ： DPPC 3 ： 1

[0536] iV)POPC ： DPPC 1 ： 1

[0537] V)DPPC ： SM 9 ： 1

[0538] 在 80mg/kg，在 0.5h 内对兔进行静脉内输注（n ＝ 3 只兔 / 测试化合物），随后在输注后 96h 内进行系列血液取样。

[0539] 用 ELISA 分析载脂蛋白水平：

[0540] - 药物水平

[0541] - 关于肝酶、胆固醇、胆固醇酯的血浆值的数据。

[0542] 对于所有测试组合物，血浆浓度是非常相似的，显示极不明显的起始“分布”相，随后是浓度的对数线性下降（图 7，表 3）。

[0543] 表 3 ：药物代谢动力学数据 .

[0544]

具有下述的四连蛋白-载脂蛋白 A-I	C_L [ml/h/kg]	V_{ss} [ml/kg]	$T_{1/2}$ [h]	C_{max} [mg/m]
100 % POPC/ 0 % DPPC	0.897 ± 0.216	45.0 ± 2.5	36.9 ± 8.2	2.40 ± 0.19
0 % POPC/ 100 % DPPC	0.922 ± 0.098	37.8 ± 4.9	30.2 ± 7.7	2.29 ± 0.19
75 % POPC/ 25 % DPPC	0.815 ± 0.064	37.8 ± 5.6	34.2 ± 4.5	2.65 ± 0.28
50 % POPC/ 50 % DPPC	0.850 ± 0.135	43.1 ± 5.9	38.6 ± 10.6	2.34 ± 0.31
90 % DPPC/ 10 % SM	1.28 ± 0.62	50.7 ± 8.7	31.3 ± 8.2	1.91 ± 0.33

[0545] 对于所有测试化合物，测定的药物代谢动力学 (PK) 参数是相似的。此外，还发现了低个体内可变性。测定的半衰期接近于 1.5 天，即与野生型载脂蛋白 A-I 相比增加。分布的体积与血浆体积相似（在兔中，大约 40ml/kg）。

[0546] f) 胆固醇动员

[0547] 在血浆中动员和酯化胆固醇。甚至在四连蛋白 - 载脂蛋白 A-I 已经开始减少后，血浆胆固醇酯水平的确继续增加。当血浆四连蛋白 - 载脂蛋白 A-I 水平已经减少到 0.5mg/ml（约正常野生型载脂蛋白 A-I 的约 50％），仍旧可检测到增加的胆固醇酯水平（图 8）。

[0548] g) 肝酶释放

[0549] 包含含有 POPC 的四连蛋白 - 载脂蛋白 A-I 的脂质颗粒不诱导肝酶释放（图 1）。类似于兔，单一静脉内注射根据本发明的包含 POPC 或 POPC/DPPC 混合物的四连蛋白 - 载脂蛋白 A-I 在小鼠中是安全的。包含摩尔比为 1 ： 3 的 DPPC ： POPC 的载脂蛋白组合物与单独的 POPC 是相当的（图 9）。

[0550] 没有观察到明显的溶血作用，直到在五个制备物的任一种中输注后 2 小时。通过光电比色测定溶血作用为在静脉内施用四连蛋白 - 载脂蛋白 A-I 后 2 小时获得的血浆样品中的红色。将 100％的全血溶血作用（通过 0.44％ Triton X-100- 最终浓度产生）用于校准（图 10）。

[0551] h) 四连蛋白 - 载脂蛋白 A-I 对人脐静脉内皮细胞的抗炎作用

[0552] 将 5-10 代 HUVECs（人脐静脉内皮细胞）在各个四连蛋白 - 载脂蛋白 A-I 制备物中温育 16 小时，并用 TNF α 刺激最后 4 小时。通过 FACS，用特异性抗体检测 VCAM1 表面表达。

[0553] 实施例 9

[0554] 脂质颗粒稳定性

[0555] 将包含 N- 端组氨酸 - 标记和 IgA 蛋白酶切割位点的野生型载脂蛋白 A-I 在大肠杆菌中表达并通过柱色谱法纯化，如在上述实施例中所报道的。通过 IgA 蛋白酶切割去除组氨酸 - 标记。使用 1 ： 150 比率的蛋白与 Lipoid S100 大豆磷脂混合物装配脂质颗粒 (HDL 颗粒）。将所述颗粒贮存在包含 5mM 磷酸钠和 1％蔗糖，pH 值为 7.3 的缓冲液中。SE-HPLC 揭示在脂质化后温育和温育 10 天后三个独立的峰。在 40℃温育后，可以检测到在 10.8 分

钟保留时间的主要的峰(47%的总蛋白),这在贮存于5℃的样品中不存在。10.8分钟的峰显示由于蛋白不稳定,形成可溶性大分子量装配体。

[0556] 从POPC ：DPPC混合物(POPC与DPPC比率为3 ：1)起始获得的如本文报道的包含四连蛋白-载脂蛋白A-I的HDL颗粒也在5℃和40℃温育。在升温的温育导致轻微程度的前锋形成,但是没有显著的向在10.8分钟的高分子量装配体的移动(在11分钟＜2%增加)。这说明,与包含野生型载脂蛋白A-I的颗粒相比,提高的HDL颗粒稳定性。

[0557] 实施例10

[0558] 胆固醇动员

[0559] 在体内施用载脂蛋白后,通过比较总胆固醇的各次排出与载脂蛋白浓度能够测定胆固醇动员到血液中的效率。关于定量评估,计算总胆固醇的基线校正的在浓度-时间曲线下面积(AUC)与载脂蛋白的在浓度-时间曲线下的面积的商。

[0560] 在本实验中,分析下述物质：

[0561] -将包含N-端组氨酸-标记和IgA蛋白酶切割位点的野生型载脂蛋白A-I在大肠杆菌中表达并通过柱色谱法纯化,如在上述实施例中所报道的;通过IgA蛋白酶切割去除组氨酸-标记;使用1 ：150比率的蛋白与Lipoid S100大豆磷脂混合物装配脂质颗粒(HDL颗粒)；

[0562] -载脂蛋白A-I Milano变体:使用1 ：40比率的蛋白与POPC装配脂质颗粒(HDL颗粒),

[0563] -如本文报道的四连蛋白-载脂蛋白A-I;使用1 ：60比率的蛋白与(POPC和DPPC)(POPC和DPPC比率为3 ：1)装配脂质颗粒(HDL颗粒)。

[0564] 将三种HDL颗粒施加于大鼠。见关于各AUC比率获得的值显示在表30中。

[0565] 表30:胆固醇动员

[0566]

	脂质	AUC(在血液中胆固醇的时间依赖性浓度) --- AUC (在血液中时间依赖性载脂蛋白 A-I 浓度)
野生型载脂蛋白A-I	大豆磷脂混合物	0.0002 (mmol/l)/(μg/ml)).
载脂蛋白A-I Milano 变体	POPC	0.0004 (mmol/l)/(μg/ml)).
如本文报道的四连蛋白-载脂蛋白A-I	POPC:DPPC 3:1	0.0013 (mmol/l)/(μg/ml)

[0001]

序列表

<110> 霍夫曼-拉罗奇有限公司

<120> 产生脂质颗粒的方法，脂质颗粒本身及其应用

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<151> 2010-08-30

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Glu Glu Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu
20 25 30

Leu Lys Glu Gln Gln Ala Leu Gln Thr Val Asp Glu Pro Pro Gln Ser
35 40 45

Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu
50 55 60

Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu
65 70 75 80

Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr
85 90 95

Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu
100 105 110

Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met
115 120 125

Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp
130 135 140

Asp Phe Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys
145 150 155 160

Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu
165 170 175

His Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp
180 185 190

Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr
195 200 205

[0002]

Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys
210 215 220

Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu
225 230 235 240

His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu
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35 40 45

Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
50 55 60

Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
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Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
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Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
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Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
115 120 125

Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
130 135 140

Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
145 150 155 160

Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu
165 170 175

Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala
180 185 190

[0003]

Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
195 200 205

Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
210 215 220

Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu
225 230 235 240

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln
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Leu Lys Gln Lys Leu Lys
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Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
35 40 45

Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
50 55 60

Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
65 70 75 80

Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
85 90 95

Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
100 105 110

Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
115 120 125

Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
130 135 140

Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu
145 150 155 160

Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala
165 170 175

Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
180 185 190

Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
195 200 205

Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu
210 215 220

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln
225 230 235 240

Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala
245 250 255

Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
260 265

<210> 7
<211> 100
<212> PRT
<213> 智人 (Homo sapiens)

<400> 7

[0005]

```
Met Lys Leu Leu Ala Ala Thr Val Leu Leu Leu Thr Ile Cys Ser Leu
1               5                  10                  15

Glu Gly Ala Leu Val Arg Arg Gln Ala Lys Glu Pro Cys Val Glu Ser
            20                  25                  30

Leu Val Ser Gln Tyr Phe Gln Thr Val Thr Asp Tyr Gly Lys Asp Leu
        35                  40                  45

Met Glu Lys Val Lys Ser Pro Glu Leu Gln Ala Glu Ala Lys Ser Tyr
    50                  55                  60

Phe Glu Lys Ser Lys Glu Gln Leu Thr Pro Leu Ile Lys Lys Ala Gly
65                  70                  75                  80

Thr Glu Leu Val Asn Phe Leu Ser Tyr Phe Val Glu Leu Gly Thr Gln
                85                  90                  95

Pro Ala Thr Gln
            100
```

<210> 8
<211> 396
<212> PRT
<213> 智人（Homo sapiens）

<400> 8

```
Met Phe Leu Lys Ala Val Val Leu Thr Leu Ala Leu Val Ala Val Ala
1               5                  10                  15

Gly Ala Arg Ala Glu Val Ser Ala Asp Gln Val Ala Thr Val Met Trp
            20                  25                  30

Asp Tyr Phe Ser Gln Leu Ser Asn Asn Ala Lys Glu Ala Val Glu His
        35                  40                  45

Leu Gln Lys Ser Glu Leu Thr Gln Gln Leu Asn Ala Leu Phe Gln Asp
    50                  55                  60

Lys Leu Gly Glu Val Asn Thr Tyr Ala Gly Asp Leu Gln Lys Lys Leu
65                  70                  75                  80

Val Pro Phe Ala Thr Glu Leu His Glu Arg Leu Ala Lys Asp Ser Glu
                85                  90                  95

Lys Leu Lys Glu Glu Ile Gly Lys Glu Leu Glu Glu Leu Arg Ala Arg
            100                 105                 110

Leu Leu Pro His Ala Asn Glu Val Ser Gln Lys Ile Gly Asp Asn Leu
        115                 120                 125

Arg Glu Leu Gln Gln Arg Leu Glu Pro Tyr Ala Asp Gln Leu Arg Thr
    130                 135                 140

Gln Val Asn Thr Gln Ala Glu Gln Leu Arg Arg Gln Leu Thr Pro Tyr
145                 150                 155                 160

Ala Gln Arg Met Glu Arg Val Leu Arg Glu Asn Ala Asp Ser Leu Gln
                165                 170                 175
```

[0006]

Ala Ser Leu Arg Pro His Ala Asp Glu Leu Lys Ala Lys Ile Asp Gln
180 185 190

Asn Val Glu Glu Leu Lys Gly Arg Leu Thr Pro Tyr Ala Asp Glu Phe
195 200 205

Lys Val Lys Ile Asp Gln Thr Val Glu Glu Leu Arg Arg Ser Leu Ala
210 215 220

Pro Tyr Ala Gln Asp Thr Gln Glu Lys Leu Asn His Gln Leu Glu Gly
225 230 235 240

Leu Thr Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Lys Ala Arg Ile
245 250 255

Ser Ala Ser Ala Glu Glu Leu Arg Gln Arg Leu Ala Pro Leu Ala Glu
260 265 270

Asp Val Arg Gly Asn Leu Arg Gly Asn Thr Glu Gly Leu Gln Lys Ser
275 280 285

Leu Ala Glu Leu Gly Gly His Leu Asp Gln Gln Val Glu Glu Phe Arg
290 295 300

Arg Arg Val Glu Pro Tyr Gly Glu Asn Phe Asn Lys Ala Leu Val Gln
305 310 315 320

Gln Met Glu Gln Leu Arg Gln Lys Leu Gly Pro His Ala Gly Asp Val
325 330 335

Glu Gly His Leu Ser Phe Leu Glu Lys Asp Leu Arg Asp Lys Val Asn
340 345 350

Ser Phe Phe Ser Thr Phe Lys Glu Lys Glu Ser Gln Asp Lys Thr Leu
355 360 365

Ser Leu Pro Glu Leu Glu Gln Gln Gln Glu Gln Gln Gln Glu Gln Gln
370 375 380

Gln Glu Gln Val Gln Met Leu Ala Pro Leu Glu Ser
385 390 395

<210> 9
<211> 366
<212> PRT
<213> 智人 (Homo sapiens)

<400> 9

Met Ala Ser Met Ala Ala Val Leu Thr Trp Ala Leu Ala Leu Leu Ser
1 5 10 15

Ala Phe Ser Ala Thr Gln Ala Arg Lys Gly Phe Trp Asp Tyr Phe Ser
20 25 30

Gln Thr Ser Gly Asp Lys Gly Arg Val Glu Gln Ile His Gln Gln Lys
35 40 45

[0007]

Met Ala Arg Glu Pro Ala Thr Leu Lys Asp Ser Leu Glu Gln Asp Leu
50 55 60

Asn Asn Met Asn Lys Phe Leu Glu Lys Leu Arg Pro Leu Ser Gly Ser
65 70 75 80

Glu Ala Pro Arg Leu Pro Gln Asp Pro Val Gly Met Arg Arg Gln Leu
85 90 95

Gln Glu Glu Leu Glu Glu Val Lys Ala Arg Leu Gln Pro Tyr Met Ala
100 105 110

Glu Ala His Glu Leu Val Gly Trp Asn Leu Glu Gly Leu Arg Gln Gln
115 120 125

Leu Lys Pro Tyr Thr Met Asp Leu Met Glu Gln Val Ala Leu Arg Val
130 135 140

Gln Glu Leu Gln Glu Gln Leu Arg Val Val Gly Glu Asp Thr Lys Ala
145 150 155 160

Gln Leu Leu Gly Gly Val Asp Glu Ala Trp Ala Leu Leu Gln Gly Leu
165 170 175

Gln Ser Arg Val Val His His Thr Gly Arg Phe Lys Glu Leu Phe His
180 185 190

Pro Tyr Ala Glu Ser Leu Val Ser Gly Ile Gly Arg His Val Gln Glu
195 200 205

Leu His Arg Ser Val Ala Pro His Ala Pro Ala Ser Pro Ala Arg Leu
210 215 220

Ser Arg Cys Val Gln Val Leu Ser Arg Lys Leu Thr Leu Lys Ala Lys
225 230 235 240

Ala Leu His Ala Arg Ile Gln Gln Asn Leu Asp Gln Leu Arg Glu Glu
245 250 255

Leu Ser Arg Ala Phe Ala Gly Thr Gly Thr Glu Glu Gly Ala Gly Pro
260 265 270

Asp Pro Gln Met Leu Ser Glu Glu Val Arg Gln Arg Leu Gln Ala Phe
275 280 285

Arg Gln Asp Thr Tyr Leu Gln Ile Ala Ala Phe Thr Arg Ala Ile Asp
290 295 300

Gln Glu Thr Glu Glu Val Gln Gln Gln Leu Ala Pro Pro Pro Pro Gly
305 310 315 320

His Ser Ala Phe Ala Pro Glu Phe Gln Gln Thr Asp Ser Gly Lys Val
325 330 335

Leu Ser Lys Leu Gln Ala Arg Leu Asp Asp Leu Trp Glu Asp Ile Thr
340 345 350

His Ser Leu His Asp Gln Gly His Ser His Leu Gly Asp Pro

[0008]

355 360 365

<210> 10
<211> 83
<212> PRT
<213> 智人 (Homo sapiens)

<400> 10

Met Arg Leu Phe Leu Ser Leu Pro Val Leu Val Val Val Leu Ser Ile
1 5 10 15

Val Leu Glu Gly Pro Ala Pro Ala Gln Gly Thr Pro Asp Val Ser Ser
20 25 30

Ala Leu Asp Lys Leu Lys Glu Phe Gly Asn Thr Leu Glu Asp Lys Ala
35 40 45

Arg Glu Leu Ile Ser Arg Ile Lys Gln Ser Glu Leu Ser Ala Lys Met
50 55 60

Arg Glu Trp Phe Ser Glu Thr Phe Gln Lys Val Lys Glu Lys Leu Lys
65 70 75 80

Ile Asp Ser

<210> 11
<211> 101
<212> PRT
<213> 智人 (Homo sapiens)

<400> 11

Met Gly Thr Arg Leu Leu Pro Ala Leu Phe Leu Val Leu Leu Val Leu
1 5 10 15

Gly Phe Glu Val Gln Gly Thr Gln Gln Pro Gln Gln Asp Glu Met Pro
20 25 30

Ser Pro Thr Phe Leu Thr Gln Val Lys Glu Ser Leu Ser Ser Tyr Trp
35 40 45

Glu Ser Ala Lys Thr Ala Ala Gln Asn Leu Tyr Glu Lys Thr Tyr Leu
50 55 60

Pro Ala Val Asp Glu Lys Leu Arg Asp Leu Tyr Ser Lys Ser Thr Ala
65 70 75 80

Ala Met Ser Thr Tyr Thr Gly Ile Phe Thr Asp Gln Val Leu Ser Val
85 90 95

Leu Lys Gly Glu Glu
100

<210> 12
<211> 99
<212> PRT
<213> 智人 (Homo sapiens)

<400> 12

Met Gln Pro Arg Val Leu Leu Val Val Ala Leu Leu Ala Leu Leu Ala

[0009]

1 5 10 15

Ser Ala Arg Ala Ser Glu Ala Glu Asp Ala Ser Leu Leu Ser Phe Met
20 25 30

Gln Gly Tyr Met Lys His Ala Thr Lys Thr Ala Lys Asp Ala Leu Ser
35 40 45

Ser Val Gln Glu Ser Gln Val Ala Gln Gln Ala Arg Gly Trp Val Thr
50 55 60

Asp Gly Phe Ser Ser Leu Lys Asp Tyr Trp Ser Thr Val Lys Asp Lys
65 70 75 80

Phe Ser Glu Phe Trp Asp Leu Asp Pro Glu Val Arg Pro Thr Ser Ala
85 90 95

Val Ala Ala

<210> 13
<211> 127
<212> PRT
<213> 智人 (Homo sapiens)

<400> 13

Met Ser Leu Leu Arg Asn Arg Leu Gln Ala Leu Pro Ala Leu Cys Leu
1 5 10 15

Cys Val Leu Val Leu Ala Cys Ile Gly Ala Cys Gln Pro Glu Ala Gln
20 25 30

Glu Gly Thr Leu Ser Pro Pro Pro Lys Leu Lys Met Ser Arg Trp Ser
35 40 45

Leu Val Arg Gly Arg Met Lys Glu Leu Leu Glu Thr Val Val Asn Arg
50 55 60

Thr Arg Asp Gly Trp Gln Trp Phe Trp Ser Pro Ser Thr Phe Arg Gly
65 70 75 80

Phe Met Gln Thr Tyr Tyr Asp Asp His Leu Arg Asp Leu Gly Pro Leu
85 90 95

Thr Lys Ala Trp Phe Leu Glu Ser Lys Asp Ser Leu Leu Lys Lys Thr
100 105 110

His Ser Leu Cys Pro Arg Leu Val Cys Gly Asp Lys Asp Gln Gly
115 120 125

<210> 14
<211> 189
<212> PRT
<213> 智人 (Homo sapiens)

<400> 14

Met Val Met Leu Leu Leu Leu Leu Ser Ala Leu Ala Gly Leu Phe Gly
1 5 10 15

[0010]

Ala Ala Glu Gly Gln Ala Phe His Leu Gly Lys Cys Pro Asn Pro Pro
20 25 30

Val Gln Glu Asn Phe Asp Val Asn Lys Tyr Leu Gly Arg Trp Tyr Glu
35 40 45

Ile Glu Lys Ile Pro Thr Thr Phe Glu Asn Gly Arg Cys Ile Gln Ala
50 55 60

Asn Tyr Ser Leu Met Glu Asn Gly Lys Ile Lys Val Leu Asn Gln Glu
65 70 75 80

Leu Arg Ala Asp Gly Thr Val Asn Gln Ile Glu Gly Glu Ala Thr Pro
85 90 95

Val Asn Leu Thr Glu Pro Ala Lys Leu Glu Val Lys Phe Ser Trp Phe
100 105 110

Met Pro Ser Ala Pro Tyr Trp Ile Leu Ala Thr Asp Tyr Glu Asn Tyr
115 120 125

Ala Leu Val Tyr Ser Cys Thr Cys Ile Ile Gln Leu Phe His Val Asp
130 135 140

Phe Ala Trp Ile Leu Ala Arg Asn Pro Asn Leu Pro Pro Glu Thr Val
145 150 155 160

Asp Ser Leu Lys Asn Ile Leu Thr Ser Asn Asn Ile Asp Val Lys Lys
165 170 175

Met Thr Val Thr Asp Gln Val Asn Cys Pro Lys Leu Ser
180 185

<210> 15
<211> 317
<212> PRT
<213> 智人 (Homo sapiens)

<400> 15

Met Lys Val Leu Trp Ala Ala Leu Leu Val Thr Phe Leu Ala Gly Cys
1 5 10 15

Gln Ala Lys Val Glu Gln Ala Val Glu Thr Glu Pro Glu Pro Glu Leu
20 25 30

Arg Gln Gln Thr Glu Trp Gln Ser Gly Gln Arg Trp Glu Leu Ala Leu
35 40 45

Gly Arg Phe Trp Asp Tyr Leu Arg Trp Val Gln Thr Leu Ser Glu Gln
50 55 60

Val Gln Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala
65 70 75 80

Leu Met Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu
85 90 95

Glu Glu Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser
100 105 110

[0011]

Lys Glu Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp
115 120 125

Val Cys Gly Arg Leu Val Gln Tyr Arg Gly Glu Val Gln Ala Met Leu
130 135 140

Gly Gln Ser Thr Glu Glu Leu Arg Val Arg Leu Ala Ser His Leu Arg
145 150 155 160

Lys Leu Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg
165 170 175

Leu Ala Val Tyr Gln Ala Gly Ala Arg Glu Gly Ala Glu Arg Gly Leu
180 185 190

Ser Ala Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val
195 200 205

Arg Ala Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu Arg
210 215 220

Ala Gln Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met Gly
225 230 235 240

Ser Arg Thr Arg Asp Arg Leu Asp Glu Val Lys Glu Gln Val Ala Glu
245 250 255

Val Arg Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala
260 265 270

Glu Ala Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu
275 280 285

Asp Met Gln Arg Gln Trp Ala Gly Leu Val Glu Lys Val Gln Ala Ala
290 295 300

Val Gly Thr Ser Ala Ala Pro Val Pro Ser Asp Asn His
305 310 315

<210> 16
<211> 308
<212> PRT
<213> 智人 (Homo sapiens)

<400> 16

Met Ile Pro Val Glu Leu Leu Leu Cys Tyr Leu Leu Leu His Pro Val
1 5 10 15

Asp Ala Thr Ser Tyr Gly Lys Gln Thr Asn Val Leu Met His Phe Pro
20 25 30

Leu Ser Leu Glu Ser Gln Thr Pro Ser Ser Asp Pro Leu Ser Cys Gln
35 40 45

Phe Leu His Pro Lys Ser Leu Pro Gly Phe Ser His Met Ala Pro Leu
50 55 60

[0012]

Pro Lys Phe Leu Val Ser Leu Ala Leu Arg Asn Ala Leu Glu Glu Ala
65 70 75 80

Gly Cys Gln Ala Asp Val Trp Ala Leu Gln Leu Gln Leu Tyr Arg Gln
85 90 95

Gly Gly Val Asn Ala Thr Gln Val Leu Ile Gln His Leu Arg Gly Leu
100 105 110

Gln Lys Gly Arg Ser Thr Glu Arg Asn Val Ser Val Glu Ala Leu Ala
115 120 125

Ser Ala Leu Gln Leu Leu Ala Arg Glu Gln Gln Ser Thr Gly Arg Val
130 135 140

Gly Arg Ser Leu Pro Thr Glu Asp Cys Glu Asn Glu Lys Glu Gln Ala
145 150 155 160

Val His Asn Val Val Gln Leu Leu Pro Gly Val Gly Thr Phe Tyr Asn
165 170 175

Leu Gly Thr Ala Leu Tyr Tyr Ala Thr Gln Asn Cys Leu Gly Lys Ala
180 185 190

Arg Glu Arg Gly Arg Asp Gly Ala Ile Asp Leu Gly Tyr Asp Leu Leu
195 200 205

Met Thr Met Ala Gly Met Ser Gly Gly Pro Met Gly Leu Ala Ile Ser
210 215 220

Ala Ala Leu Lys Pro Ala Leu Arg Ser Gly Val Gln Gln Leu Ile Gln
225 230 235 240

Tyr Tyr Gln Asp Gln Lys Asp Ala Asn Ile Ser Gln Pro Glu Thr Thr
245 250 255

Lys Glu Gly Leu Arg Ala Ile Ser Asp Val Ser Asp Leu Glu Glu Thr
260 265 270

Thr Thr Leu Ala Ser Phe Ile Ser Glu Val Val Ser Ser Ala Pro Tyr
275 280 285

Trp Gly Trp Ala Ile Ile Lys Ser Tyr Asp Leu Asp Pro Gly Ala Gly
290 295 300

Ser Leu Glu Ile
305

<210> 17
<211> 345
<212> PRT
<213> 智人 (Homo sapiens)

<400> 17

Met Ile Ser Pro Val Leu Ile Leu Phe Ser Ser Phe Leu Cys His Val
1 5 10 15

Ala Ile Ala Gly Arg Thr Cys Pro Lys Pro Asp Asp Leu Pro Phe Ser
20 25 30

[0013]

Thr Val Val Pro Leu Lys Thr Phe Tyr Glu Pro Gly Glu Glu Ile Thr
35 40 45

Tyr Ser Cys Lys Pro Gly Tyr Val Ser Arg Gly Gly Met Arg Lys Phe
50 55 60

Ile Cys Pro Leu Thr Gly Leu Trp Pro Ile Asn Thr Leu Lys Cys Thr
65 70 75 80

Pro Arg Val Cys Pro Phe Ala Gly Ile Leu Glu Asn Gly Ala Val Arg
85 90 95

Tyr Thr Thr Phe Glu Tyr Pro Asn Thr Ile Ser Phe Ser Cys Asn Thr
100 105 110

Gly Phe Tyr Leu Asn Gly Ala Asp Ser Ala Lys Cys Thr Glu Glu Gly
115 120 125

Lys Trp Ser Pro Glu Leu Pro Val Cys Ala Pro Ile Ile Cys Pro Pro
130 135 140

Pro Ser Ile Pro Thr Phe Ala Thr Leu Arg Val Tyr Lys Pro Ser Ala
145 150 155 160

Gly Asn Asn Ser Leu Tyr Arg Asp Thr Ala Val Phe Glu Cys Leu Pro
165 170 175

Gln His Ala Met Phe Gly Asn Asp Thr Ile Thr Cys Thr Thr His Gly
180 185 190

Asn Trp Thr Lys Leu Pro Glu Cys Arg Glu Val Lys Cys Pro Phe Pro
195 200 205

Ser Arg Pro Asp Asn Gly Phe Val Asn Tyr Pro Ala Lys Pro Thr Leu
210 215 220

Tyr Tyr Lys Asp Lys Ala Thr Phe Gly Cys His Asp Gly Tyr Ser Leu
225 230 235 240

Asp Gly Pro Glu Glu Ile Glu Cys Thr Lys Leu Gly Asn Trp Ser Ala
245 250 255

Met Pro Ser Cys Lys Ala Ser Cys Lys Val Pro Val Lys Lys Ala Thr
260 265 270

Val Val Tyr Gln Gly Glu Arg Val Lys Ile Gln Glu Lys Phe Lys Asn
275 280 285

Gly Met Leu His Gly Asp Lys Val Ser Phe Phe Cys Lys Asn Lys Glu
290 295 300

Lys Lys Cys Ser Tyr Thr Glu Asp Ala Gln Cys Ile Asp Gly Thr Ile
305 310 315 320

Glu Val Pro Lys Cys Phe Lys Glu His Ser Ser Leu Ala Phe Trp Lys
325 330 335

[0014]

Thr Asp Ala Ser Asp Val Lys Pro Cys
340 345

<210> 18
<211> 398
<212> PRT
<213> 智人 (Homo sapiens)

<400> 18

Met Glu Gly Ala Ala Leu Leu Arg Val Ser Val Leu Cys Ile Trp Met
1 5 10 15

Ser Ala Leu Phe Leu Gly Val Gly Val Arg Ala Glu Glu Ala Gly Ala
20 25 30

Arg Val Gln Gln Asn Val Pro Ser Gly Thr Asp Thr Gly Asp Pro Gln
35 40 45

Ser Lys Pro Leu Gly Asp Trp Ala Ala Gly Thr Met Asp Pro Glu Ser
50 55 60

Ser Ile Phe Ile Glu Asp Ala Ile Lys Tyr Phe Lys Glu Lys Val Ser
65 70 75 80

Thr Gln Asn Leu Leu Leu Leu Leu Thr Asp Asn Glu Ala Trp Asn Gly
85 90 95

Phe Val Ala Ala Ala Glu Leu Pro Arg Asn Glu Ala Asp Glu Leu Arg
100 105 110

Lys Ala Leu Asp Asn Leu Ala Arg Gln Met Ile Met Lys Asp Lys Asn
115 120 125

Trp His Asp Lys Gly Gln Gln Tyr Arg Asn Trp Phe Leu Lys Glu Phe
130 135 140

Pro Arg Leu Lys Ser Glu Leu Glu Asp Asn Ile Arg Arg Leu Arg Ala
145 150 155 160

Leu Ala Asp Gly Val Gln Lys Val His Lys Gly Thr Thr Ile Ala Asn
165 170 175

Val Val Ser Gly Ser Leu Ser Ile Ser Ser Gly Ile Leu Thr Leu Val
180 185 190

Gly Met Gly Leu Ala Pro Phe Thr Glu Gly Gly Ser Leu Val Leu Leu
195 200 205

Glu Pro Gly Met Glu Leu Gly Ile Thr Ala Ala Leu Thr Gly Ile Thr
210 215 220

Ser Ser Thr Met Asp Tyr Gly Lys Lys Trp Trp Thr Gln Ala Gln Ala
225 230 235 240

His Asp Leu Val Ile Lys Ser Leu Asp Lys Leu Lys Glu Val Arg Glu
245 250 255

Phe Leu Gly Glu Asn Ile Ser Asn Phe Leu Ser Leu Ala Gly Asn Thr

[0015]

260 265 270

Tyr Gln Leu Thr Arg Gly Ile Gly Lys Asp Ile Arg Ala Leu Arg Arg
275 280 285

Ala Arg Ala Asn Leu Gln Ser Val Pro His Ala Ser Ala Ser Arg Pro
290 295 300

Arg Val Thr Glu Pro Ile Ser Ala Glu Ser Gly Glu Gln Val Glu Arg
305 310 315 320

Val Asn Glu Pro Ser Ile Leu Glu Met Ser Arg Gly Val Lys Leu Thr
325 330 335

Asp Val Ala Pro Val Ser Phe Phe Leu Val Leu Asp Val Val Tyr Leu
340 345 350

Val Tyr Glu Ser Lys His Leu His Glu Gly Ala Lys Ser Glu Thr Ala
355 360 365

Glu Glu Leu Lys Lys Val Ala Gln Glu Leu Glu Glu Lys Leu Asn Ile
370 375 380

Leu Asn Asn Asn Tyr Lys Ile Leu Gln Ala Asp Gln Glu Leu
385 390 395

<210> 19
<211> 337
<212> PRT
<213> 智人（Homo sapiens）

<400> 19

Met Asn Pro Glu Ser Ser Ile Phe Ile Glu Asp Tyr Leu Lys Tyr Phe
1 5 10 15

Gln Asp Gln Val Ser Arg Glu Asn Leu Leu Gln Leu Leu Thr Asp Asp
20 25 30

Glu Ala Trp Asn Gly Phe Val Ala Ala Ala Glu Leu Pro Arg Asp Glu
35 40 45

Ala Asp Glu Leu Arg Lys Ala Leu Asn Lys Leu Ala Ser His Met Val
50 55 60

Met Lys Asp Lys Asn Arg His Asp Lys Asp Gln Gln His Arg Gln Trp
65 70 75 80

Phe Leu Lys Glu Phe Pro Arg Leu Lys Arg Glu Leu Glu Asp His Ile
85 90 95

Arg Lys Leu Arg Ala Leu Ala Glu Glu Val Glu Gln Val His Arg Gly
100 105 110

Thr Thr Ile Ala Asn Val Val Ser Asn Ser Val Gly Thr Thr Ser Gly
115 120 125

Ile Leu Thr Leu Leu Gly Leu Gly Leu Ala Pro Phe Thr Glu Gly Ile
130 135 140

[0016]

Ser Phe Val Leu Leu Asp Thr Gly Met Gly Leu Gly Ala Ala Ala Ala
145 150 155 160

Val Ala Gly Ile Thr Cys Ser Val Val Glu Leu Val Asn Lys Leu Arg
165 170 175

Ala Arg Ala Gln Ala Arg Asn Leu Asp Gln Ser Gly Thr Asn Val Ala
180 185 190

Lys Val Met Lys Glu Phe Val Gly Gly Asn Thr Pro Asn Val Leu Thr
195 200 205

Leu Val Asp Asn Trp Tyr Gln Val Thr Gln Gly Ile Gly Arg Asn Ile
210 215 220

Arg Ala Ile Arg Arg Ala Arg Ala Asn Pro Gln Leu Gly Ala Tyr Ala
225 230 235 240

Pro Pro Pro His Ile Ile Gly Arg Ile Ser Ala Glu Gly Gly Glu Gln
245 250 255

Val Glu Arg Val Val Glu Gly Pro Ala Gln Ala Met Ser Arg Gly Thr
260 265 270

Met Ile Val Gly Ala Ala Thr Gly Gly Ile Leu Leu Leu Leu Asp Val
275 280 285

Val Ser Leu Ala Tyr Glu Ser Lys His Leu Leu Glu Gly Ala Lys Ser
290 295 300

Glu Ser Ala Glu Glu Leu Lys Lys Arg Ala Gln Glu Leu Glu Gly Lys
305 310 315 320

Leu Asn Phe Leu Thr Lys Ile His Glu Met Leu Gln Pro Gly Gln Asp
325 330 335

Gln

<210> 20
<211> 402
<212> PRT
<213> 智人 (Homo sapiens)

<400> 20

Met Gly Leu Gly Gln Gly Trp Gly Trp Glu Ala Ser Cys Phe Ala Cys
1 5 10 15

Leu Ile Arg Ser Cys Cys Gln Val Val Thr Phe Thr Phe Pro Phe Gly
20 25 30

Phe Gln Gly Ile Ser Gln Ser Leu Glu Asn Val Ser Gly Tyr Tyr Ala
35 40 45

Asp Ala Arg Leu Glu Val Gly Ser Thr Gln Leu Arg Thr Ala Gly Ser
50 55 60

Cys Ser His Ser Phe Lys Arg Ser Phe Leu Glu Lys Lys Arg Phe Thr

[0017]

65 70 75 80

Glu Glu Ala Thr Lys Tyr Phe Arg Glu Arg Val Ser Pro Val His Leu
85 90 95

Gln Ile Leu Leu Thr Asn Asn Glu Ala Trp Lys Arg Phe Val Thr Ala
100 105 110

Ala Glu Leu Pro Arg Asp Glu Ala Asp Ala Leu Tyr Glu Ala Leu Lys
115 120 125

Lys Leu Arg Thr Tyr Ala Ala Ile Glu Asp Glu Tyr Val Gln Gln Lys
130 135 140

Asp Glu Gln Phe Arg Glu Trp Phe Leu Lys Glu Phe Pro Gln Val Lys
145 150 155 160

Arg Lys Ile Gln Glu Ser Ile Glu Lys Leu Arg Ala Leu Ala Asn Gly
165 170 175

Ile Glu Glu Val His Arg Gly Cys Thr Ile Ser Asn Val Val Ser Ser
180 185 190

Ser Thr Gly Ala Ala Ser Gly Ile Met Ser Leu Ala Gly Leu Val Leu
195 200 205

Ala Pro Phe Thr Ala Gly Thr Ser Leu Ala Leu Thr Ala Ala Gly Val
210 215 220

Gly Leu Gly Ala Ala Ser Ala Val Thr Gly Ile Thr Thr Ser Ile Val
225 230 235 240

Glu His Ser Tyr Thr Ser Ser Ala Glu Ala Glu Ala Ser Arg Leu Thr
245 250 255

Ala Thr Ser Ile Asp Arg Leu Lys Val Phe Lys Glu Val Met Arg Asp
260 265 270

Ile Thr Pro Asn Leu Leu Ser Leu Leu Asn Asn Tyr Tyr Glu Ala Thr
275 280 285

Gln Thr Ile Gly Ser Glu Ile Arg Ala Ile Arg Gln Ala Arg Ala Arg
290 295 300

Ala Arg Leu Pro Val Thr Thr Trp Arg Ile Ser Ala Gly Ser Gly Gly
305 310 315 320

Gln Ala Glu Arg Thr Ile Ala Gly Thr Thr Arg Ala Val Ser Arg Gly
325 330 335

Ala Arg Ile Leu Ser Ala Thr Thr Ser Gly Ile Phe Leu Ala Leu Asp
340 345 350

Val Val Asn Leu Val Tyr Glu Ser Lys His Leu His Glu Gly Ala Lys
355 360 365

Ser Ala Ser Ala Glu Glu Leu Arg Arg Gln Ala Gln Glu Leu Glu Glu
370 375 380

[0018]

Asn Leu Met Glu Leu Thr Gln Ile Tyr Gln Arg Leu Asn Pro Cys His
385 390 395 400

Thr His

<210> 21
<211> 351
<212> PRT
<213> 智人 (Homo sapiens)

<400> 21

Met Glu Gly Ala Ala Leu Leu Lys Ile Phe Val Val Cys Ile Trp Val
1 5 10 15

Gln Gln Asn His Pro Gly Trp Thr Val Ala Gly Gln Phe Gln Glu Lys
20 25 30

Lys Arg Phe Thr Glu Glu Val Ile Glu Tyr Phe Gln Lys Lys Val Ser
35 40 45

Pro Val His Leu Lys Ile Leu Leu Thr Ser Asp Glu Ala Trp Lys Arg
50 55 60

Phe Val Arg Val Ala Glu Leu Pro Arg Glu Glu Ala Asp Ala Leu Tyr
65 70 75 80

Glu Ala Leu Lys Asn Leu Thr Pro Tyr Val Ala Ile Glu Asp Lys Asp
85 90 95

Met Gln Gln Lys Glu Gln Gln Phe Arg Glu Trp Phe Leu Lys Glu Phe
100 105 110

Pro Gln Ile Arg Trp Lys Ile Gln Glu Ser Ile Glu Arg Leu Arg Val
115 120 125

Ile Ala Asn Glu Ile Glu Lys Val His Arg Gly Cys Val Ile Ala Asn
130 135 140

Val Val Ser Gly Ser Thr Gly Ile Leu Ser Val Ile Gly Val Met Leu
145 150 155 160

Ala Pro Phe Thr Ala Gly Leu Ser Leu Ser Ile Thr Ala Ala Gly Val
165 170 175

Gly Leu Gly Ile Ala Ser Ala Thr Ala Gly Ile Ala Ser Ser Ile Val
180 185 190

Glu Asn Thr Tyr Thr Arg Ser Ala Glu Leu Thr Ala Ser Arg Leu Thr
195 200 205

Ala Thr Ser Thr Asp Gln Leu Glu Ala Leu Arg Asp Ile Leu Arg Asp
210 215 220

Ile Thr Pro Asn Val Leu Ser Phe Ala Leu Asp Phe Asp Glu Ala Thr
225 230 235 240

[0019]

Lys Met Ile Ala Asn Asp Val His Thr Leu Arg Arg Ser Lys Ala Thr
245 250 255

Val Gly Arg Pro Leu Ile Ala Trp Arg Tyr Val Pro Ile Asn Val Val
260 265 270

Glu Thr Leu Arg Thr Arg Gly Ala Pro Thr Arg Ile Val Arg Lys Val
275 280 285

Ala Arg Asn Leu Gly Lys Ala Thr Ser Gly Val Leu Val Val Leu Asp
290 295 300

Val Val Asn Leu Val Gln Asp Ser Leu Asp Leu His Lys Gly Ala Lys
305 310 315 320

Ser Glu Ser Ala Glu Ser Leu Arg Gln Trp Ala Gln Glu Leu Glu Glu
325 330 335

Asn Leu Asn Glu Leu Thr His Ile His Gln Ser Leu Lys Ala Gly
340 345 350

<210> 22
<211> 433
<212> PRT
<213> 智人（Homo sapiens）

<400> 22

Met Pro Cys Gly Lys Gln Gly Asn Leu Gln Val Pro Gly Ser Lys Val
1 5 10 15

Leu Pro Gly Leu Gly Glu Gly Cys Lys Glu Met Trp Leu Arg Lys Val
20 25 30

Ile Tyr Gly Gly Glu Val Trp Gly Lys Ser Pro Glu Pro Glu Phe Pro
35 40 45

Ser Leu Val Asn Leu Cys Gln Ser Trp Lys Ile Asn Asn Leu Met Ser
50 55 60

Thr Val His Ser Asp Glu Ala Gly Met Leu Ser Tyr Phe Leu Phe Glu
65 70 75 80

Glu Leu Met Arg Cys Asp Lys Asp Ser Met Pro Asp Gly Asn Leu Ser
85 90 95

Glu Glu Glu Lys Leu Phe Leu Ser Tyr Phe Pro Leu His Lys Phe Glu
100 105 110

Leu Glu Gln Asn Ile Lys Glu Leu Asn Thr Leu Ala Asp Gln Val Asp
115 120 125

Thr Thr His Glu Leu Leu Thr Lys Thr Ser Leu Val Ala Ser Ser Ser
130 135 140

Gly Ala Val Ser Gly Val Met Asn Ile Leu Gly Leu Ala Leu Ala Pro
145 150 155 160

Val Thr Ala Gly Gly Ser Leu Met Leu Ser Ala Thr Gly Thr Gly Leu
165 170 175

[0020]

Gly Ala Ala Ala Ala Ile Thr Asn Ile Val Thr Asn Val Leu Glu Asn
180 185 190

Arg Ser Asn Ser Ala Ala Arg Asp Lys Ala Ser Arg Leu Gly Pro Leu
195 200 205

Thr Thr Ser His Glu Ala Phe Gly Gly Ile Asn Trp Ser Glu Ile Glu
210 215 220

Ala Ala Gly Phe Cys Val Asn Lys Cys Val Lys Ala Ile Gln Gly Ile
225 230 235 240

Lys Asp Leu His Ala Tyr Gln Met Ala Lys Ser Asn Ser Gly Phe Met
245 250 255

Ala Met Val Lys Asn Phe Val Ala Lys Arg His Ile Pro Phe Trp Thr
260 265 270

Ala Arg Gly Val Gln Arg Ala Phe Glu Gly Thr Thr Leu Ala Met Thr
275 280 285

Asn Gly Ala Trp Val Met Gly Ala Ala Gly Ala Gly Phe Leu Leu Met
290 295 300

Lys Asp Met Ser Ser Phe Leu Gln Ser Trp Lys His Leu Glu Asp Gly
305 310 315 320

Ala Arg Thr Glu Thr Ala Glu Glu Leu Arg Ala Leu Ala Lys Lys Leu
325 330 335

Glu Gln Glu Leu Asp Arg Leu Thr Gln His His Arg His Leu Pro Gln
340 345 350

Lys Ala Ser Gln Thr Cys Ser Ser Ser Arg Gly Arg Ala Val Arg Gly
355 360 365

Ser Arg Val Val Lys Pro Glu Gly Ser Arg Ser Pro Leu Pro Trp Pro
370 375 380

Val Val Glu His Gln Pro Arg Leu Gly Pro Gly Val Ala Leu Arg Thr
385 390 395 400

Pro Lys Arg Thr Val Ser Ala Pro Arg Met Leu Gly His Gln Pro Ala
405 410 415

Pro Pro Ala Pro Ala Arg Lys Gly Arg Gln Ala Pro Gly Arg His Arg
420 425 430

Gln

<210> 23
<211> 343
<212> PRT
<213> 智人 (Homo sapiens)

<400> 23

[0021]

```
Met Asp Asn Gln Ala Glu Arg Glu Ser Glu Ala Gly Val Gly Leu Gln
1               5                  10                  15

Arg Asp Glu Asp Asp Ala Pro Leu Cys Glu Asp Val Glu Leu Gln Asp
            20                  25                  30

Gly Asp Leu Ser Pro Glu Glu Lys Ile Phe Leu Arg Glu Phe Pro Arg
        35                  40                  45

Leu Lys Glu Asp Leu Lys Gly Asn Ile Asp Lys Leu Arg Ala Leu Ala
    50                  55                  60

Asp Asp Ile Asp Lys Thr His Lys Lys Phe Thr Lys Ala Asn Met Val
65                  70                  75                  80

Ala Thr Ser Thr Ala Val Ile Ser Gly Val Met Ser Leu Leu Gly Leu
                85                  90                  95

Ala Leu Ala Pro Ala Thr Gly Gly Gly Ser Leu Leu Leu Ser Thr Ala
            100                 105                 110

Gly Gln Gly Leu Ala Thr Ala Ala Gly Val Thr Ser Ile Val Ser Gly
        115                 120                 125

Thr Leu Glu Arg Ser Lys Asn Lys Glu Ala Gln Ala Arg Ala Glu Asp
    130                 135                 140

Ile Leu Pro Thr Tyr Asp Gln Glu Asp Arg Glu Asp Glu Glu Glu Lys
145                 150                 155                 160

Ala Asp Tyr Val Thr Ala Ala Gly Lys Ile Ile Tyr Asn Leu Arg Asn
                165                 170                 175

Thr Leu Lys Tyr Ala Lys Lys Asn Val Arg Ala Phe Trp Lys Leu Arg
            180                 185                 190

Ala Asn Pro Arg Leu Ala Asn Ala Thr Lys Arg Leu Leu Thr Thr Gly
        195                 200                 205

Gln Val Ser Ser Arg Ser Arg Val Gln Val Gln Lys Ala Phe Ala Gly
    210                 215                 220

Thr Thr Leu Ala Met Thr Lys Asn Ala Arg Val Leu Gly Gly Val Met
225                 230                 235                 240

Ser Ala Phe Ser Leu Gly Tyr Asp Leu Ala Thr Leu Ser Lys Glu Trp
                245                 250                 255

Lys His Leu Lys Glu Gly Ala Arg Thr Lys Phe Ala Glu Glu Leu Arg
            260                 265                 270

Ala Lys Ala Leu Glu Leu Glu Arg Lys Leu Thr Glu Leu Thr Gln Leu
        275                 280                 285

Tyr Lys Ser Leu Gln Gln Lys Val Arg Ser Arg Ala Arg Gly Val Gly
    290                 295                 300

Lys Asp Leu Thr Gly Thr Cys Glu Thr Glu Ala Tyr Trp Lys Glu Leu
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[0022]

305 310 315 320

Arg Glu His Val Trp Met Trp Leu Trp Leu Cys Val Cys Leu Cys Val
325 330 335

Cys Val Tyr Val Gln Phe Thr
340

<210> 24
<211> 188
<212> PRT
<213> 智人 (Homo sapiens)

<400> 24

Met Phe His Gln Ile Trp Ala Ala Leu Leu Tyr Phe Tyr Gly Ile Ile
1 5 10 15

Leu Asn Ser Ile Tyr Gln Cys Pro Glu His Ser Gln Leu Thr Thr Leu
20 25 30

Gly Val Asp Gly Lys Glu Phe Pro Glu Val His Leu Gly Gln Trp Tyr
35 40 45

Phe Ile Ala Gly Ala Ala Pro Thr Lys Glu Glu Leu Ala Thr Phe Asp
50 55 60

Pro Val Asp Asn Ile Val Phe Asn Met Ala Ala Gly Ser Ala Pro Met
65 70 75 80

Gln Leu His Leu Arg Ala Thr Ile Arg Met Lys Asp Gly Leu Cys Val
85 90 95

Pro Arg Lys Trp Ile Tyr His Leu Thr Glu Gly Ser Thr Asp Leu Arg
100 105 110

Thr Glu Gly Arg Pro Asp Met Lys Thr Glu Leu Phe Ser Ser Ser Cys
115 120 125

Pro Gly Gly Ile Met Leu Asn Glu Thr Gly Gln Gly Tyr Gln Arg Phe
130 135 140

Leu Leu Tyr Asn Arg Ser Pro His Pro Pro Glu Lys Cys Val Glu Glu
145 150 155 160

Phe Lys Ser Leu Thr Ser Cys Leu Asp Ser Lys Ala Phe Leu Leu Thr
165 170 175

Pro Arg Asn Gln Glu Ala Cys Glu Leu Ser Asn Asn
180 185

<210> 25
<211> 198
<212> PRT
<213> 智人 (Homo sapiens)

<400> 25

Met Phe Lys Val Ile Gln Arg Ser Val Gly Pro Ala Ser Leu Ser Leu
1 5 10 15

[0023]

Leu Thr Phe Lys Val Tyr Ala Ala Pro Lys Lys Asp Ser Pro Pro Lys
20 25 30

Asn Ser Val Lys Val Asp Glu Leu Ser Leu Tyr Ser Val Pro Glu Gly
35 40 45

Gln Ser Lys Tyr Val Glu Glu Ala Arg Ser Gln Leu Glu Glu Ser Ile
50 55 60

Ser Gln Leu Arg His Tyr Cys Glu Pro Tyr Thr Thr Trp Cys Gln Glu
65 70 75 80

Thr Tyr Ser Gln Thr Lys Pro Lys Met Gln Ser Leu Val Gln Trp Gly
85 90 95

Leu Asp Ser Tyr Asp Tyr Leu Gln Asn Ala Pro Pro Gly Phe Phe Pro
100 105 110

Arg Leu Gly Val Ile Gly Phe Ala Gly Leu Ile Gly Leu Leu Leu Ala
115 120 125

Arg Gly Ser Lys Ile Lys Lys Leu Val Tyr Pro Pro Gly Phe Met Gly
130 135 140

Leu Ala Ala Ser Leu Tyr Tyr Pro Gln Gln Ala Ile Val Phe Ala Gln
145 150 155 160

Val Ser Gly Glu Arg Leu Tyr Asp Trp Gly Leu Arg Gly Tyr Ile Val
165 170 175

Ile Glu Asp Leu Trp Lys Glu Asn Phe Gln Lys Pro Gly Asn Val Lys
180 185 190

Asn Ser Pro Gly Thr Lys
195

<210> 26
<211> 268
<212> PRT
<213> 智人 (Homo sapiens)

<400> 26

Met Ala Ala Ile Arg Met Gly Lys Leu Thr Thr Met Pro Ala Gly Leu
1 5 10 15

Ile Tyr Ala Ser Val Ser Val His Ala Ala Lys Gln Glu Glu Ser Lys
20 25 30

Lys Gln Leu Val Lys Pro Glu Gln Leu Pro Ile Tyr Thr Ala Pro Pro
35 40 45

Leu Gln Ser Lys Tyr Val Glu Glu Gln Pro Gly His Leu Gln Met Gly
50 55 60

Phe Ala Ser Ile Arg Thr Ala Thr Gly Cys Tyr Ile Gly Trp Cys Lys
65 70 75 80

Gly Val Tyr Val Phe Val Lys Asn Gly Ile Met Asp Thr Val Gln Phe
85 90 95

[0024]

Gly Lys Asp Ala Tyr Val Tyr Leu Lys Asn Pro Pro Arg Asp Phe Leu
100 105 110

Pro Lys Met Gly Val Ile Thr Val Ser Gly Leu Ala Gly Leu Val Ser
115 120 125

Ala Arg Lys Gly Ser Lys Phe Lys Lys Ile Thr Tyr Pro Leu Gly Leu
130 135 140

Ala Thr Leu Gly Ala Thr Val Cys Tyr Pro Val Gln Ser Val Ile Ile
145 150 155 160

Ala Lys Val Thr Ala Lys Lys Val Tyr Ala Thr Ser Gln Gln Ile Phe
165 170 175

Gly Ala Val Lys Ser Leu Trp Thr Lys Ser Ser Lys Glu Glu Ser Leu
180 185 190

Pro Lys Pro Lys Glu Lys Thr Lys Leu Gly Ser Ser Ser Glu Ile Glu
195 200 205

Val Pro Ala Lys Thr Thr His Val Leu Lys His Ser Val Pro Leu Pro
210 215 220

Thr Glu Leu Ser Ser Glu Ala Lys Thr Lys Ser Glu Ser Thr Ser Gly
225 230 235 240

Ala Thr Gln Phe Met Pro Asp Pro Lys Leu Met Asp His Gly Gln Ser
245 250 255

His Pro Glu Asp Ile Asp Met Tyr Ser Thr Arg Ser
260 265

<210> 27
<211> 449
<212> PRT
<213> 智人 (Homo sapiens)

<400> 27

Met Met Lys Thr Leu Leu Leu Phe Val Gly Leu Leu Leu Thr Trp Glu
1 5 10 15

Ser Gly Gln Val Leu Gly Asp Gln Thr Val Ser Asp Asn Glu Leu Gln
20 25 30

Glu Met Ser Asn Gln Gly Ser Lys Tyr Val Asn Lys Glu Ile Gln Asn
35 40 45

Ala Val Asn Gly Val Lys Gln Ile Lys Thr Leu Ile Glu Lys Thr Asn
50 55 60

Glu Glu Arg Lys Thr Leu Leu Ser Asn Leu Glu Glu Ala Lys Lys Lys
65 70 75 80

Lys Glu Asp Ala Leu Asn Glu Thr Arg Glu Ser Glu Thr Lys Leu Lys
85 90 95

[0025]

Glu Leu Pro Gly Val Cys Asn Glu Thr Met Met Ala Leu Trp Glu Glu
100 105 110

Cys Lys Pro Cys Leu Lys Gln Thr Cys Met Lys Phe Tyr Ala Arg Val
115 120 125

Cys Arg Ser Gly Ser Gly Leu Val Gly Arg Gln Leu Glu Glu Phe Leu
130 135 140

Asn Gln Ser Ser Pro Phe Tyr Phe Trp Met Asn Gly Asp Arg Ile Asp
145 150 155 160

Ser Leu Leu Glu Asn Asp Arg Gln Gln Thr His Met Leu Asp Val Met
165 170 175

Gln Asp His Phe Ser Arg Ala Ser Ser Ile Ile Asp Glu Leu Phe Gln
180 185 190

Asp Arg Phe Phe Thr Arg Glu Pro Gln Asp Thr Tyr His Tyr Leu Pro
195 200 205

Phe Ser Leu Pro His Arg Arg Pro His Phe Phe Phe Pro Lys Ser Arg
210 215 220

Ile Val Arg Ser Leu Met Pro Phe Ser Pro Tyr Glu Pro Leu Asn Phe
225 230 235 240

His Ala Met Phe Gln Pro Phe Leu Glu Met Ile His Glu Ala Gln Gln
245 250 255

Ala Met Asp Ile His Phe His Ser Pro Ala Phe Gln His Pro Pro Thr
260 265 270

Glu Phe Ile Arg Glu Gly Asp Asp Asp Arg Thr Val Cys Arg Glu Ile
275 280 285

Arg His Asn Ser Thr Gly Cys Leu Arg Met Lys Asp Gln Cys Asp Lys
290 295 300

Cys Arg Glu Ile Leu Ser Val Asp Cys Ser Thr Asn Asn Pro Ser Gln
305 310 315 320

Ala Lys Leu Arg Arg Glu Leu Asp Glu Ser Leu Gln Val Ala Glu Arg
325 330 335

Leu Thr Arg Lys Tyr Asn Glu Leu Leu Lys Ser Tyr Gln Trp Lys Met
340 345 350

Leu Asn Thr Ser Ser Leu Leu Glu Gln Leu Asn Glu Gln Phe Asn Trp
355 360 365

Val Ser Arg Leu Ala Asn Leu Thr Gln Gly Glu Asp Gln Tyr Tyr Leu
370 375 380

Arg Val Thr Thr Val Ala Ser His Thr Ser Asp Ser Asp Val Pro Ser
385 390 395 400

Gly Val Thr Glu Val Val Val Lys Leu Phe Asp Ser Asp Pro Ile Thr

[0026]

405 410 415

Val Thr Val Pro Val Glu Val Ser Arg Lys Asn Pro Lys Phe Met Glu
420 425 430

Thr Val Ala Glu Lys Ala Leu Gln Glu Tyr Arg Lys Lys His Arg Glu
435 440 445

Glu

<210> 28
<211> 267
<212> PRT
<213> 智人 (Homo sapiens)

<400> 28

Met Lys Ala Ala Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Pro Gln Ser Pro Trp
20 25 30

Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
35 40 45

Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
50 55 60

Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
65 70 75 80

Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
85 90 95

Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
100 105 110

Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
115 120 125

Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
130 135 140

Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu
145 150 155 160

Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala
165 170 175

Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
180 185 190

Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
195 200 205

Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu
210 215 220

[0027]

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln
225 230 235 240

Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala
245 250 255

Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
260 265

<210> 29
<211> 267
<212> PRT
<213> 智人（Homo sapiens）

<400> 29

Met Lys Ala Ala Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Pro Gln Ser Pro Trp
20 25 30

Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
35 40 45

Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
50 55 60

Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
65 70 75 80

Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
85 90 95

Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
100 105 110

Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
115 120 125

Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
130 135 140

Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu
145 150 155 160

Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala
165 170 175

Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
180 185 190

Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
195 200 205

Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu
210 215 220

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln

[0028]

225 230 235 240

Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala
245 250 255

Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
260 265

<210> 30
<211> 267
<212> PRT
<213> 智人（Homo sapiens）

<400> 30

Met Lys Ala Thr Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Pro Gln Thr Pro Trp
20 25 30

Asp Arg Val Lys Asp Leu Val Thr Val Tyr Val Glu Ala Leu Lys Asp
35 40 45

Ser Gly Lys Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
50 55 60

Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
65 70 75 80

Val Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
85 90 95

Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
100 105 110

Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
115 120 125

Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
130 135 140

Pro Leu Arg Ala Glu Leu His Glu Gly Thr Arg Gln Lys Leu His Glu
145 150 155 160

Leu His Glu Lys Leu Ser Pro Leu Gly Glu Glu Val Arg Asp Arg Ala
165 170 175

Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
180 185 190

Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
195 200 205

Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Ser Glu His Leu
210 215 220

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln
225 230 235 240

[0029]

Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala
245 250 255

Leu Glu Glu Tyr Thr Lys Lys Leu Ser Thr Gln
260 265

<210> 31
<211> 265
<212> PRT
<213> 智人 (Homo sapiens)

<400> 31

Met Lys Ala Val Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Asp Pro Gln Ser Ser Trp Asp
20 25 30

Arg Val Lys Asp Phe Ala Thr Val Tyr Val Glu Ala Ile Lys Asp Ser
35 40 45

Gly Arg Asp Tyr Val Ala Gln Phe Glu Ala Ser Ala Leu Gly Lys Gln
50 55 60

Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Thr Leu Ala Ser Thr Leu
65 70 75 80

Ser Lys Val Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp
85 90 95

Asn Leu Glu Lys Glu Thr Ala Ser Leu Arg Gln Glu Met His Lys Asp
100 105 110

Leu Glu Glu Val Lys Gln Lys Val Gln Pro Tyr Leu Asp Glu Phe Gln
115 120 125

Lys Lys Trp His Glu Glu Val Glu Ile Tyr Arg Gln Lys Val Ala Pro
130 135 140

Leu Gly Glu Glu Phe Arg Glu Gly Ala Arg Gln Lys Val Gln Glu Leu
145 150 155 160

Gln Asp Lys Leu Ser Pro Leu Ala Gln Glu Leu Arg Asp Arg Ala Arg
165 170 175

Ala His Val Glu Thr Leu Arg Gln Gln Leu Ala Pro Tyr Ser Asp Asp
180 185 190

Leu Arg Gln Arg Leu Thr Ala Arg Leu Glu Ala Leu Lys Glu Gly Gly
195 200 205

Gly Ser Leu Ala Glu Tyr His Ala Lys Ala Ser Glu Gln Leu Lys Ala
210 215 220

Leu Gly Glu Lys Ala Lys Pro Val Leu Glu Asp Leu Arg Gln Gly Leu
225 230 235 240

Leu Pro Val Leu Glu Ser Leu Lys Val Ser Ile Leu Ala Ala Ile Asp

[0030]

245 250 255

Glu Ala Ser Lys Lys Leu Asn Ala Gln
260 265

<210> 32
<211> 264
<212> PRT
<213> 智人 (Homo sapiens)

<400> 32

Met Lys Ala Trp Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser Gln
1 5 10 15

Ala Arg His Phe Trp Gln Gln Asp Asp Pro Gln Ser Pro Trp Asp Arg
20 25 30

Val Lys Asp Phe Ala Thr Val Tyr Val Asp Ala Ile Lys Asp Ser Gly
35 40 45

Arg Asp Tyr Val Ala Gln Phe Glu Ala Ser Ala Leu Gly Lys His Leu
50 55 60

Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Leu Gly Ser Thr Phe Thr
65 70 75 80

Lys Val Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn
85 90 95

Leu Glu Lys Glu Thr Glu Ala Leu Arg Gln Glu Met Ser Lys Asp Leu
100 105 110

Glu Glu Val Lys Lys Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln Asn
115 120 125

Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Met Ala Pro Leu
130 135 140

Gly Ala Glu Phe Arg Glu Gly Ala Arg Gln Lys Val Gln Glu Leu Gln
145 150 155 160

Glu Lys Leu Ser Pro Leu Ala Glu Glu Leu Arg Asp Arg Leu Arg Ala
165 170 175

His Val Glu Ala Leu Arg Gln His Val Ala Pro Tyr Ser Asp Asp Leu
180 185 190

Arg Gln Arg Met Ala Ala Arg Phe Glu Ala Leu Lys Glu Gly Gly Gly
195 200 205

Ser Leu Ala Glu Tyr Gln Ala Lys Ala Gln Glu Gln Leu Lys Ala Leu
210 215 220

Gly Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu
225 230 235 240

Pro Val Leu Glu Asn Leu Lys Val Ser Ile Leu Ala Ala Ile Asp Glu
245 250 255

[0031]

Ala Ser Lys Lys Leu Asn Ala Gln
260

<210> 33
<211> 266
<212> PRT
<213> 智人（Homo sapiens）

<400> 33

Met Lys Ala Ala Leu Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Gln Ser Pro Trp Asp
20 25 30

Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Ala Val Lys Asp Ser
35 40 45

Gly Arg Asp Tyr Val Ala Gln Phe Glu Ala Ser Ala Leu Gly Lys Gln
50 55 60

Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Leu Ser Ser Thr Val
65 70 75 80

Thr Lys Leu Arg Glu Gln Ile Gly Pro Val Thr Gln Glu Phe Trp Asp
85 90 95

Asn Leu Glu Lys Glu Thr Glu Val Leu Arg Gln Glu Met Ser Lys Asp
100 105 110

Leu Glu Glu Val Lys Gln Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln
115 120 125

Lys Lys Trp Gln Glu Glu Val Glu Leu Tyr Arg Gln Lys Val Ala Pro
130 135 140

Leu Gly Ser Glu Leu Arg Glu Gly Ala Arg Gln Lys Leu Gln Glu Leu
145 150 155 160

Gln Glu Lys Leu Ser Pro Leu Ala Glu Glu Leu Arg Asp Arg Ala Arg
165 170 175

Thr His Val Asp Ala Leu Arg Ala Gln Leu Ala Pro Tyr Ser Asp Asp
180 185 190

Leu Arg Glu Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Gly Gly
195 200 205

Gly Ala Ser Leu Ala Glu Tyr His Ala Arg Ala Ser Glu Gln Leu Ser
210 215 220

Ala Leu Gly Glu Lys Ala Arg Pro Ala Leu Glu Asp Leu Arg Gln Gly
225 230 235 240

Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Leu Leu Ala Ala Ile
245 250 255

Asp Glu Ala Thr Lys Lys Leu Asn Ala Gln

[0032]

260 265

<210> 34
<211> 206
<212> PRT
<213> 智人 (Homo sapiens)

<400> 34

Met Lys Ala Val Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Arg Asp Glu Pro Arg Ser Ser Trp Asp
20 25 30

Lys Ile Lys Asp Phe Ala Thr Val Tyr Val Asp Thr Val Lys Asp Ser
35 40 45

Gly Arg Glu Tyr Val Ala Gln Phe Glu Ala Ser Ala Phe Gly Lys Gln
50 55 60

Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Leu Ser Ser Thr Val
65 70 75 80

Ser Lys Leu Gln Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp
85 90 95

Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Glu Glu Met Asn Lys Asp
100 105 110

Leu Gln Glu Val Arg Gln Lys Val Gln Pro Tyr Leu Asp Glu Phe Gln
115 120 125

Lys Lys Trp Gln Glu Glu Val Glu Arg Tyr Arg Gln Lys Val Glu Pro
130 135 140

Leu Gly Ala Glu Leu Arg Glu Ser Ala Arg Gln Lys Leu Thr Glu Leu
145 150 155 160

Gln Glu Lys Leu Ser Pro Leu Ala Glu Glu Leu Arg Asp Ser Ala Arg
165 170 175

Thr His Val Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Ala Ser Val
180 185 190

Gln Asn Val Leu Asp Glu Ala Thr Lys Lys Leu Asn Thr Gln
195 200 205

<210> 35
<211> 265
<212> PRT
<213> 智人 (Homo sapiens)

<400> 35

Met Lys Ala Val Val Leu Thr Leu Ala Val Leu Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Gln Ser Ser Trp Asp
20 25 30

[0033]

Arg Val Arg Asp Leu Ala Asn Val Tyr Val Asp Ala Val Lys Glu Ser
35 40 45

Gly Arg Glu Tyr Val Ser Gln Leu Glu Ala Ser Ala Leu Gly Lys Gln
50 55 60

Leu Asn Leu Lys Leu Val Asp Asn Trp Asp Thr Leu Gly Ser Thr Phe
65 70 75 80

Gln Lys Val His Glu His Leu Gly Pro Val Ala Gln Glu Phe Trp Glu
85 90 95

Lys Leu Glu Lys Glu Thr Glu Glu Leu Arg Arg Glu Ile Asn Lys Asp
100 105 110

Leu Glu Asp Val Arg Gln Lys Thr Gln Pro Phe Leu Asp Glu Ile Gln
115 120 125

Lys Lys Trp Gln Glu Asp Leu Glu Arg Tyr Arg Gln Lys Val Glu Pro
130 135 140

Leu Ser Ala Gln Leu Arg Glu Gly Ala Arg Gln Lys Leu Met Glu Leu
145 150 155 160

Gln Glu Gln Val Thr Pro Leu Gly Glu Asp Leu Arg Asp Ser Val Arg
165 170 175

Ala Tyr Ala Asp Thr Leu Arg Thr Gln Leu Ala Pro Tyr Ser Glu Gln
180 185 190

Met Arg Lys Thr Leu Gly Ala Arg Leu Glu Ala Ile Lys Glu Gly Gly
195 200 205

Ser Ala Ser Leu Ala Glu Tyr His Ala Lys Ala Ser Glu Gln Leu Ser
210 215 220

Ala Leu Gly Glu Lys Ala Lys Pro Val Leu Glu Asp Ile His Gln Gly
225 230 235 240

Leu Met Pro Met Trp Glu Ser Phe Lys Thr Gly Val Leu Asn Val Ile
245 250 255

Asp Glu Ala Ala Lys Lys Leu Thr Ala
260 265

<210> 36
<211> 264
<212> PRT
<213> 智人 (Homo sapiens)

<400> 36

Met Lys Ala Val Val Leu Ala Val Ala Leu Val Phe Leu Thr Gly Ser
1 5 10 15

Gln Ala Trp His Val Trp Gln Gln Asp Glu Pro Gln Ser Gln Trp Asp
20 25 30

Lys Val Lys Asp Phe Ala Asn Val Tyr Val Asp Ala Val Lys Asp Ser
35 40 45

[0034]

Gly Arg Asp Tyr Val Ser Gln Phe Glu Ser Ser Ser Leu Gly Gln Gln
50 55 60

Leu Asn Leu Asn Leu Leu Glu Asn Trp Asp Thr Leu Gly Ser Thr Val
65 70 75 80

Ser Gln Leu Gln Glu Arg Leu Gly Pro Leu Thr Arg Asp Phe Trp Asp
85 90 95

Asn Leu Glu Lys Glu Thr Asp Trp Val Arg Gln Glu Met Asn Lys Asp
100 105 110

Leu Glu Glu Val Lys Gln Lys Val Gln Pro Tyr Leu Asp Glu Phe Gln
115 120 125

Lys Lys Trp Lys Glu Asp Val Glu Leu Tyr Arg Gln Lys Val Ala Pro
130 135 140

Leu Gly Ala Glu Leu Gln Glu Ser Ala Arg Gln Lys Leu Gln Glu Leu
145 150 155 160

Gln Gly Arg Leu Ser Pro Val Ala Glu Glu Phe Arg Asp Arg Met Arg
165 170 175

Thr His Val Asp Ser Leu Arg Thr Gln Leu Ala Pro His Ser Glu Gln
180 185 190

Met Arg Glu Ser Leu Ala Gln Arg Leu Ala Glu Leu Lys Ser Asn Pro
195 200 205

Thr Leu Asn Glu Tyr His Thr Arg Ala Lys Thr His Leu Lys Thr Leu
210 215 220

Gly Glu Lys Ala Arg Pro Ala Leu Glu Asp Leu Arg His Ser Leu Met
225 230 235 240

Pro Met Leu Glu Thr Leu Lys Thr Lys Ala Gln Ser Val Ile Asp Lys
245 250 255

Ala Ser Glu Thr Leu Thr Ala Gln
260

<210> 37
<211> 259
<212> PRT
<213> 智人(Homo sapiens)

<400> 37

Met Lys Ala Ala Val Leu Ala Val Ala Leu Val Phe Leu Thr Gly Cys
1 5 10 15

Gln Ala Trp Glu Phe Trp Gln Gln Asp Glu Pro Gln Ser Gln Trp Asp
20 25 30

Arg Val Lys Asp Phe Ala Thr Val Tyr Val Asp Ala Val Lys Asp Ser
35 40 45

[0035]

Gly Arg Asp Tyr Val Ser Gln Phe Glu Ser Ser Thr Leu Gly Lys Gln
50 55 60

Leu Asn Leu Asn Leu Leu Asp Asn Trp Asp Thr Leu Gly Ser Thr Val
65 70 75 80

Gly Arg Leu Gln Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Ala
85 90 95

Asn Leu Glu Lys Glu Thr Asp Trp Leu Arg Asn Glu Met Asn Lys Asp
100 105 110

Leu Glu Asn Val Lys Gln Lys Met Gln Pro His Leu Asp Glu Phe Gln
115 120 125

Glu Lys Trp Asn Glu Glu Val Glu Ala Tyr Arg Gln Lys Leu Glu Pro
130 135 140

Leu Gly Thr Glu Leu His Lys Asn Ala Lys Glu Met Gln Arg His Leu
145 150 155 160

Lys Val Val Ala Glu Glu Phe Arg Asp Arg Met Arg Val Asn Ala Asp
165 170 175

Ala Leu Arg Ala Lys Phe Gly Leu Tyr Ser Asp Gln Met Arg Glu Asn
180 185 190

Leu Ala Gln Arg Leu Thr Glu Ile Arg Asn His Pro Thr Leu Ile Glu
195 200 205

Tyr His Thr Lys Ala Gly Asp His Leu Arg Thr Leu Gly Glu Lys Ala
210 215 220

Lys Pro Ala Leu Asp Asp Leu Gly Gln Gly Leu Met Pro Val Leu Glu
225 230 235 240

Ala Trp Lys Ala Lys Ile Met Ser Met Ile Asp Glu Ala Lys Lys Lys
245 250 255

Leu Asn Ala

<210> 38
<211> 241
<212> PRT
<213> 智人 (Homo sapiens)

<400> 38

Asp Glu Ala Lys Ser Tyr Trp Asp Gln Ile Lys Asp Met Leu Thr Val
1 5 10 15

Tyr Val Asp Thr Ala Lys Asp Ser Gly Lys Asp Tyr Leu Thr Ser Leu
20 25 30

Asp Thr Ser Ala Leu Gly Gln Gln Leu Asn Lys Lys Leu Ala Asp Asn
35 40 45

Trp Asp Thr Val Ser Ser Ala Leu Leu Lys Ala Arg Glu Gln Met Lys
50 55 60

[0036]

Pro Ile Ala Met Glu Phe Trp Gly Asn Leu Glu Lys Asp Thr Glu Gly
65 70 75 80

Leu Arg Gln Thr Val Ser Lys Asp Leu Glu Leu Val Lys Glu Lys Val
85 90 95

Gln Pro Tyr Leu Asp Ser Phe Gln Lys Lys Val Glu Glu Glu Leu Glu
100 105 110

Leu Tyr Arg Gln Lys Val Ala Pro Leu Ser Ala Glu Trp Arg Glu Gln
115 120 125

Ala Arg Gln Lys Ala Gln Glu Leu Gln Gln Lys Ala Gly Glu Leu Gly
130 135 140

Gln Gln His Arg Asp Arg Val Arg Thr His Val Asp Ala Leu Arg Thr
145 150 155 160

Asp Leu Ala Pro Tyr Gly Glu Glu Ala Arg Lys Leu Leu Leu Gln Arg
165 170 175

Leu Gln Asp Ile Lys Ala Lys Ser Gly Asp Leu Ala Glu Tyr Gln Thr
180 185 190

Lys Leu Ser Glu His Leu Lys Ser Phe Gly Glu Lys Ala Gln Pro Thr
195 200 205

Leu Gln Asp Leu Arg His Gly Leu Glu Pro Leu Trp Glu Gly Ile Lys
210 215 220

Ala Gly Ala Met Ser Met Leu Glu Glu Leu Gly Lys Lys Leu Asn Ser
225 230 235 240

Gln

<210> 39
<211> 264
<212> PRT
<213> 智人 (Homo sapiens)

<400> 39

Met Arg Gly Val Leu Val Thr Leu Ala Val Leu Phe Leu Thr Gly Thr
1 5 10 15

Gln Ala Arg Ser Phe Trp Gln His Asp Glu Pro Gln Thr Pro Leu Asp
20 25 30

Arg Ile Arg Asp Met Val Asp Val Tyr Leu Glu Thr Val Lys Ala Ser
35 40 45

Gly Lys Asp Ala Ile Ala Gln Phe Glu Ser Ser Ala Val Gly Lys Gln
50 55 60

Leu Asp Leu Lys Leu Ala Asp Asn Leu Asp Thr Leu Ser Ala Ala Ala
65 70 75 80

[0037]

Ala Lys Leu Arg Glu Asp Met Ala Pro Tyr Tyr Lys Glu Val Arg Glu
85 90 95

Met Trp Leu Lys Asp Thr Glu Ala Leu Arg Ala Glu Leu Thr Lys Asp
100 105 110

Leu Glu Glu Val Lys Glu Lys Ile Arg Pro Phe Leu Asp Gln Phe Ser
115 120 125

Ala Lys Trp Thr Glu Glu Leu Glu Gln Tyr Arg Gln Arg Leu Thr Pro
130 135 140

Val Ala Gln Glu Leu Lys Glu Leu Thr Lys Gln Lys Val Glu Leu Met
145 150 155 160

Gln Ala Lys Leu Thr Pro Val Ala Glu Glu Ala Arg Asp Arg Leu Arg
165 170 175

Gly His Val Glu Glu Leu Arg Lys Asn Leu Ala Pro Tyr Ser Asp Glu
180 185 190

Leu Arg Gln Lys Leu Ser Gln Lys Leu Glu Glu Ile Arg Glu Lys Gly
195 200 205

Ile Pro Gln Ala Ser Glu Tyr Gln Ala Lys Val Met Glu Gln Leu Ser
210 215 220

Asn Leu Arg Glu Lys Met Thr Pro Leu Val Gln Glu Phe Arg Glu Arg
225 230 235 240

Leu Thr Pro Tyr Ala Glu Asn Leu Lys Asn Arg Leu Ile Ser Phe Leu
245 250 255

Asp Glu Leu Gln Lys Ser Val Ala
260

<210> 40
<211> 264
<212> PRT
<213> 智人 (Homo sapiens)

<400> 40

Met Arg Gly Val Leu Val Thr Leu Ala Val Leu Phe Leu Thr Gly Thr
1 5 10 15

Gln Ala Arg Ser Phe Trp Gln His Asp Asp Pro Gln Thr Pro Leu Asp
20 25 30

Arg Ile Arg Asp Met Leu Asp Val Tyr Leu Glu Thr Val Lys Ala Ser
35 40 45

Gly Lys Asp Ala Ile Ser Gln Phe Glu Ser Ser Ala Val Gly Lys Gln
50 55 60

Leu Asp Leu Lys Leu Ala Asp Asn Leu Asp Thr Leu Ser Ala Ala Ala
65 70 75 80

Ala Lys Leu Arg Glu Asp Met Thr Pro Tyr Tyr Arg Glu Val Arg Glu
85 90 95

[0038]

Met Trp Leu Lys Asp Thr Glu Ala Leu Arg Ala Glu Leu Thr Lys Asp
100 105 110

Leu Glu Glu Val Lys Glu Lys Ile Arg Pro Phe Leu Asp Gln Phe Ser
115 120 125

Ala Lys Trp Thr Glu Glu Val Glu Gln Tyr Arg Gln Arg Leu Ala Pro
130 135 140

Val Ala Gln Glu Leu Lys Asp Leu Thr Lys Gln Lys Val Glu Leu Met
145 150 155 160

Gln Ala Lys Leu Thr Pro Val Ala Glu Glu Val Arg Asp Arg Leu Arg
165 170 175

Glu Gln Val Glu Glu Leu Arg Lys Asn Leu Ala Pro Tyr Ser Ser Glu
180 185 190

Leu Arg Gln Lys Leu Ser Gln Lys Leu Glu Glu Ile Arg Glu Arg Gly
195 200 205

Ile Pro Gln Ala Ser Glu Tyr Gln Ala Lys Val Val Glu Gln Leu Ser
210 215 220

Asn Leu Arg Glu Lys Met Thr Pro Leu Val Gln Glu Phe Lys Glu Arg
225 230 235 240

Leu Thr Pro Tyr Ala Glu Asn Leu Lys Asn Arg Leu Ile Asp Leu Leu
245 250 255

Asp Glu Val Gln Lys Thr Met Ala
260

<210> 41
<211> 264
<212> PRT
<213> 智人 (Homo sapiens)

<400> 41

Met Arg Val Val Val Val Thr Leu Ala Leu Leu Phe Leu Thr Gly Thr
1 5 10 15

Gln Ala Arg Tyr Phe Trp Gln His Asp Glu Pro Gln Ala Pro Leu Asp
20 25 30

Arg Leu Arg Asp Leu Val Asp Val Tyr Leu Glu Thr Val Lys Ala Ser
35 40 45

Gly Lys Asp Ala Ile Ala Gln Phe Glu Ala Ser Ala Val Gly Lys Gln
50 55 60

Leu Asp Leu Lys Leu Ala Asp Asn Leu Asp Thr Leu Gly Ala Ala Ala
65 70 75 80

Ala Lys Leu Arg Glu Asp Met Ala Pro Tyr Tyr Lys Glu Val Arg Glu
85 90 95

[0039]

Met Trp Leu Lys Asp Thr Glu Ser Leu Arg Ala Glu Leu Thr Lys Asp
100 105 110

Leu Glu Glu Val Lys Glu Lys Ile Arg Pro Phe Leu Asp Gln Phe Ser
115 120 125

Ala Lys Trp Thr Glu Glu Leu Glu Gln Tyr Arg Gln Arg Leu Ala Pro
130 135 140

Val Ala Glu Glu Leu Lys Glu Leu Thr Lys Gln Lys Val Glu Leu Met
145 150 155 160

Gln Gln Lys Leu Thr Pro Val Ala Glu Glu Ala Arg Asp Arg Leu Arg
165 170 175

Gly His Val Glu Glu Leu Arg Lys Asn Leu Ala Pro Tyr Ser Asp Glu
180 185 190

Leu Arg Gln Lys Leu Ser Gln Lys Leu Glu Glu Ile Arg Glu Lys Gly
195 200 205

Ile Pro Gln Ala Ala Glu Tyr Gln Ala Lys Val Val Glu Gln Leu Ser
210 215 220

Asn Leu Arg Glu Lys Met Thr Pro Leu Val Gln Asp Phe Lys Glu Arg
225 230 235 240

Leu Thr Pro Tyr Ala Glu Asn Leu Lys Thr Arg Phe Ile Ser Leu Leu
245 250 255

Asp Glu Leu Gln Lys Thr Val Ala
260

<210> 42
<211> 262
<212> PRT
<213> 智人 (Homo sapiens)

<400> 42

Met Lys Phe Leu Ala Leu Ala Leu Thr Ile Leu Leu Ala Ala Gly Thr
1 5 10 15

Gln Ala Phe Pro Met Gln Ala Asp Ala Pro Ser Gln Leu Glu His Val
20 25 30

Lys Ala Ala Leu Ser Met Tyr Ile Ala Gln Val Lys Leu Thr Ala Gln
35 40 45

Arg Ser Ile Asp Leu Leu Asp Asp Thr Glu Tyr Lys Glu Tyr Lys Met
50 55 60

Gln Leu Thr Gln Ser Leu Asp Asn Leu Gln Gln Tyr Ala Asp Ala Thr
65 70 75 80

Ser Gln Ser Leu Ala Pro Tyr Ser Glu Ala Phe Gly Thr Gln Leu Thr
85 90 95

Asp Ala Thr Ala Ala Val Arg Ala Glu Val Met Lys Asp Val Glu Glu
100 105 110

[0040]

Leu Arg Ser Gln Leu Glu Pro Lys Arg Ala Glu Leu Lys Glu Val Leu
115 120 125

Asp Lys His Ile Asp Glu Tyr Arg Lys Lys Leu Glu Pro Leu Ile Lys
130 135 140

Glu His Ile Glu Leu Arg Arg Thr Glu Met Glu Ala Phe Arg Ala Lys
145 150 155 160

Met Glu Pro Ile Val Glu Glu Leu Arg Ala Lys Val Ala Ile Asn Val
165 170 175

Glu Glu Thr Lys Thr Lys Leu Met Pro Ile Val Glu Ile Val Arg Ala
180 185 190

Lys Leu Thr Glu Arg Leu Glu Glu Leu Arg Thr Leu Ala Ala Pro Tyr
195 200 205

Ala Glu Glu Tyr Lys Glu Gln Met Ile Lys Ala Val Gly Glu Val Arg
210 215 220

Glu Lys Val Ser Pro Leu Ser Glu Asp Phe Lys Gly Gln Val Gly Pro
225 230 235 240

Ala Ala Glu Gln Ala Lys Gln Lys Leu Leu Ala Phe Tyr Glu Thr Ile
245 250 255

Ser Gln Ala Met Lys Ala
260

<210> 43
<211> 262
<212> PRT
<213> 智人 (Homo sapiens)

<400> 43

Met Lys Phe Leu Ala Leu Ala Leu Thr Ile Leu Leu Ala Ala Ala Thr
1 5 10 15

Gln Ala Val Pro Met Gln Ala Asp Ala Pro Ser Gln Leu Glu His Val
20 25 30

Lys Val Ala Met Met Glu Tyr Met Ala Gln Val Lys Glu Thr Gly Gln
35 40 45

Arg Ser Ile Asp Leu Leu Asp Asp Thr Glu Phe Lys Glu Tyr Lys Val
50 55 60

Gln Leu Ser Gln Ser Leu Asp Asn Leu Gln Gln Tyr Ala Gln Thr Thr
65 70 75 80

Ser Gln Ser Leu Ala Pro Tyr Ser Glu Ala Phe Gly Ala Gln Leu Thr
85 90 95

Asp Ala Ala Ala Ala Val Arg Ala Glu Val Met Lys Asp Val Glu Asp
100 105 110

[0041]

Val Arg Thr Gln Leu Glu Pro Lys Arg Ala Glu Leu Lys Glu Val Leu
115 120 125

Asp Lys His Ile Asp Glu Tyr Arg Lys Lys Leu Glu Pro Leu Ile Lys
130 135 140

Glu Ile Val Glu Gln Arg Arg Thr Glu Leu Glu Ala Phe Arg Val Lys
145 150 155 160

Met Glu Pro Val Val Glu Glu Met Arg Ala Lys Val Ser Thr Asn Val
165 170 175

Glu Glu Thr Lys Ala Lys Leu Met Pro Ile Val Glu Thr Val Arg Ala
180 185 190

Lys Leu Thr Glu Arg Leu Glu Glu Leu Arg Thr Leu Ala Ala Pro Tyr
195 200 205

Ala Glu Glu Tyr Lys Glu Gln Met Phe Lys Ala Val Gly Glu Val Arg
210 215 220

Glu Lys Val Gly Pro Leu Thr Asn Asp Phe Lys Gly Gln Val Gly Pro
225 230 235 240

Ala Ala Glu Gln Ala Lys Glu Lys Leu Met Asp Phe Tyr Glu Thr Ile
245 250 255

Ser Gln Ala Met Lys Ala
260

<210> 44
<211> 258
<212> PRT
<213> 智人 (Homo sapiens)

<400> 44

Met Lys Phe Leu Val Leu Ala Leu Thr Ile Leu Leu Ala Ala Gly Thr
1 5 10 15

Gln Ala Phe Pro Met Gln Ala Asp Ala Pro Ser Gln Leu Glu His Val
20 25 30

Lys Ala Ala Leu Asn Met Tyr Ile Ala Gln Val Lys Leu Thr Ala Gln
35 40 45

Arg Ser Ile Asp Leu Leu Asp Asp Thr Glu Tyr Lys Glu Tyr Lys Met
50 55 60

Gln Leu Ser Gln Ser Leu Asp Asn Leu Gln Gln Phe Ala Asp Ser Thr
65 70 75 80

Ser Lys Ser Trp Pro Pro Thr Pro Arg Ser Ser Ala Pro Ser Cys Asp
85 90 95

Ala Thr Ala Thr Val Arg Ala Glu Val Met Lys Asp Val Glu Asp Val
100 105 110

Arg Thr Gln Leu Glu Pro Lys Arg Ala Glu Leu Thr Glu Val Leu Asn
115 120 125

[0042]

Lys His Ile Asp Glu Tyr Arg Lys Lys Leu Glu Pro Leu Ile Lys Gln
130 135 140

His Ile Glu Leu Arg Arg Thr Glu Met Asp Ala Phe Arg Ala Lys Ile
145 150 155 160

Asp Pro Val Val Glu Glu Met Arg Ala Lys Val Ala Val Asn Val Glu
165 170 175

Glu Thr Lys Thr Lys Leu Met Pro Ile Val Glu Ile Val Arg Ala Lys
180 185 190

Leu Thr Glu Arg Leu Glu Glu Leu Arg Thr Leu Ala Ala Pro Tyr Ala
195 200 205

Glu Glu Tyr Lys Glu Gln Met Phe Lys Ala Val Gly Glu Val Arg Glu
210 215 220

Lys Val Ala Pro Leu Ser Glu Asp Phe Lys Ala Arg Trp Ala Pro Pro
225 230 235 240

Pro Arg Arg Pro Ser Lys Ser Ser Trp Leu Ser Thr Arg Pro Ser Ala
245 250 255

Arg Pro

<210> 45
<211> 262
<212> PRT
<213> 智人（Homo sapiens）

<400> 45

Met Lys Phe Val Ala Leu Ala Leu Thr Leu Leu Leu Ala Leu Gly Ser
1 5 10 15

Gln Ala Asn Leu Phe Gln Ala Asp Ala Pro Thr Gln Leu Glu His Tyr
20 25 30

Lys Ala Ala Ala Leu Val Tyr Leu Asn Gln Val Lys Asp Gln Ala Glu
35 40 45

Lys Ala Leu Asp Asn Leu Asp Gly Thr Asp Tyr Glu Gln Tyr Lys Leu
50 55 60

Gln Leu Ser Glu Ser Leu Thr Lys Leu Gln Glu Tyr Ala Gln Thr Thr
65 70 75 80

Ser Gln Ala Leu Thr Pro Tyr Ala Glu Thr Ile Ser Thr Gln Leu Met
85 90 95

Glu Asn Thr Lys Gln Leu Arg Glu Arg Val Met Thr Asp Val Glu Asp
100 105 110

Leu Arg Ser Lys Leu Glu Pro His Arg Ala Glu Leu Tyr Thr Ala Leu
115 120 125

[0043]

Gln Lys His Ile Asp Glu Tyr Arg Glu Lys Leu Glu Pro Val Phe Gln
130 135 140

Glu Tyr Ser Ala Leu Asn Arg Gln Asn Ala Glu Gln Leu Arg Ala Lys
145 150 155 160

Leu Glu Pro Leu Met Asp Asp Ile Arg Lys Ala Phe Glu Ser Asn Ile
165 170 175

Glu Glu Thr Lys Ser Lys Val Val Pro Met Val Glu Ala Val Arg Thr
180 185 190

Lys Leu Thr Glu Arg Leu Glu Asp Leu Arg Thr Met Ala Ala Pro Tyr
195 200 205

Ala Glu Glu Tyr Lys Glu Gln Leu Val Lys Ala Val Glu Glu Ala Arg
210 215 220

Glu Lys Ile Ala Pro His Thr Gln Asp Leu Gln Thr Arg Met Glu Pro
225 230 235 240

Tyr Met Glu Asn Val Arg Thr Thr Phe Ala Gln Met Tyr Glu Thr Ile
245 250 255

Ala Lys Ala Ile Gln Ala
260

<210> 46
<211> 260
<212> PRT
<213> 智人 (Homo sapiens)

<400> 46

Met Lys Phe Ala Ala Leu Ala Leu Ala Leu Leu Leu Ala Val Gly Ser
1 5 10 15

His Ala Ala Ser Met Gln Ala Asp Ala Pro Ser Gln Leu Asp His Ala
20 25 30

Arg Ala Val Leu Asp Val Tyr Leu Thr Gln Val Lys Asp Met Ser Leu
35 40 45

Arg Ala Val Asn Gln Leu Asp Asp Pro Gln Tyr Ala Glu Phe Lys Thr
50 55 60

Asn Leu Ala Gln Arg Ile Glu Glu Met Tyr Thr Gln Ile Lys Thr Leu
65 70 75 80

Gln Gly Ser Val Ser Pro Met Thr Asp Ser Phe Tyr Asn Thr Val Met
85 90 95

Glu Val Thr Lys Asp Thr Arg Glu Ser Leu Asn Val Asp Leu Glu Ala
100 105 110

Leu Lys Ser Ser Leu Ala Pro Gln Asn Glu Gln Leu Lys Gln Val Ile
115 120 125

Glu Lys His Leu Asn Asp Tyr Arg Thr Leu Leu Thr Pro Ile Tyr Asn
130 135 140

[0044]

```
Asp Tyr Lys Thr Lys His Asp Glu Glu Met Ala Ala Leu Lys Thr Arg
145                 150                 155                 160

Leu Glu Pro Val Met Glu Glu Leu Arg Thr Lys Ile Gln Ala Asn Val
                165                 170                 175

Glu Glu Thr Lys Ala Val Leu Met Pro Met Val Glu Thr Val Arg Thr
            180                 185                 190

Lys Val Thr Glu Arg Leu Glu Ser Leu Arg Glu Val Val Gln Pro Tyr
        195                 200                 205

Val Gln Glu Tyr Lys Glu Gln Met Lys Gln Met Tyr Asp Gln Ala Gln
    210                 215                 220

Thr Val Asp Thr Asp Ala Leu Arg Thr Lys Ile Thr Pro Leu Val Glu
225                 230                 235                 240

Glu Ile Lys Val Lys Met Asn Ala Ile Phe Glu Ile Ile Ala Ala Ser
                245                 250                 255

Val Thr Lys Ser
            260
```

<210> 47
<211> 396
<212> PRT
<213> 智人 (Homo sapiens)

<400> 47

```
Met Phe Leu Lys Ala Val Val Leu Thr Leu Ala Leu Val Ala Val Ala
1               5                   10                  15

Gly Ala Arg Ala Glu Val Ser Ala Asp Gln Val Ala Thr Val Met Trp
            20                  25                  30

Asp Tyr Phe Ser Gln Leu Ser Asn Asn Ala Lys Glu Ala Val Glu His
        35                  40                  45

Leu Gln Lys Ser Glu Leu Thr Gln Gln Leu Asn Ala Leu Phe Gln Asp
    50                  55                  60

Lys Leu Gly Glu Val Asn Thr Tyr Ala Gly Asp Leu Gln Lys Lys Leu
65                  70                  75                  80

Val Pro Phe Ala Thr Glu Leu His Glu Arg Leu Ala Lys Asp Ser Glu
                85                  90                  95

Lys Leu Lys Glu Glu Ile Gly Lys Glu Leu Glu Glu Leu Arg Ala Arg
            100                 105                 110

Leu Leu Pro His Ala Asn Glu Val Ser Gln Lys Ile Gly Asp Asn Leu
        115                 120                 125

Arg Glu Leu Gln Gln Arg Leu Glu Pro Tyr Ala Asp Gln Leu Arg Thr
    130                 135                 140
```

[0045]

```
Gln Val Asn Thr Gln Ala Glu Gln Leu Arg Arg Gln Leu Thr Pro Tyr
145                 150                 155                 160

Ala Gln Arg Met Glu Arg Val Leu Arg Glu Asn Ala Asp Ser Leu Gln
                165                 170                 175

Ala Ser Leu Arg Pro His Ala Asp Glu Leu Lys Ala Lys Ile Asp Gln
            180                 185                 190

Asn Val Glu Glu Leu Lys Gly Arg Leu Thr Pro Tyr Ala Asp Glu Phe
        195                 200                 205

Lys Val Lys Ile Asp Gln Thr Val Glu Glu Leu Arg Arg Ser Leu Ala
    210                 215                 220

Pro Tyr Ala Gln Asp Thr Gln Glu Lys Leu Asn His Gln Leu Glu Gly
225                 230                 235                 240

Leu Thr Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Lys Ala Arg Ile
                245                 250                 255

Ser Ala Ser Ala Glu Glu Leu Arg Gln Arg Leu Ala Pro Leu Ala Glu
            260                 265                 270

Asp Val Arg Gly Asn Leu Lys Gly Asn Thr Glu Gly Leu Gln Lys Ser
        275                 280                 285

Leu Ala Glu Leu Gly Gly His Leu Asp Gln Gln Val Glu Glu Phe Arg
    290                 295                 300

Arg Arg Val Glu Pro Tyr Gly Glu Asn Phe Asn Lys Ala Leu Val Gln
305                 310                 315                 320

Gln Met Glu Gln Leu Arg Gln Lys Leu Gly Pro His Ala Gly Asp Val
                325                 330                 335

Glu Gly His Leu Ser Phe Leu Glu Lys Asp Leu Arg Asp Lys Val Asn
            340                 345                 350

Ser Phe Phe Ser Thr Phe Lys Glu Lys Glu Ser Gln Asp Lys Thr Leu
        355                 360                 365

Ser Leu Pro Glu Leu Glu Gln Gln Gln Glu Gln Gln Gln Glu Gln Gln
    370                 375                 380

Gln Glu Gln Val Gln Met Leu Ala Pro Leu Glu Ser
385                 390                 395


<210>  48
<211>  429
<212>  PRT
<213>  智人 (Homo sapiens)

<400>  48

Met Phe Leu Lys Ala Val Val Leu Thr Leu Ala Leu Val Ala Val Thr
1               5                   10                  15

Gly Ala Arg Ala Glu Val Ser Ala Asp Gln Val Ala Thr Val Met Trp
            20                  25                  30
```

[0046]

Asp Tyr Phe Ser Gln Leu Ser Ser Asn Ala Lys Glu Ala Val Glu His
35 40 45

Leu Gln Lys Ser Glu Leu Thr Gln Gln Leu Asn Ala Leu Phe Gln Asp
50 55 60

Lys Leu Gly Glu Val Asn Thr Tyr Ala Gly Asp Leu Gln Lys Lys Leu
65 70 75 80

Val Pro Phe Ala Thr Glu Leu His Glu Arg Leu Ala Lys Asp Ser Glu
85 90 95

Lys Leu Lys Glu Glu Ile Arg Lys Glu Leu Glu Glu Val Arg Ala Arg
100 105 110

Leu Leu Pro His Ala Asn Glu Val Ser Gln Lys Ile Gly Glu Asn Val
115 120 125

Arg Glu Leu Gln Gln Arg Leu Glu Pro Tyr Thr Asp Gln Leu Arg Thr
130 135 140

Gln Val Asn Thr Gln Thr Glu Gln Leu Arg Arg Gln Leu Thr Pro Tyr
145 150 155 160

Ala Gln Arg Met Glu Arg Val Leu Arg Glu Asn Ala Asp Ser Leu Gln
165 170 175

Thr Ser Leu Arg Pro His Ala Asp Gln Leu Lys Ala Lys Ile Asp Gln
180 185 190

Asn Val Glu Glu Leu Lys Glu Arg Leu Thr Pro Tyr Ala Asp Glu Phe
195 200 205

Lys Val Lys Ile Asp Gln Thr Val Glu Glu Leu Arg Arg Ser Leu Ala
210 215 220

Pro Tyr Ala Gln Asp Ala Gln Glu Lys Leu Asn His Gln Leu Glu Gly
225 230 235 240

Leu Ala Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Lys Ala Arg Ile
245 250 255

Ser Ala Ser Ala Glu Glu Leu Arg Gln Arg Leu Ala Pro Leu Ala Glu
260 265 270

Asp Met Arg Gly Asn Leu Arg Gly Asn Thr Glu Gly Leu Gln Lys Ser
275 280 285

Leu Ala Glu Leu Gly Gly His Leu Asp Arg His Val Glu Glu Phe Arg
290 295 300

Leu Arg Val Glu Pro Tyr Gly Glu Asn Phe Asn Lys Ala Leu Val Gln
305 310 315 320

Gln Met Glu Gln Leu Arg Gln Lys Leu Gly Pro His Ala Gly Asp Val
325 330 335

[0047]

Glu Gly His Leu Ser Phe Leu Glu Lys Asp Leu Arg Asp Lys Val Asn
340 345 350

Ser Phe Phe Ser Thr Phe Lys Glu Lys Glu Ser Gln Asp Asn Thr Leu
355 360 365

Ser Leu Pro Glu Pro Glu Gln Gln Arg Glu Gln Gln Gln Glu Gln Gln
370 375 380

Gln Glu Gln Glu Gln Glu Gln Gln Gln Gln Gln Glu Gln Gln Gln Gln
385 390 395 400

Gln Glu Gln Gln Arg Glu Gln Gln Gln Gln Glu Gln Gln Gln Glu Gln
405 410 415

Gln Gln Glu Gln Val Gln Met Leu Ala Pro Leu Glu Ser
420 425

<210> 49
<211> 395
<212> PRT
<213> 智人（Homo sapiens）

<400> 49

Met Phe Leu Lys Ala Ala Val Leu Thr Leu Ala Leu Val Ala Ile Thr
1 5 10 15

Gly Thr Arg Ala Glu Val Thr Ser Asp Gln Val Ala Asn Val Val Trp
20 25 30

Asp Tyr Phe Thr Gln Leu Ser Asn Asn Ala Lys Glu Ala Val Glu Gln
35 40 45

Phe Gln Lys Thr Asp Val Thr Gln Gln Leu Ser Thr Leu Phe Gln Asp
50 55 60

Lys Leu Gly Asp Ala Ser Thr Tyr Ala Asp Gly Val His Asn Lys Leu
65 70 75 80

Val Pro Phe Val Val Gln Leu Ser Gly His Leu Ala Lys Glu Thr Glu
85 90 95

Arg Val Lys Glu Glu Ile Lys Lys Glu Leu Glu Asp Leu Arg Asp Arg
100 105 110

Met Met Pro His Ala Asn Lys Val Thr Gln Thr Phe Gly Glu Asn Met
115 120 125

Gln Lys Leu Gln Glu His Leu Lys Pro Tyr Ala Val Asp Leu Gln Asp
130 135 140

Gln Ile Asn Thr Gln Thr Gln Glu Met Lys Leu Gln Leu Thr Pro Tyr
145 150 155 160

Ile Gln Arg Met Gln Thr Thr Ile Lys Glu Asn Val Asp Asn Leu His
165 170 175

Thr Ser Met Met Pro Leu Ala Thr Asn Leu Lys Asp Lys Phe Asn Arg

[0048]

180 185 190

Asn Met Glu Glu Leu Lys Gly His Leu Thr Pro Arg Ala Asn Glu Leu
195 200 205

Lys Ala Thr Ile Asp Gln Asn Leu Glu Asp Leu Arg Arg Ser Leu Ala
210 215 220

Pro Leu Thr Val Gly Val Gln Glu Lys Leu Asn His Gln Met Glu Gly
225 230 235 240

Leu Ala Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Gln Thr Lys Val
245 250 255

Ser Ala Lys Ile Asp Gln Leu Gln Lys Asn Leu Ala Pro Leu Val Glu
260 265 270

Asp Val Gln Ser Lys Val Lys Gly Asn Thr Glu Gly Leu Gln Lys Ser
275 280 285

Leu Glu Asp Leu Asn Arg Gln Leu Glu Gln Gln Val Glu Glu Phe Arg
290 295 300

Arg Thr Val Glu Pro Met Gly Glu Met Phe Asn Lys Ala Leu Val Gln
305 310 315 320

Gln Leu Glu Gln Phe Arg Gln Gln Leu Gly Pro Asn Ser Gly Glu Val
325 330 335

Glu Ser His Leu Ser Phe Leu Glu Lys Ser Leu Arg Glu Lys Val Asn
340 345 350

Ser Phe Met Ser Thr Leu Glu Lys Lys Gly Ser Pro Asp Gln Pro Gln
355 360 365

Ala Leu Pro Leu Pro Glu Gln Ala Gln Glu Gln Ala Gln Glu Gln Ala
370 375 380

Gln Glu Gln Val Gln Pro Lys Pro Leu Glu Ser
385 390 395

<210> 50
<211> 401
<212> PRT
<213> 智人 (Homo sapiens)

<400> 50

Gly Ala Arg Ala Glu Val Ser Ala Asp Gln Val Ala Thr Val Met Trp
1 5 10 15

Asp Tyr Phe Ser Gln Leu Ser Ser Asn Ala Lys Glu Ala Val Glu His
20 25 30

Leu Gln Lys Ser Glu Leu Thr Gln Gln Leu Asn Ala Leu Phe Gln Asp
35 40 45

Lys Leu Gly Glu Val Asn Thr Tyr Ala Gly Asp Leu Gln Lys Lys Leu
50 55 60

[0049]

Val Pro Phe Ala Thr Glu Leu His Glu Arg Leu Ala Lys Asp Ser Lys
65 70 75 80

Lys Leu Lys Glu Glu Ile Arg Lys Glu Leu Glu Glu Val Arg Ala Arg
85 90 95

Leu Leu Pro His Ala Asn Glu Val Ser Gln Lys Ile Gly Glu Asn Val
100 105 110

Arg Glu Leu Gln Gln Arg Leu Glu Pro Tyr Thr Asp Gln Leu Arg Thr
115 120 125

Gln Val Asn Thr Gln Thr Glu Gln Leu Arg Arg Gln Leu Thr Pro Tyr
130 135 140

Ala Gln Arg Met Glu Arg Val Leu Arg Glu Asn Ala Asp Ser Leu Gln
145 150 155 160

Thr Ser Leu Arg Pro His Ala Asp Gln Leu Lys Ala Lys Ile Asp Gln
165 170 175

Asn Val Glu Glu Leu Lys Gly Arg Leu Thr Pro Tyr Ala Asp Glu Phe
180 185 190

Lys Val Lys Ile Asp Gln Thr Val Glu Glu Leu Arg Arg Ser Leu Ala
195 200 205

Pro Tyr Ala Gln Asp Ala Gln Glu Lys Leu Asn His Gln Leu Glu Gly
210 215 220

Leu Ala Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Lys Ala Arg Ile
225 230 235 240

Ser Ala Ser Ala Glu Glu Leu Arg Gln Arg Leu Ala Pro Leu Ala Glu
245 250 255

Asp Met Arg Gly Asn Leu Arg Gly Asn Thr Glu Gly Leu Gln Lys Ser
260 265 270

Leu Ala Glu Leu Gly Gly His Leu Asp Arg His Val Glu Glu Phe Arg
275 280 285

Leu Arg Val Glu Pro Tyr Gly Glu Asn Phe Asn Lys Ala Leu Val Gln
290 295 300

Gln Met Glu Gln Leu Arg Gln Lys Leu Gly Pro His Ala Gly Asp Val
305 310 315 320

Glu Gly His Leu Ser Phe Leu Glu Lys Asp Leu Arg Asp Lys Val Asn
325 330 335

Ser Phe Phe Ser Thr Phe Lys Glu Lys Glu Ser Gln Asp Asn Thr Leu
340 345 350

Ser Leu Pro Glu Pro Glu Gln Gln Gln Glu Gln Gln Gln Glu Gln Glu
355 360 365

[0050]

Gln Gln Gln Glu Gln Gln Glu Glu Gln Gln Gln Gln Glu Gln Gln Gln
370 375 380

Glu Gln Glu Gln Gln Gln Glu Gln Val Gln Met Leu Ala Pro Leu Glu
385 390 395 400

Ser

<210> 51
<211> 382
<212> PRT
<213> 智人 (Homo sapiens)

<400> 51

Met Phe Leu Lys Ala Val Val Leu Ser Leu Ala Leu Val Ala Val Thr
1 5 10 15

Gly Ala Arg Ala Glu Val Asn Ala Asp Gln Val Ala Thr Val Met Trp
20 25 30

Asp Tyr Phe Ser Gln Leu Gly Ser Asn Ala Lys Lys Ala Val Glu His
35 40 45

Leu Gln Lys Ser Glu Leu Thr Gln Gln Leu Asn Thr Leu Phe Gln Asp
50 55 60

Lys Leu Gly Glu Val Asn Thr Tyr Thr Glu Asp Leu Gln Lys Lys Leu
65 70 75 80

Val Pro Phe Ala Thr Glu Leu His Glu Arg Leu Thr Lys Asp Ser Glu
85 90 95

Lys Leu Lys Glu Glu Ile Arg Arg Glu Leu Glu Glu Leu Arg Ala Arg
100 105 110

Leu Leu Pro His Ala Thr Glu Val Ser Gln Lys Ile Gly Asp Asn Val
115 120 125

Arg Glu Leu Gln Gln Arg Leu Gly Pro Phe Thr Gly Gly Leu Arg Thr
130 135 140

Gln Val Asn Thr Gln Val Gln Gln Leu Gln Arg Gln Leu Lys Pro Tyr
145 150 155 160

Ala Glu Arg Met Glu Ser Val Leu Arg Gln Asn Ile Arg Asn Leu Glu
165 170 175

Ala Ser Val Ala Pro Tyr Ala Asp Glu Phe Lys Ala Lys Ile Asp Gln
180 185 190

Asn Val Glu Glu Leu Lys Gly Ser Leu Thr Pro Tyr Ala Glu Glu Leu
195 200 205

Lys Ala Lys Ile Asp Gln Asn Val Glu Glu Leu Arg Arg Ser Leu Ala
210 215 220

Pro Tyr Ala Gln Asp Val Gln Glu Lys Leu Asn His Gln Leu Glu Gly
225 230 235 240

[0051]

Leu Ala Phe Gln Met Lys Lys Gln Ala Glu Glu Leu Lys Ala Lys Ile
245 250 255

Ser Ala Asn Ala Asp Glu Leu Arg Gln Lys Leu Val Pro Val Ala Glu
260 265 270

Asn Val His Gly His Leu Lys Gly Asn Thr Glu Gly Leu Gln Lys Ser
275 280 285

Leu Leu Glu Leu Arg Ser His Leu Asp Gln Gln Val Glu Glu Phe Arg
290 295 300

Leu Lys Val Glu Pro Tyr Gly Glu Thr Phe Asn Lys Ala Leu Val Gln
305 310 315 320

Gln Val Glu Asp Leu Arg Gln Lys Leu Gly Pro Leu Ala Gly Asp Val
325 330 335

Glu Gly His Leu Ser Phe Leu Glu Lys Asp Leu Arg Asp Lys Val Asn
340 345 350

Thr Phe Phe Ser Thr Leu Lys Glu Glu Ala Ser Gln Gly Gln Ser Gln
355 360 365

Ala Leu Pro Ala Gln Glu Lys Ala Gln Ala Pro Leu Glu Gly
370 375 380

<210> 52
<211> 391
<212> PRT
<213> 智人（Homo sapiens）

<400> 52

Met Phe Leu Lys Ala Val Val Leu Thr Val Ala Leu Val Ala Ile Thr
1 5 10 15

Gly Thr Gln Ala Glu Val Thr Ser Asp Gln Val Ala Asn Val Met Trp
20 25 30

Asp Tyr Phe Thr Gln Leu Ser Asn Asn Ala Lys Glu Ala Val Glu Gln
35 40 45

Leu Gln Lys Thr Asp Val Thr Gln Gln Leu Asn Thr Leu Phe Gln Asp
50 55 60

Lys Leu Gly Asn Ile Asn Thr Tyr Ala Asp Asp Leu Gln Asn Lys Leu
65 70 75 80

Val Pro Phe Ala Val Gln Leu Ser Gly His Leu Thr Lys Glu Thr Glu
85 90 95

Arg Val Arg Glu Glu Ile Gln Lys Glu Leu Glu Asp Leu Arg Ala Asn
100 105 110

Met Met Pro His Ala Asn Lys Val Ser Gln Met Phe Gly Asp Asn Val
115 120 125

[0052]

```
Gln Lys Leu Gln Glu His Leu Arg Pro Tyr Ala Thr Asp Leu Gln Ala
    130                 135                 140

Gln Ile Asn Ala Gln Thr Gln Asp Met Lys Arg Gln Leu Thr Pro Tyr
145                 150                 155                 160

Ile Gln Arg Met Gln Thr Thr Ile Gln Asp Asn Val Glu Asn Leu Gln
                165                 170                 175

Ser Ser Met Val Pro Phe Ala Asn Glu Leu Lys Glu Lys Phe Asn Gln
            180                 185                 190

Asn Met Glu Gly Leu Lys Gly Gln Leu Thr Pro Arg Ala Asn Glu Leu
        195                 200                 205

Lys Ala Thr Ile Asp Gln Asn Leu Glu Asp Leu Arg Ser Arg Leu Ala
    210                 215                 220

Pro Leu Ala Glu Gly Val Gln Glu Lys Leu Asn His Gln Met Glu Gly
225                 230                 235                 240

Leu Ala Phe Gln Met Lys Lys Asn Ala Glu Glu Leu Gln Thr Lys Val
                245                 250                 255

Ser Thr Asn Ile Asp Gln Leu Gln Lys Asn Leu Ala Pro Leu Val Glu
            260                 265                 270

Asp Val Gln Ser Lys Leu Lys Gly Asn Thr Glu Gly Leu Gln Lys Ser
        275                 280                 285

Leu Glu Asp Leu Asn Lys Gln Leu Asp Gln Gln Val Glu Val Phe Arg
    290                 295                 300

Arg Ala Val Glu Pro Leu Gly Asp Lys Phe Asn Met Ala Leu Val Gln
305                 310                 315                 320

Gln Met Glu Lys Phe Arg Gln Gln Leu Gly Ser Asp Ser Gly Asp Val
                325                 330                 335

Glu Ser His Leu Ser Phe Leu Glu Lys Asn Leu Arg Glu Lys Val Ser
            340                 345                 350

Ser Phe Met Ser Thr Leu Gln Lys Lys Gly Ser Pro Asp Gln Pro Leu
        355                 360                 365

Ala Leu Pro Leu Pro Glu Gln Val Gln Glu Gln Val Gln Glu Gln Val
    370                 375                 380

Gln Pro Lys Pro Leu Glu Ser
385                 390
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<210> 53
<211> 51
<212> PRT
<213> 智人 (Homo sapiens)

<300>
<302> 三聚化组件
<310> WO 98/56906
<311> 1998-06-11

[0053]

<312> 1998-12-17
<313> (1)..(51)

<400> 53

Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
1 5 10 15

Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
20 25 30

Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
35 40 45

Val Cys Leu
50

<210> 54
<211> 40
<212> PRT
<213> 智人 (Homo sapiens)

<400> 54

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
1 5 10 15

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
20 25 30

Glu Gln Gln Ala Leu Gln Thr Val
35 40

<210> 55
<211> 9
<212> PRT
<213> 智人 (Homo sapiens)

<400> 55

Cys Asp Leu Pro Gln Thr His Ser Leu
1 5

<210> 56
<211> 6
<212> PRT
<213> 人工序列

<220>
<223> 六组氨酸标记物

<400> 56

His His His His His His
1 5

<210> 57
<211> 310
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 57

Met Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser His His His His
1 5 10 15

[0054]

```
His His Gly Ser Val Val Ala Pro Pro Ala Pro Ile Val Asn Ala Lys
            20                  25                  30

Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu
        35                  40                  45

Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu
    50                  55                  60

Gln Thr Val Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp
65                  70                  75                  80

Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr
                85                  90                  95

Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys
            100                 105                 110

Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg
        115                 120                 125

Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys
    130                 135                 140

Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val
145                 150                 155                 160

Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln
                165                 170                 175

Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu
            180                 185                 190

Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu
        195                 200                 205

Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp
    210                 215                 220

Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg
225                 230                 235                 240

Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu
                245                 250                 255

Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu
            260                 265                 270

Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val
        275                 280                 285

Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr
    290                 295                 300

Lys Lys Leu Asn Thr Gln
305                 310
```

[0055]

<210> 58
<211> 35
<212> DNA
<213> 人工序列

<220>
<223> 引物N1

<400> 58
aaaaaagcgg ccgcgacaat tcgcgcgcga aggcg 35

<210> 59
<211> 36
<212> DNA
<213> 人工序列

<220>
<223> 引物N2

<400> 59
aaaaaagcgg ccgctcactg cccgctttcc agtcgg 36

<210> 60
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 60

Val Val Ala Pro Pro Ala Pro
1 5

<210> 61
<211> 5
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 61

Pro Ala Pro Ser Pro
1 5

<210> 62
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 62

Pro Pro Ser Pro
1

<210> 63
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 63

[0056]

Pro Pro Ala Pro
1

<210> 64
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 64

Pro Pro Thr Pro
1

<210> 65
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> IgA蛋白酶切割位点

<400> 65

Pro Pro Gly Pro
1

<210> 66
<211> 285
<212> PRT
<213> 人工序列

<220>
<223> 四连蛋白 载脂蛋白A I

<220>
<221> MISC_FEATURE
<223> X = A或G或S或T

<220>
<221> misc_feature
<222> (1)..(1)
<223> Xaa可以是任何天然存在的氨基酸

<400> 66

Xaa Pro Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe
1 5 10 15

Glu Glu Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu
20 25 30

Leu Lys Glu Gln Gln Ala Leu Gln Thr Val Asp Glu Pro Pro Gln Ser
35 40 45

Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu
50 55 60

Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu
65 70 75 80

Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr
85 90 95

Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu

[0057]

100 105 110

Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met
115 120 125

Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp
130 135 140

Asp Phe Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys
145 150 155 160

Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu
165 170 175

His Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp
180 185 190

Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr
195 200 205

Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys
210 215 220

Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu
225 230 235 240

His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu
245 250 255

Arg Gln Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu
260 265 270

Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
275 280 285

<210> 67
<211> 291
<212> PRT
<213> 人工序列

<220>
<223> 具有N-端His-标记物的四连蛋白-载脂蛋白A-I

<220>
<221> MISC_FEATURE
<222> (7)..(7)
<223> X =AP, GP, SP, PP, GSAP, GSGP, GSSP, GSPP, GGGS,
GGGGS, GGGSGGGS, GGGGSGGGGS, GGGSGGGSGGGS, GGGGSGGGGSGGGGS,
GGGSAP, GGGSGP, GGGSSP中的任何一个

<220>
<221> MISC_FEATURE
<222> (7)..(7)
<223> X =GGGSPP, GGGGSAP, GGGGSGP, GGGGSSP, GGGGSPP,
GGGSGGGSAP, GGGSGGGSGP, GGGSGGGSSP, GGGSGGGSPP, GGGSGGGSGGGSAP,
GGGSGGGSGGGSGP, GGGSGGGSGGGSSP, GGGSGGGSGGGSPP中的任何一个

<220>
<221> MISC_FEATURE
<222> (7)..(7)
<223> X =GGGGSAP, GGGGSGP, GGGGSSP, GGGGSPP, GGGGSGGGGSAP,
GGGGSGGGGSGP, GGGGSGGGGSSP, GGGGSGGGGSPP, GGGGSGGGGSGGGGSAP,
GGGGSGGGGSGGGGSGP, GGGGSGGGGSGGGGSSP和GGGGSGGGGSGGGGSPP中的任何一个。

[0058]

<220>
<221> misc_feature
<222> (8)..(8)
<223> Xaa可以是任何天然存在的氨基酸

<400> 67

Met His His His His His His Xaa Ile Val Asn Ala Lys Lys Asp Val
1 5 10 15

Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr Leu
20 25 30

Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr Val
35 40 45

Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala Thr
50 55 60

Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln
65 70 75 80

Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp
85 90 95

Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu
100 105 110

Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu
115 120 125

Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys
130 135 140

Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met
145 150 155 160

Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu
165 170 175

Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu
180 185 190

Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg
195 200 205

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala
210 215 220

Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr
225 230 235 240

His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys
245 250 255

Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser
260 265 270

Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu
275 280 285

[0059]

Asn Thr Gln
290

<210> 68
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 接头

<400> 68

Gly Ser Ala Pro
1

<210> 69
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 接头2

<400> 69

Gly Ser Gly Pro
1

<210> 70
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 接头3

<400> 70

Gly Ser Ser Pro
1

<210> 71
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 接头4

<400> 71

Gly Ser Pro Pro
1

<210> 72
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 接头5

<400> 72

Gly Gly Gly Ser
1

<210> 73

[0060]

<211> 5
<212> PRT
<213> 人工序列

<220>
<223> 接头6

<400> 73

Gly Gly Gly Gly Ser
1 5

<210> 74
<211> 8
<212> PRT
<213> 人工序列

<220>
<223> 接头7

<400> 74

Gly Gly Gly Ser Gly Gly Gly Ser
1 5

<210> 75
<211> 10
<212> PRT
<213> 人工序列

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<223> 接头8

<400> 75

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10

<210> 76
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 接头9

<400> 76

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser
1 5 10

<210> 77
<211> 15
<212> PRT
<213> 人工序列

<220>
<223> 接头10

<400> 77

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

<210> 78
<211> 6
<212> PRT
<213> 人工序列

<220>
<223> 接头11

[0061]

<400> 78

Gly Gly Gly Ser Ala Pro
1 5

<210> 79
<211> 6
<212> PRT
<213> 人工序列

<220>
<223> 接头12

<400> 79

Gly Gly Gly Ser Gly Pro
1 5

<210> 80
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<212> PRT
<213> 人工序列

<220>
<223> 接头13

<400> 80

Gly Gly Gly Ser Ser Pro
1 5

<210> 81
<211> 6
<212> PRT
<213> 人工序列

<220>
<223> 接头14

<400> 81

Gly Gly Gly Ser Pro Pro
1 5

<210> 82
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头15

<400> 82

Gly Gly Gly Gly Ser Ala Pro
1 5

<210> 83
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头16

<400> 83

Gly Gly Gly Gly Ser Gly Pro
1 5

<210> 84

[0062]

<210> 7
<211> PRT
<213> 人工序列

<220>
<223> 接头17

<400> 84

Gly Gly Gly Gly Ser Ser Pro
1 5

<210> 85
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头18

<400> 85

Gly Gly Gly Gly Ser Pro Pro
1 5

<210> 86
<211> 10
<212> PRT
<213> 人工序列

<220>
<223> 接头19

<400> 86

Gly Gly Gly Ser Gly Gly Gly Ser Ala Pro
1 5 10

<210> 87
<211> 10
<212> PRT
<213> 人工序列

<220>
<223> 接头20

<400> 87

Gly Gly Gly Ser Gly Gly Gly Ser Gly Pro
1 5 10

<210> 88
<211> 10
<212> PRT
<213> 人工序列

<220>
<223> 接头21

<400> 88

Gly Gly Gly Ser Gly Gly Gly Ser Ser Pro
1 5 10

<210> 89
<211> 10
<212> PRT
<213> 人工序列

<220>
<223> 接头22

[0063]

<400> 89

Gly Gly Gly Ser Gly Gly Gly Ser Pro Pro
1 5 10

<210> 90
<211> 14
<212> PRT
<213> 人工序列

<220>
<223> 接头23

<400> 90

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Ala Pro
1 5 10

<210> 91
<211> 14
<212> PRT
<213> 人工序列

<220>
<223> 接头24

<400> 91

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Pro
1 5 10

<210> 92
<211> 14
<212> PRT
<213> 人工序列

<220>
<223> 接头25

<400> 92

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Ser Pro
1 5 10

<210> 93
<211> 14
<212> PRT
<213> 人工序列

<220>
<223> 接头26

<400> 93

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Pro Pro
1 5 10

<210> 94
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头27

<400> 94

Gly Gly Gly Gly Ser Ala Pro
1 5

<210> 95

[0064]

<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头28

<400> 95

Gly Gly Gly Gly Ser Gly Pro
1 5

<210> 96
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头29

<400> 96

Gly Gly Gly Gly Ser Ser Pro
1 5

<210> 97
<211> 7
<212> PRT
<213> 人工序列

<220>
<223> 接头30

<400> 97

Gly Gly Gly Gly Ser Pro Pro
1 5

<210> 98
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 接头31

<400> 98

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala Pro
1 5 10

<210> 99
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 接头32

<400> 99

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Pro
1 5 10

<210> 100
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 接头33

[0065]

<400> 100

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Pro
1 5 10

<210> 101
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 接头34

<400> 101

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Pro Pro
1 5 10

<210> 102
<211> 17
<212> PRT
<213> 人工序列

<220>
<223> 接头35

<400> 102

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala
1 5 10 15

Pro

<210> 103
<211> 17
<212> PRT
<213> 人工序列

<220>
<223> 接头36

<400> 103

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Pro

<210> 104
<211> 17
<212> PRT
<213> 人工序列

<220>
<223> 接头37

<400> 104

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser
1 5 10 15

Pro

<210> 105
<211> 17
<212> PRT

[0066]

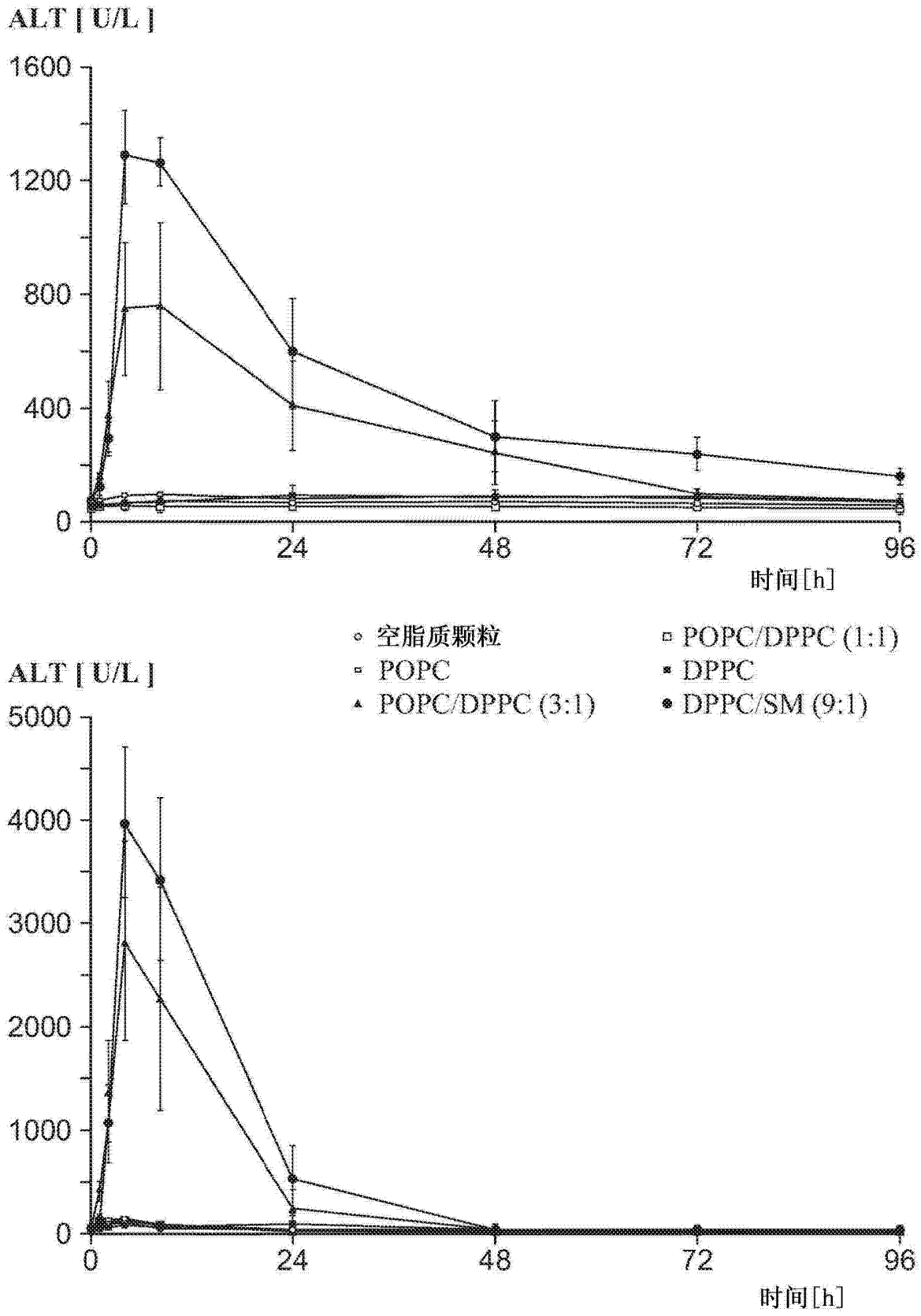
<213> 人工序列

<220>
<223> 接头38

<400> 105

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Pro
1 5 10 15

Pro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1200
800
400
0
0
24
48
72
96
时间[h]
空脂质颗粒
POPC
POPC/DPPC (3:1)
POPC/DPPC (1:1)
DPPC
DPPC/SM (9:1)
ALT [U/L]
5000
4000
3000
2000
1000
0
0
24
48
72
96
时间[h]

图 1

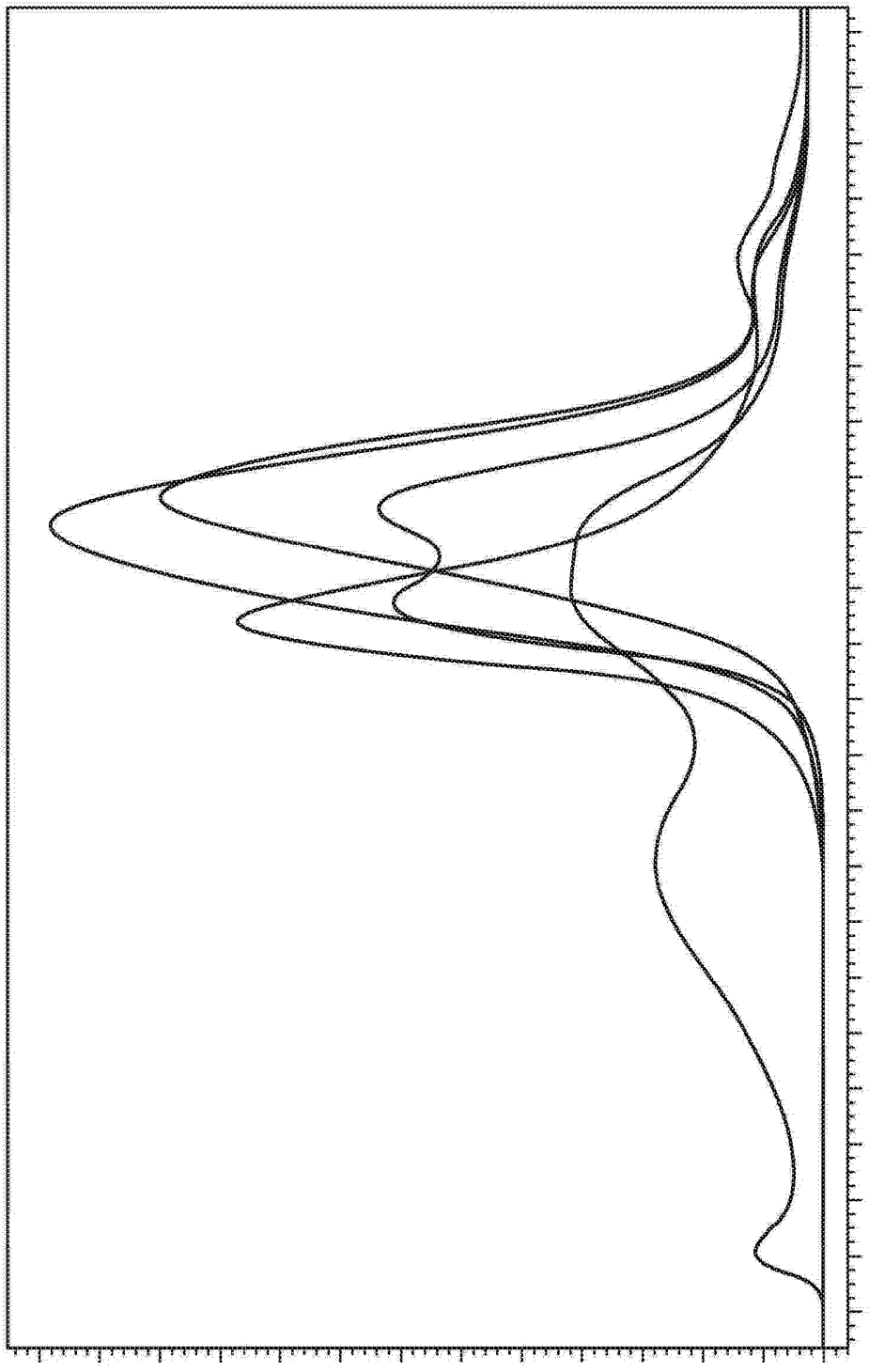


图 2

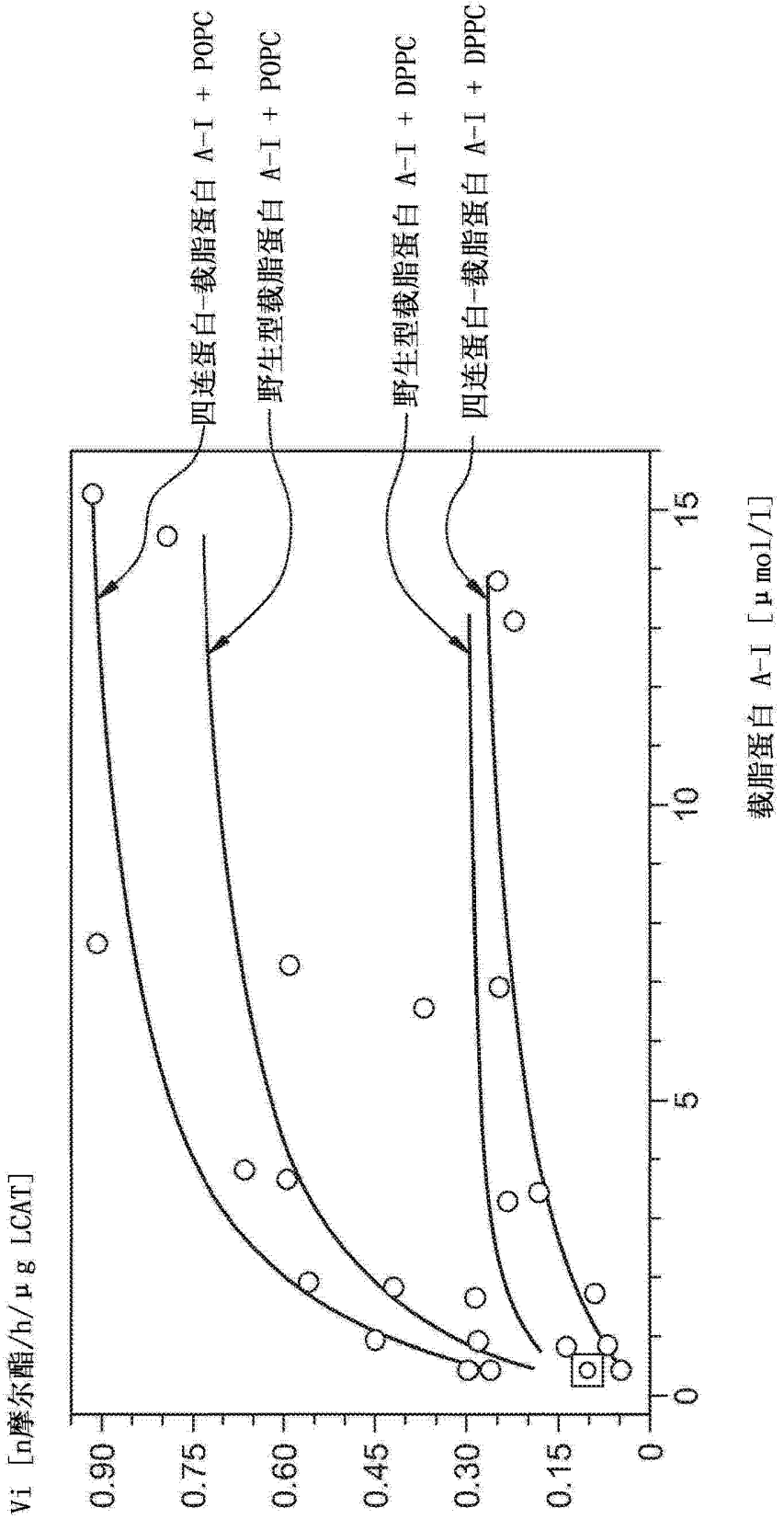

四连蛋白-载脂蛋白 A-I + POPC
野生型载脂蛋白 A-I + POPC
野生型载脂蛋白 A-I + DPPC
四连蛋白-载脂蛋白 A-I + DPPC
Vi [n摩尔酯/h/μg LCAT]
0.90
0.75
0.60
0.45
0.30
0.15
0
0
5
10
15
载脂蛋白 A-I [μmol/l]

图 3

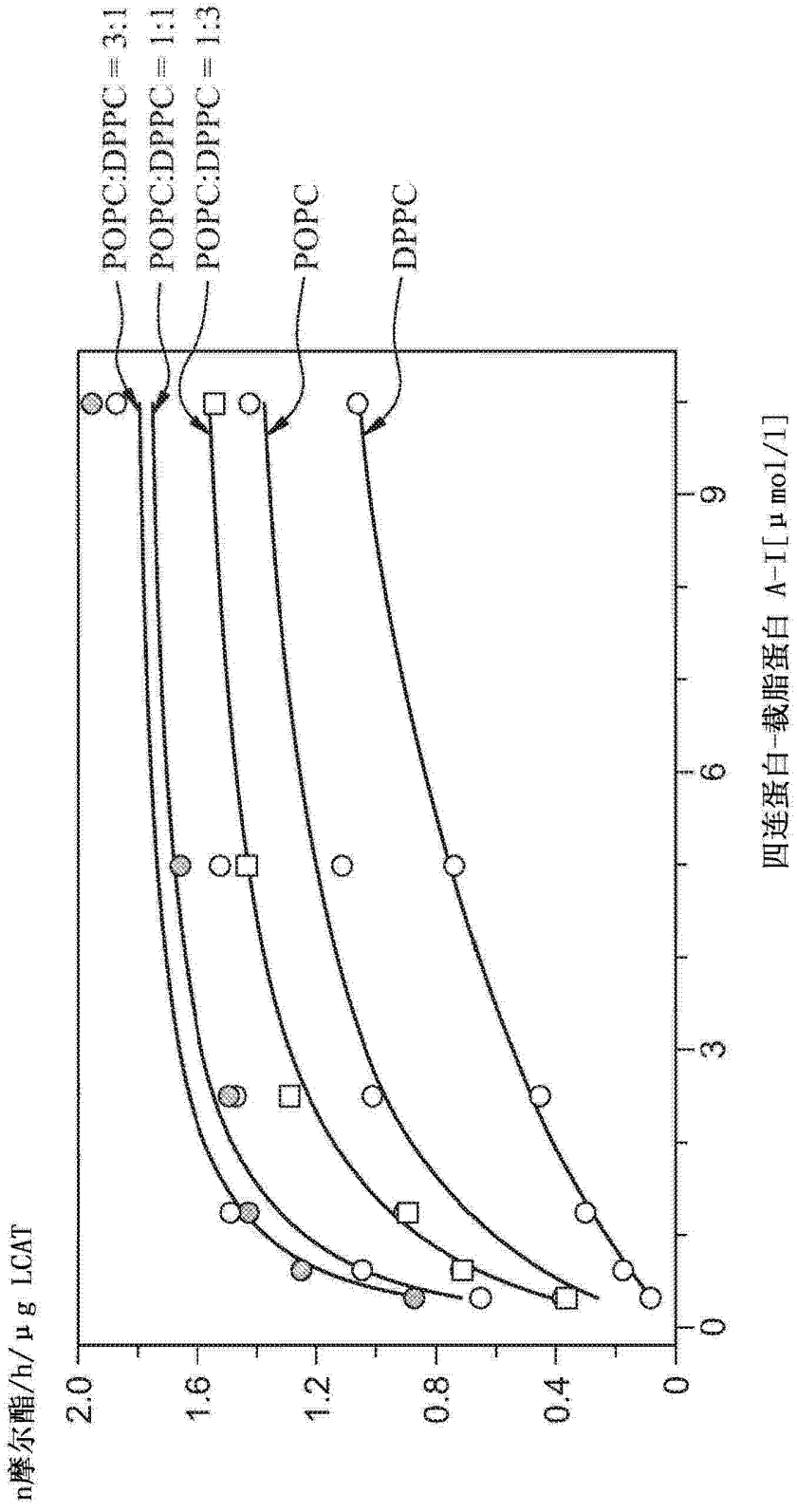

POPC:DPPC = 3:1
POPC:DPPC = 1:1
POPC:DPPC = 1:3
POPC
DPPC
n摩尔酯/h/μg LCAT
四连蛋白-载脂蛋白 A-I[μmol/l]
2.0
1.6
1.2
0.8
0.4
0
0
3
6
9

图 4

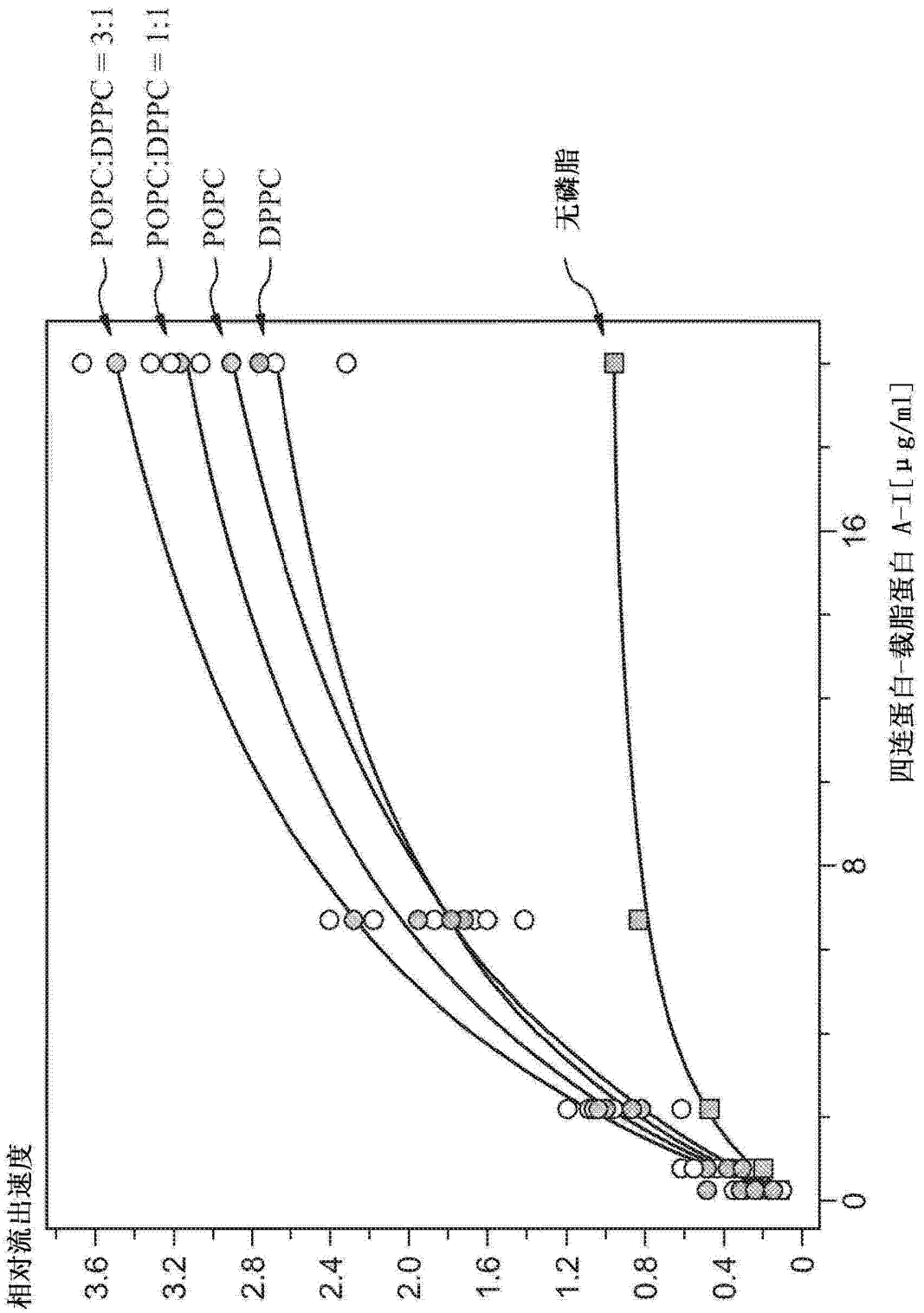

POPC:DPPC = 3:1
POPC:DPPC = 1:1
POPC
DPPC
无磷脂
相对流出速度
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
0
8
16
四连蛋白-载脂蛋白 A-I[μg/ml]

图 5

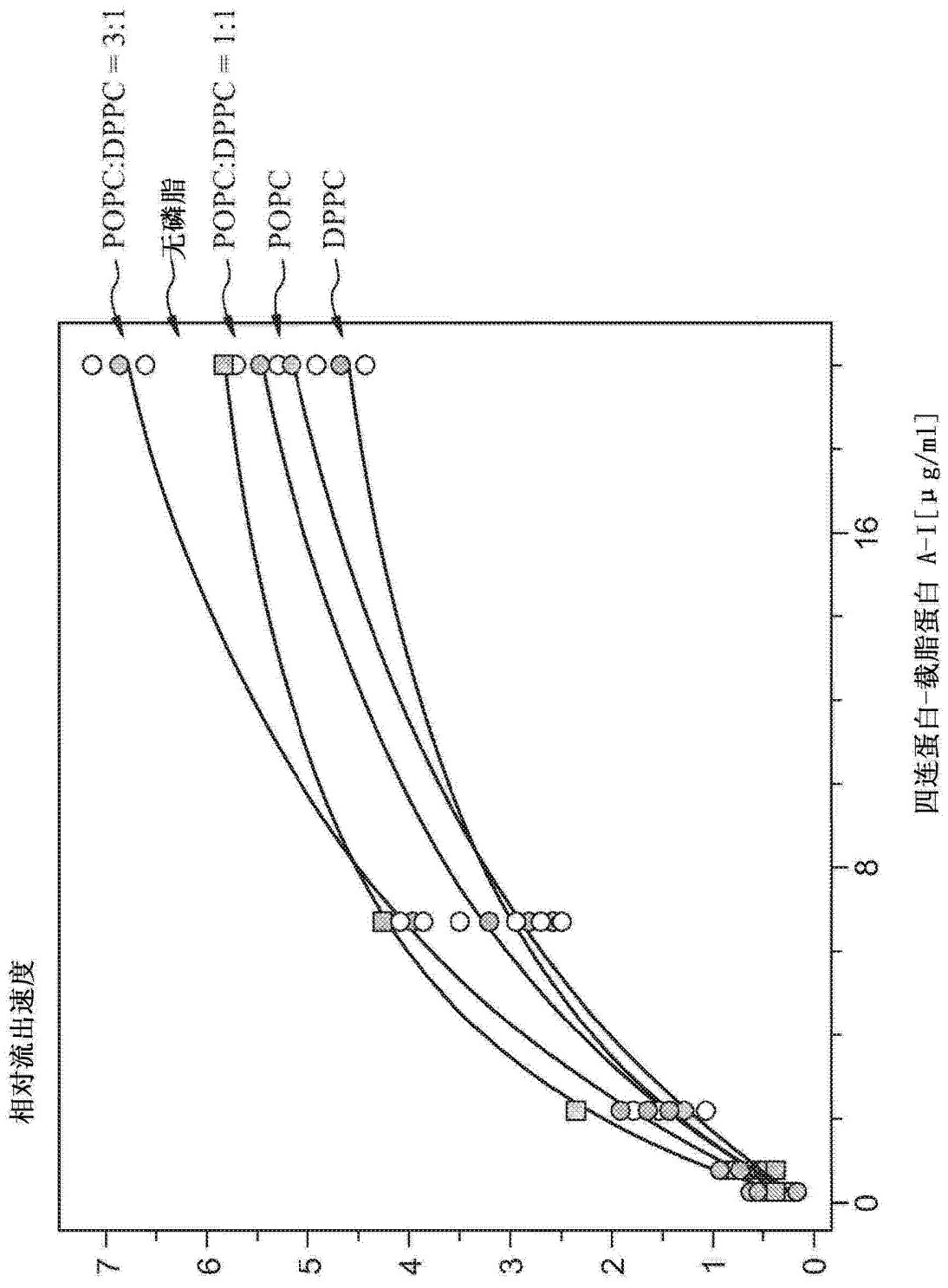

POPC:DPPC = 3:1
无磷脂
POPC:DPPC = 1:1
POPC
DPPC
相对流出速度
四连蛋白-载脂蛋白 A-I[μg/ml]
0
1
2
3
4
5
6
7
0
8
16

图 6

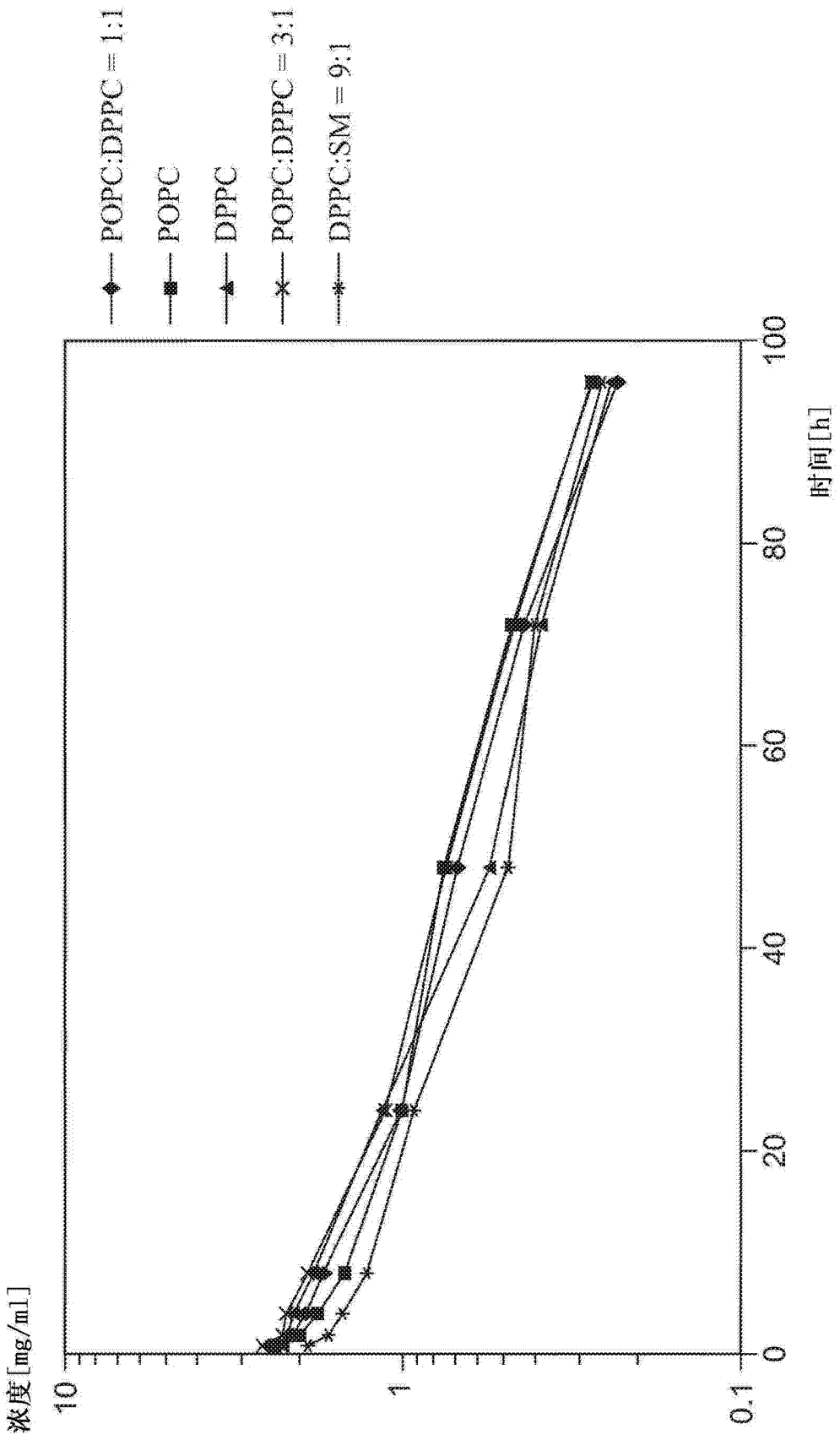

POPC:DPPC = 1:1
POPC
DPPC
POPC:DPPC = 3:1
DPPC:SM = 9:1
浓度[mg/ml]
10
1
0.1
0
20
40
60
80
100
时间[h]

图 7

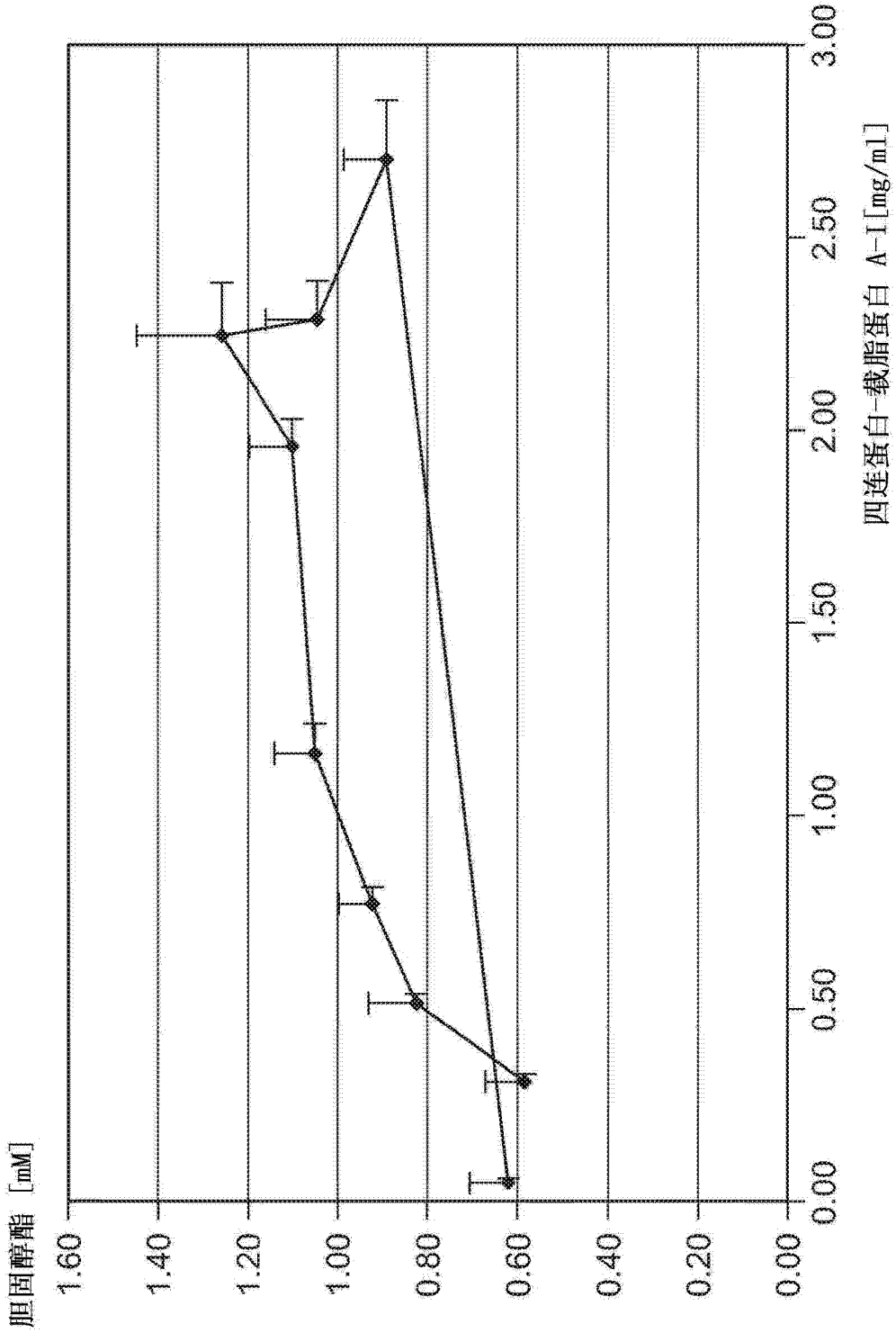
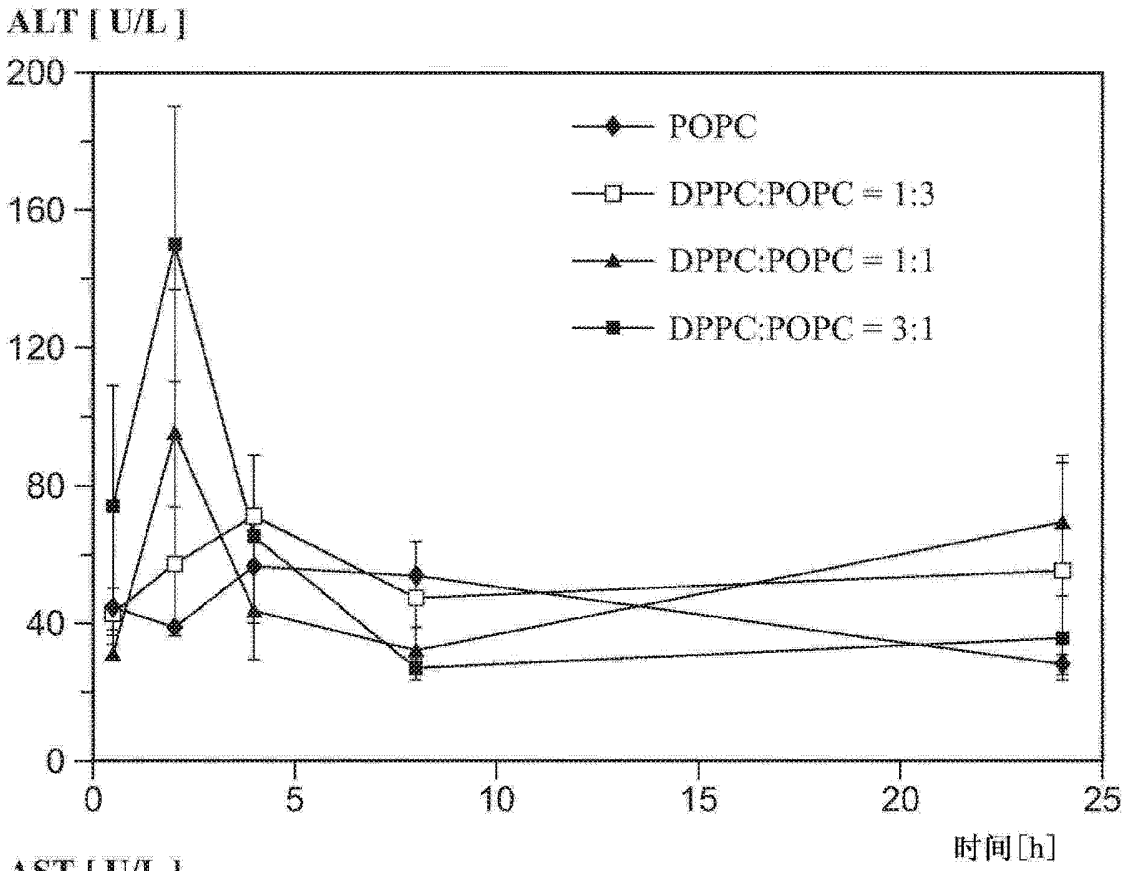

胆固醇酯 [mM]
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
四连蛋白-载脂蛋白 A-I[mg/ml]

图 8


ALT [U/L]
200
160
120
80
40
0
0
5
10
15
20
25
时间[h]
POPC
DPPC:POPC = 1:3
DPPC:POPC = 1:1
DPPC:POPC = 3:1

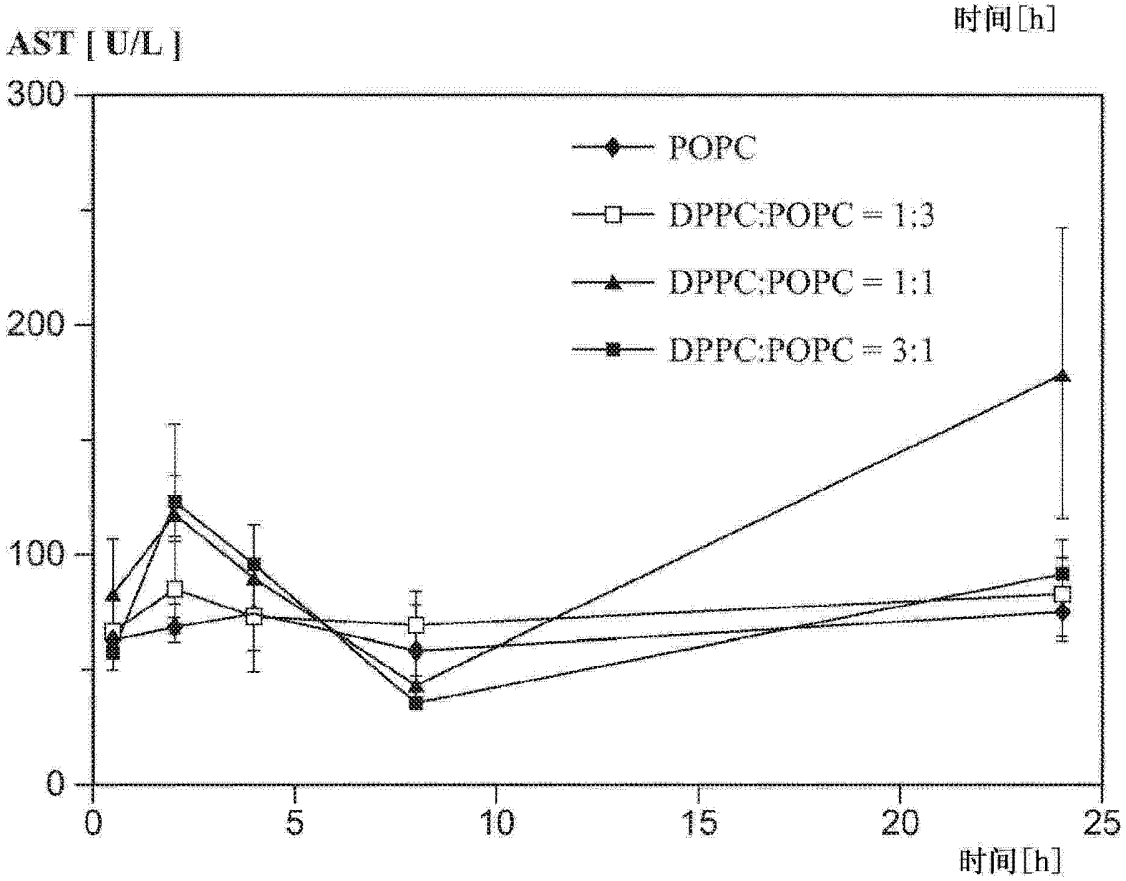

AST [U/L]
300
200
100
0
0
5
10
15
20
25
时间[h]
POPC
DPPC:POPC = 1:3
DPPC:POPC = 1:1
DPPC:POPC = 3:1

图 9

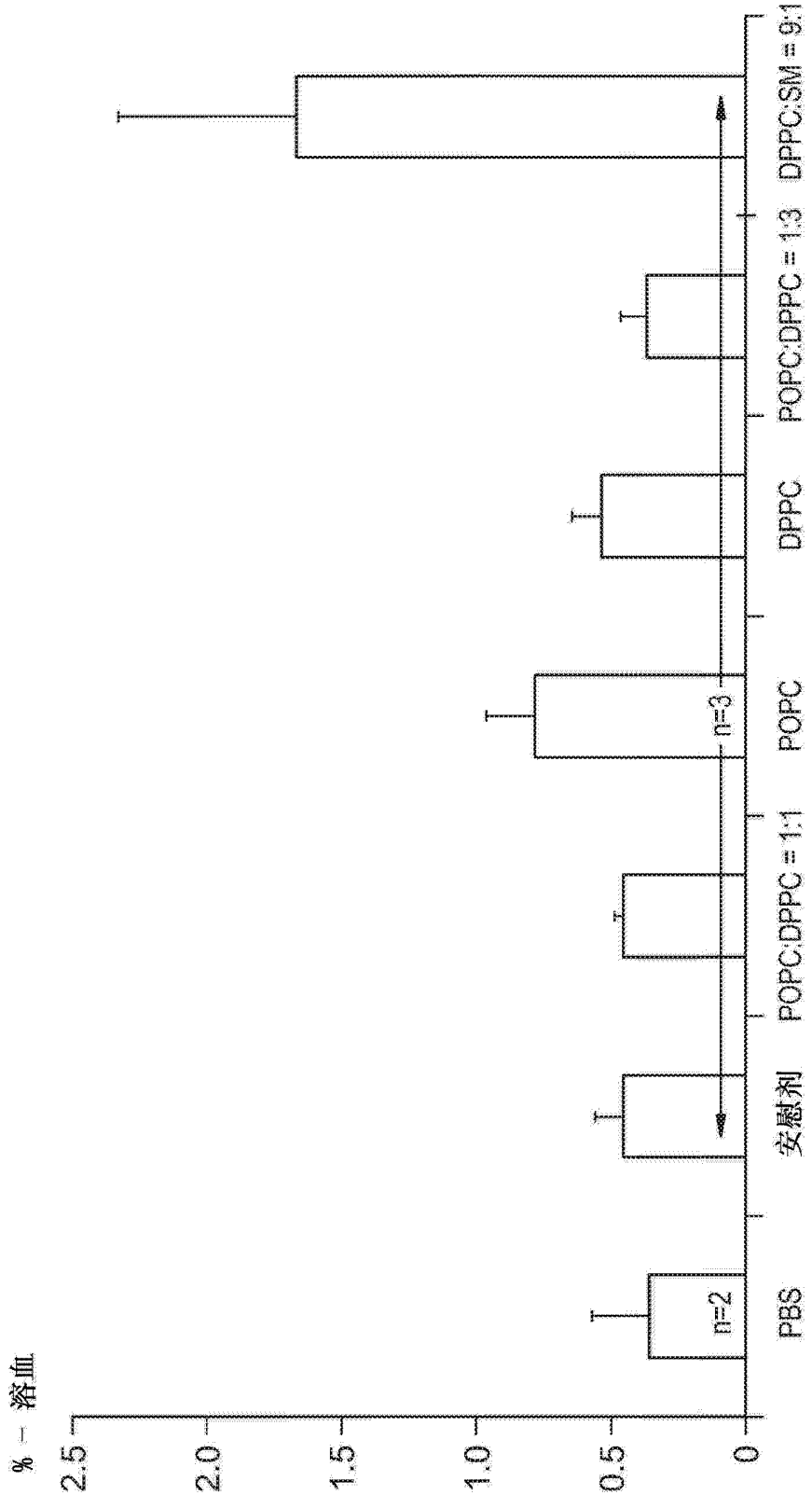

% - 溶血
2.5
2.0
1.5
1.0
0.5
0
n=2
n=3
PBS
安慰剂
POPC:DPPC = 1:1
POPC
DPPC
POPC:DPPC = 1:3
DPPC:SM = 9:1

图 10

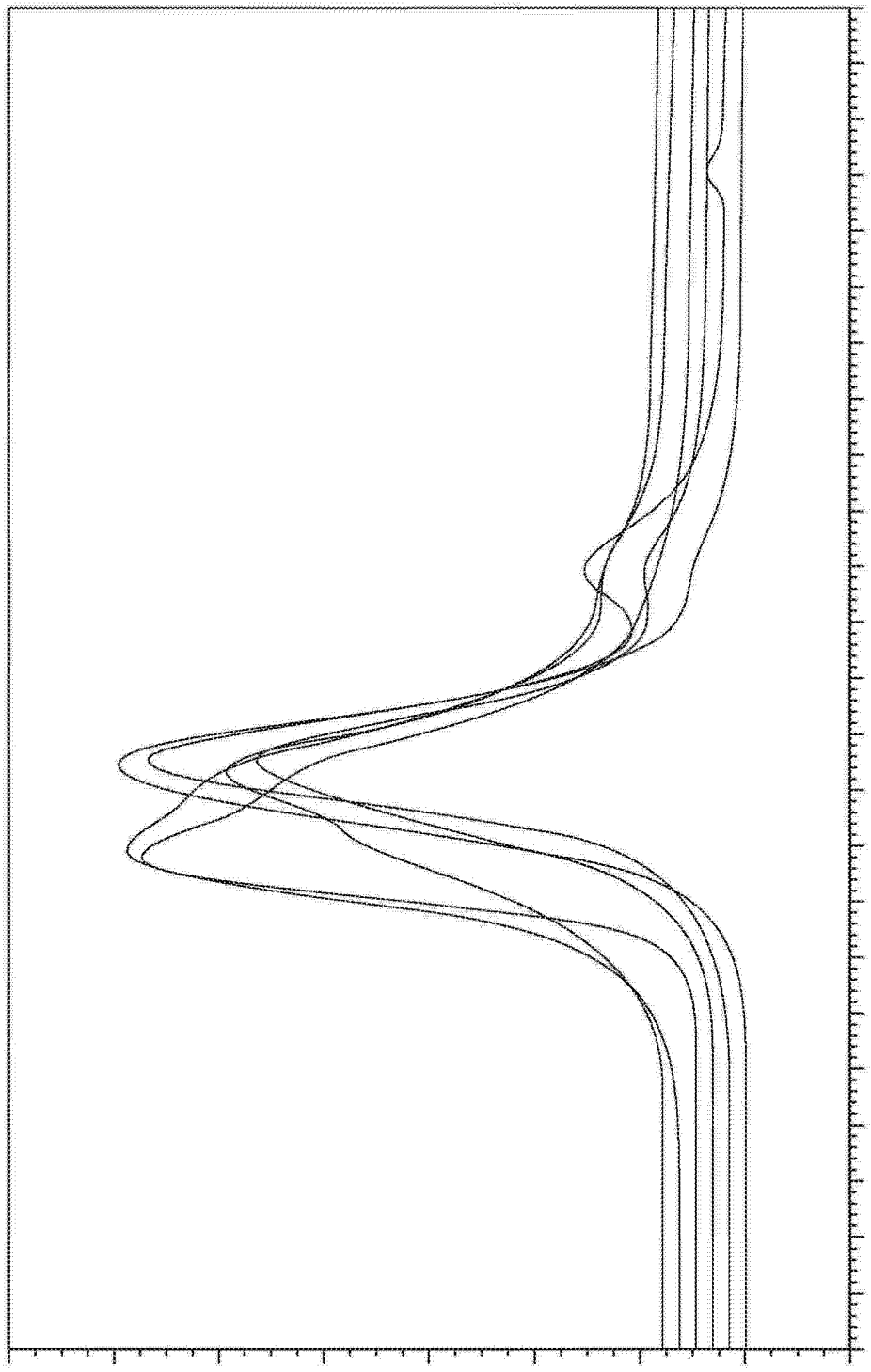


图 11

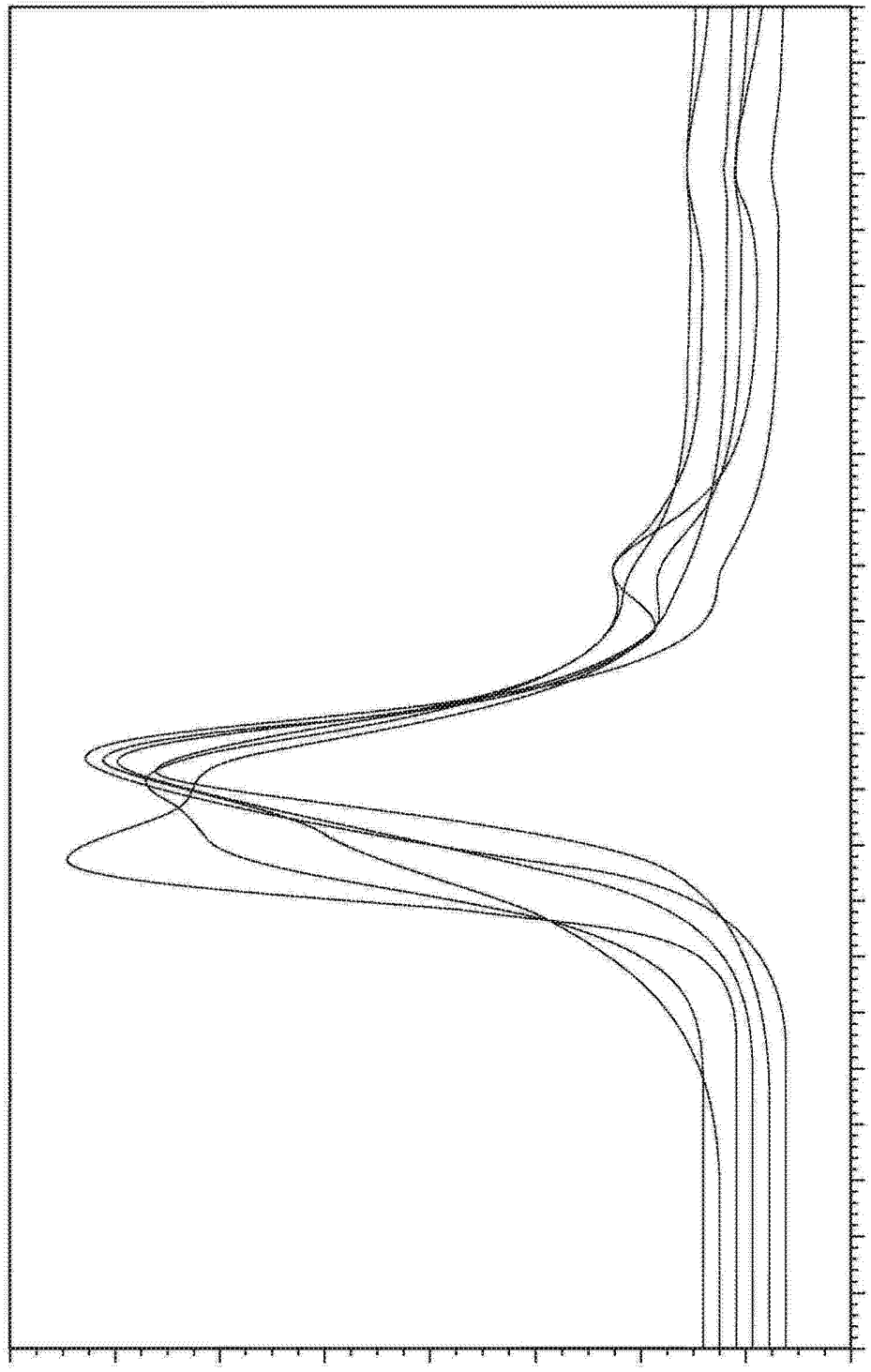


图 12

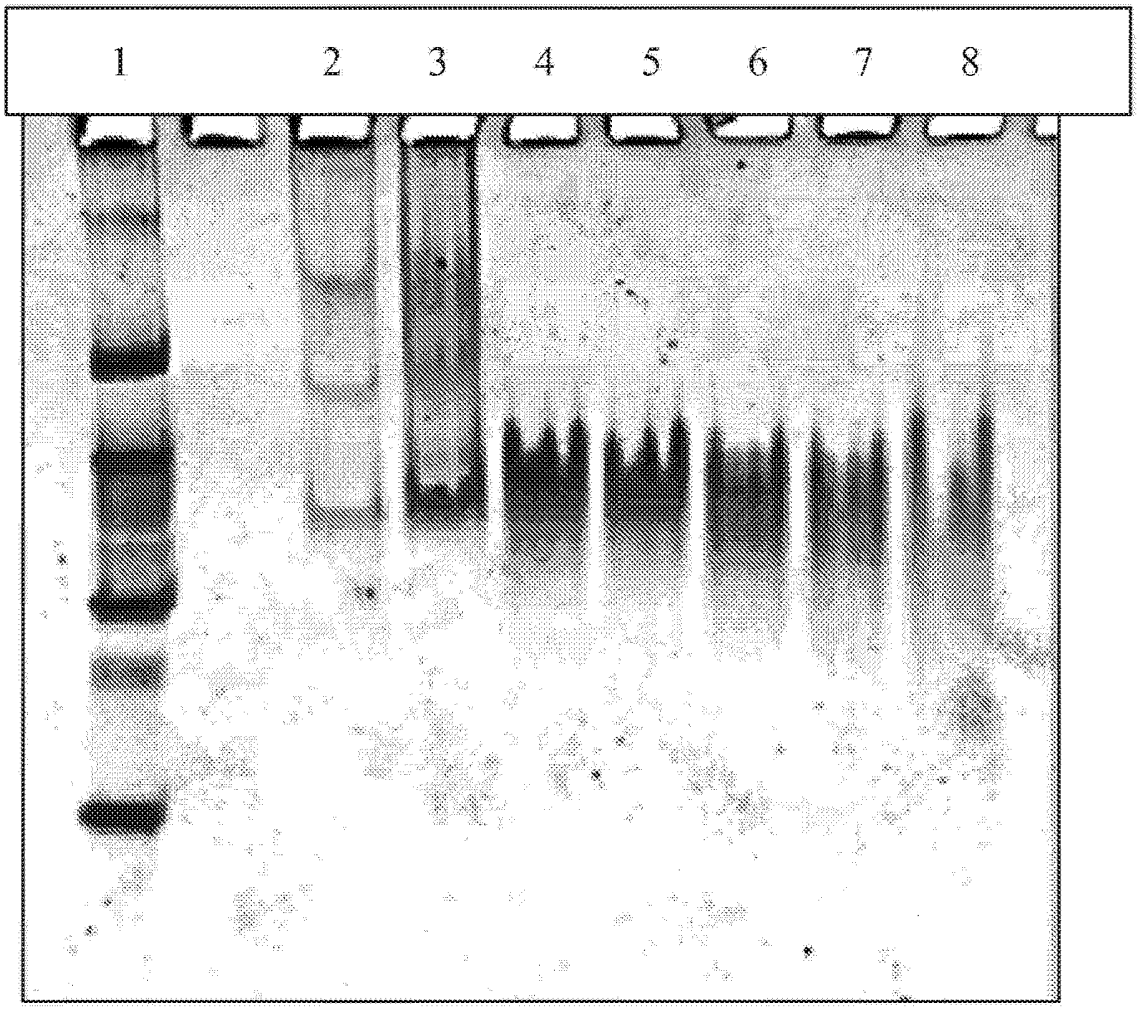


图 13

图 14

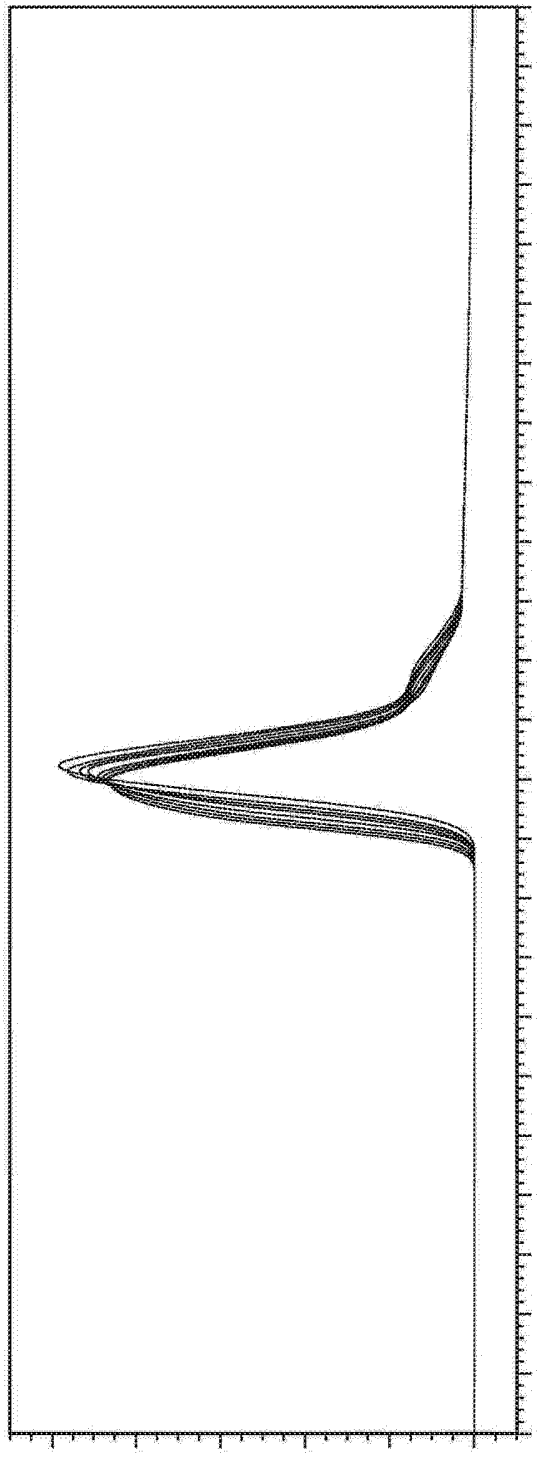


图 15

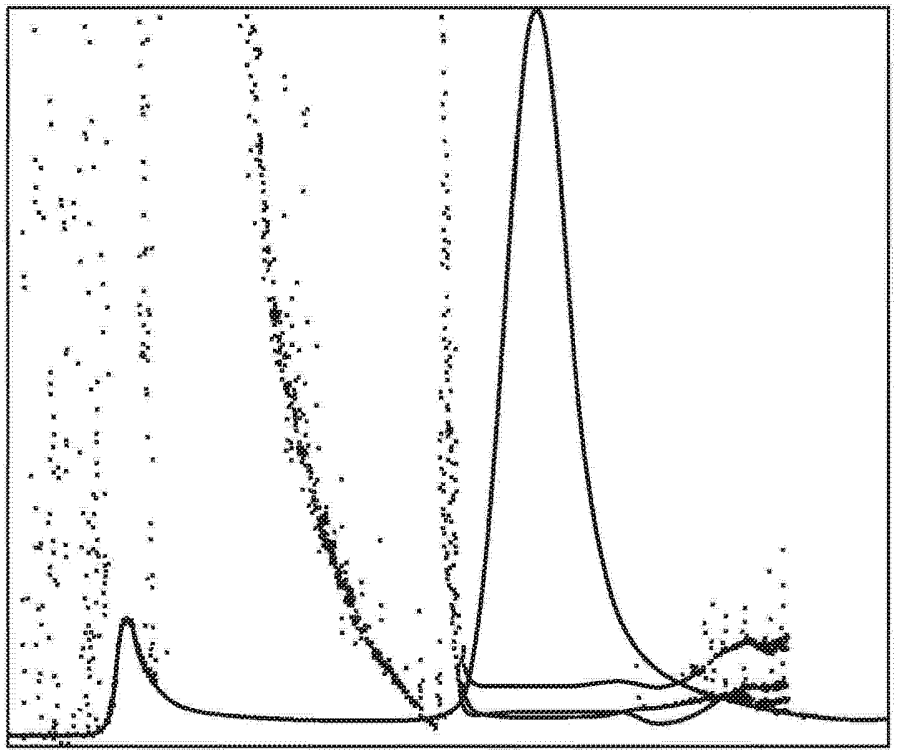


图 16

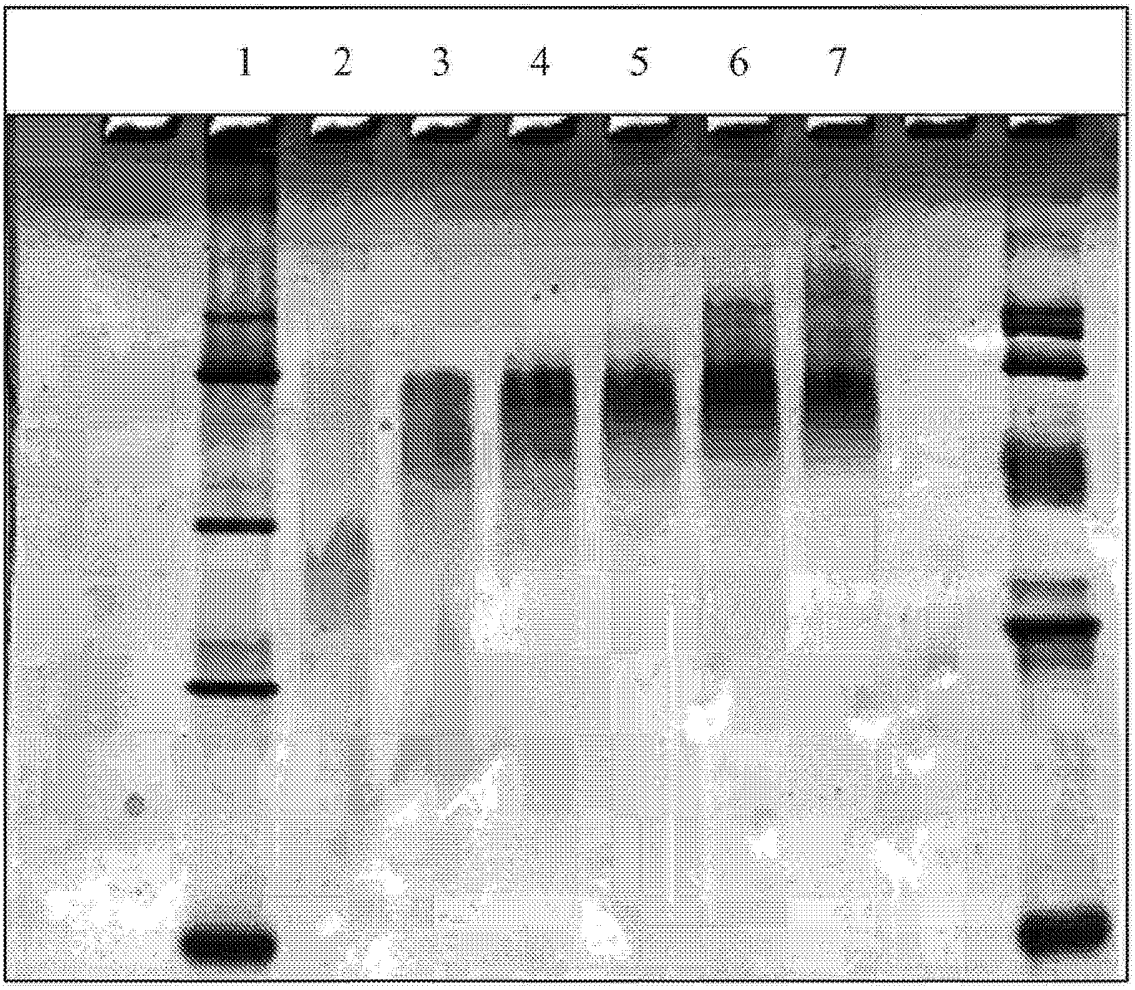


图 17

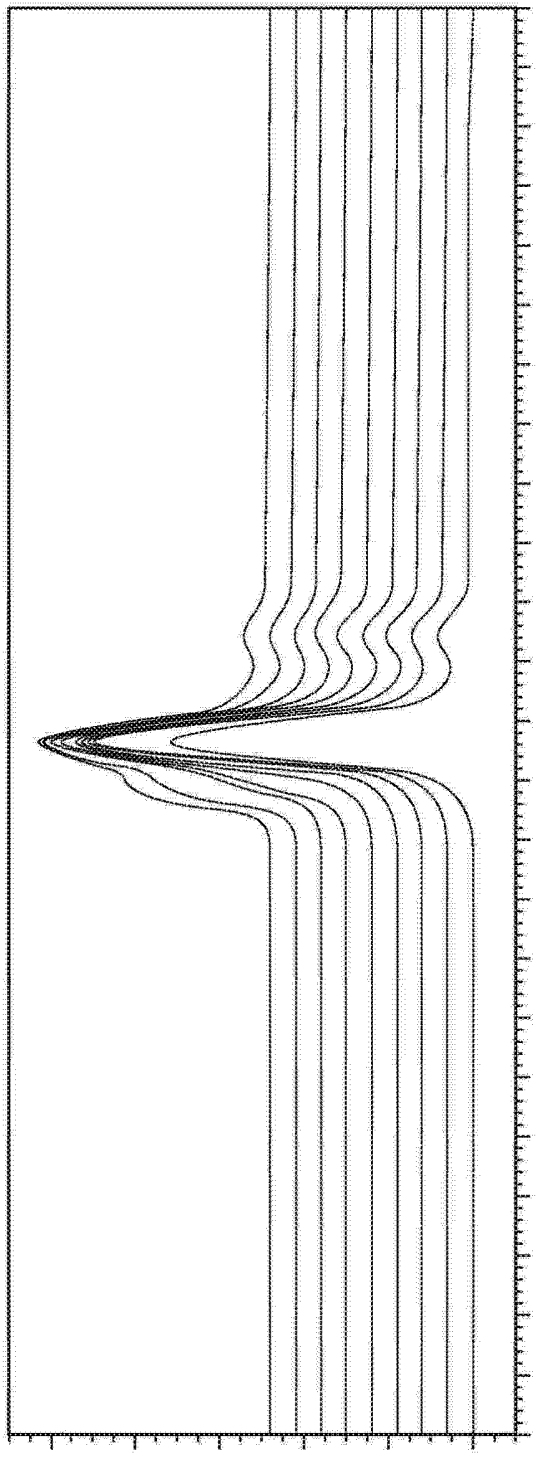


图 18

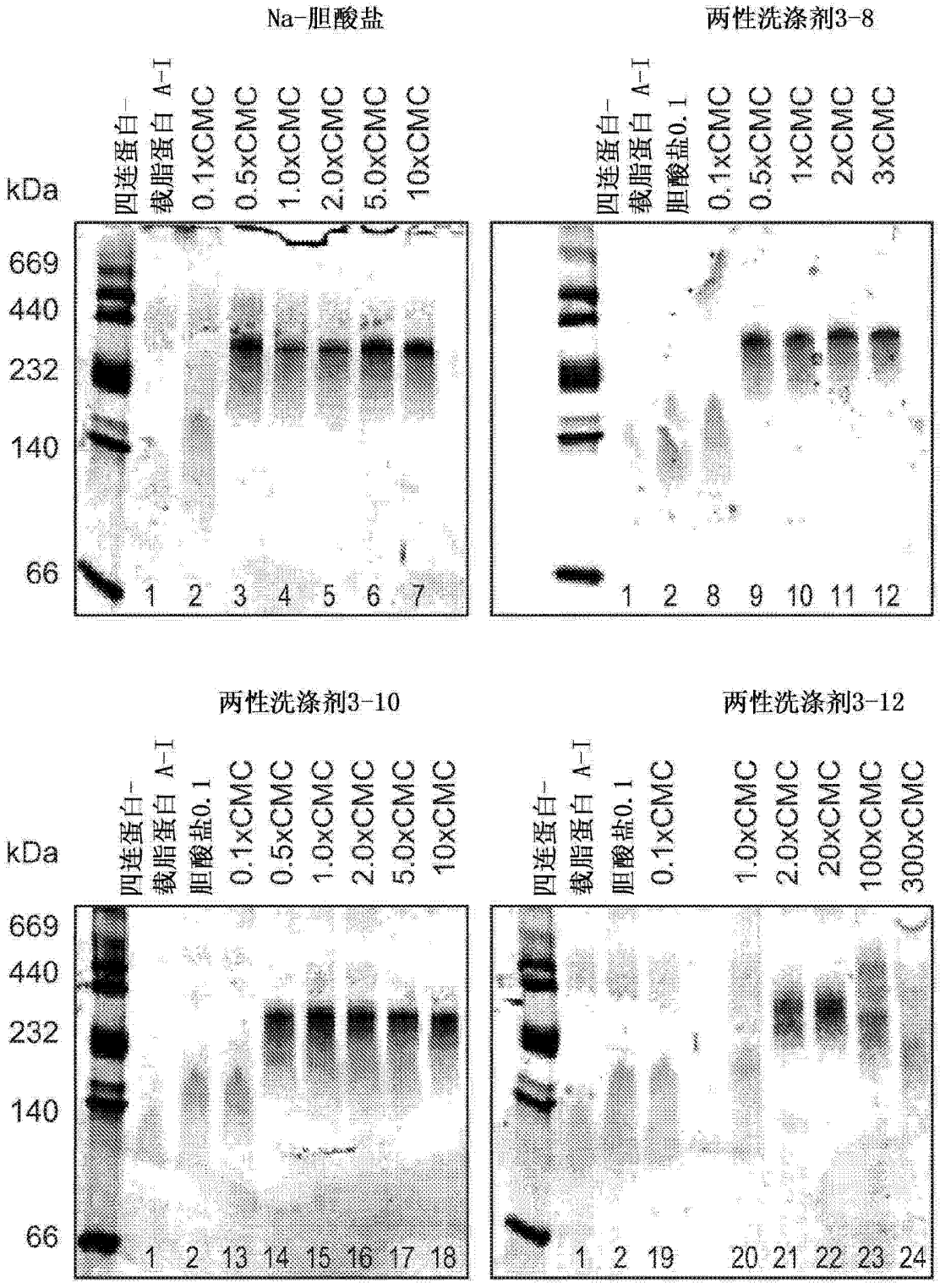

Na-胆酸盐
两性洗涤剂3-8
两性洗涤剂3-10
两性洗涤剂3-12
kDa
669
440
232
140
66
四连蛋白-载脂蛋白 A-I
0.1xCMC
0.5xCMC
1.0xCMC
2.0xCMC
5.0xCMC
10xCMC
胆酸盐0.1
1xCMC
2xCMC
3xCMC
20xCMC
100xCMC
300xCMC
1 2 3 4 5 6 7
1 2 8 9 10 11 12
1 2 13 14 15 16 17 18
1 2 19 20 21 22 23 24

图 19

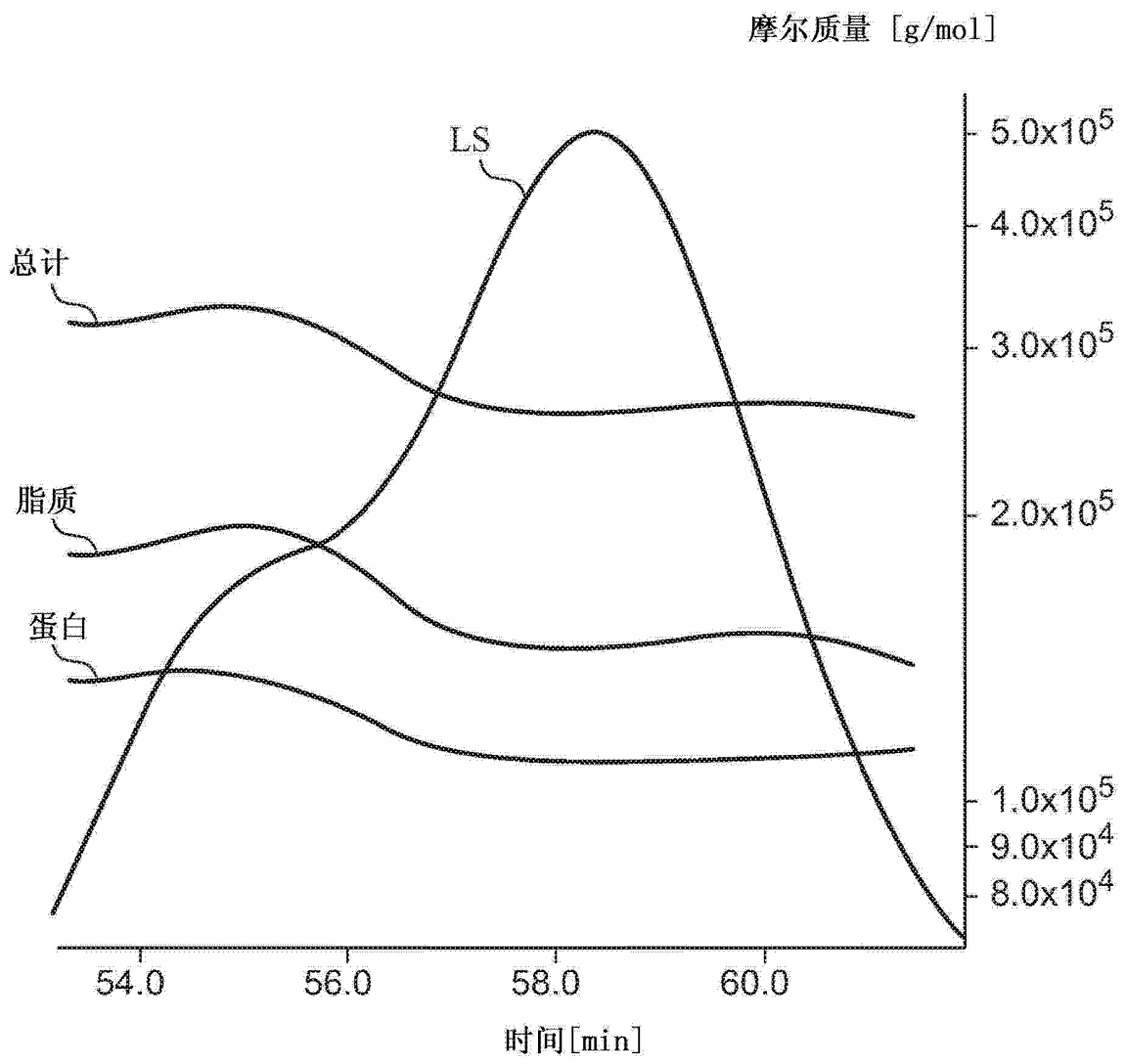

摩尔质量 [g/mol]
LS
总计
脂质
蛋白
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
9.0x10^4
8.0x10^4
54.0
56.0
58.0
60.0
时间[min]

图 20

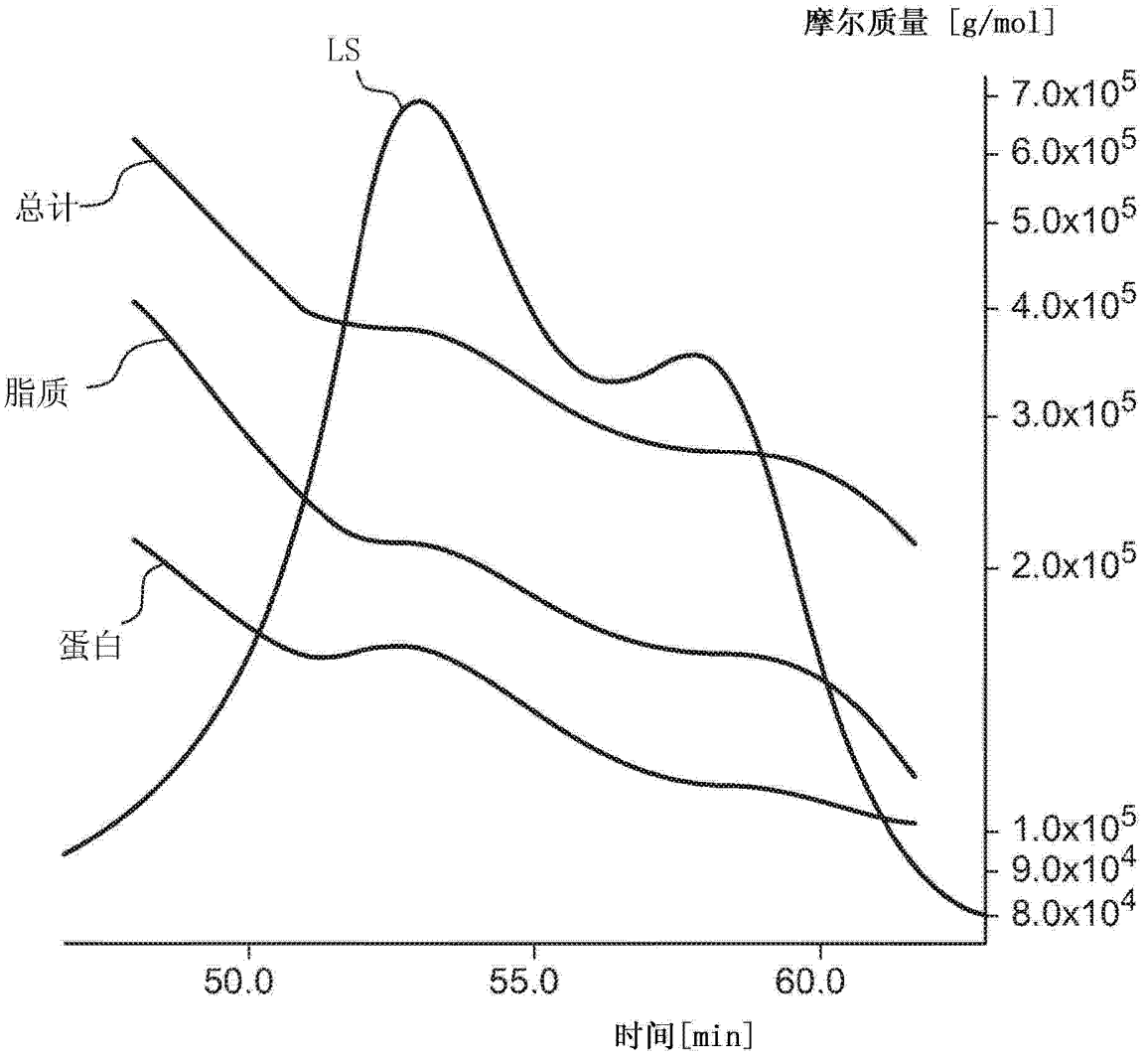

摩尔质量 [g/mol]
LS
总计
脂质
蛋白
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
9.0x10^4
8.0x10^4
50.0
55.0
60.0
时间[min]

图 21

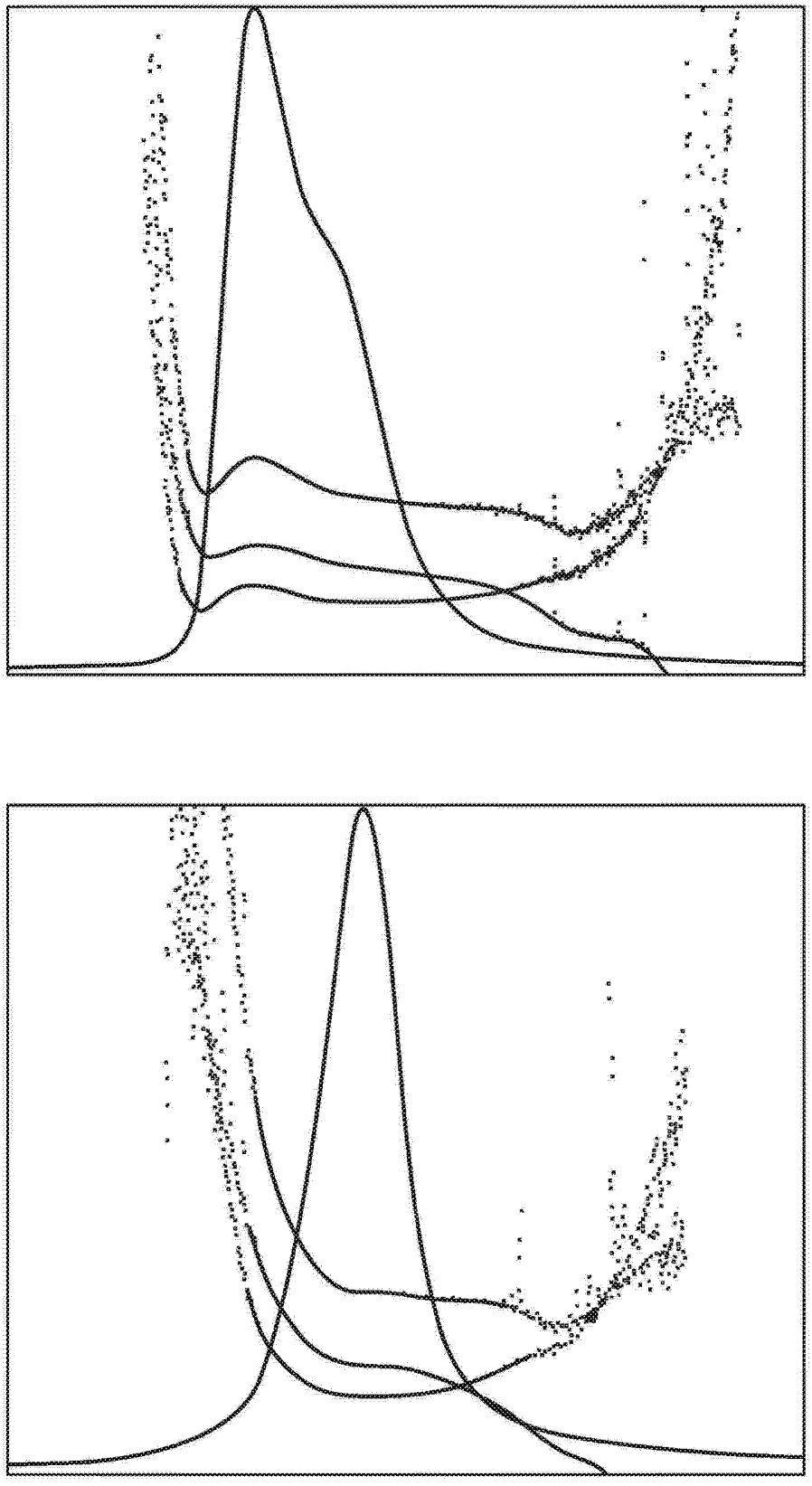


图 22

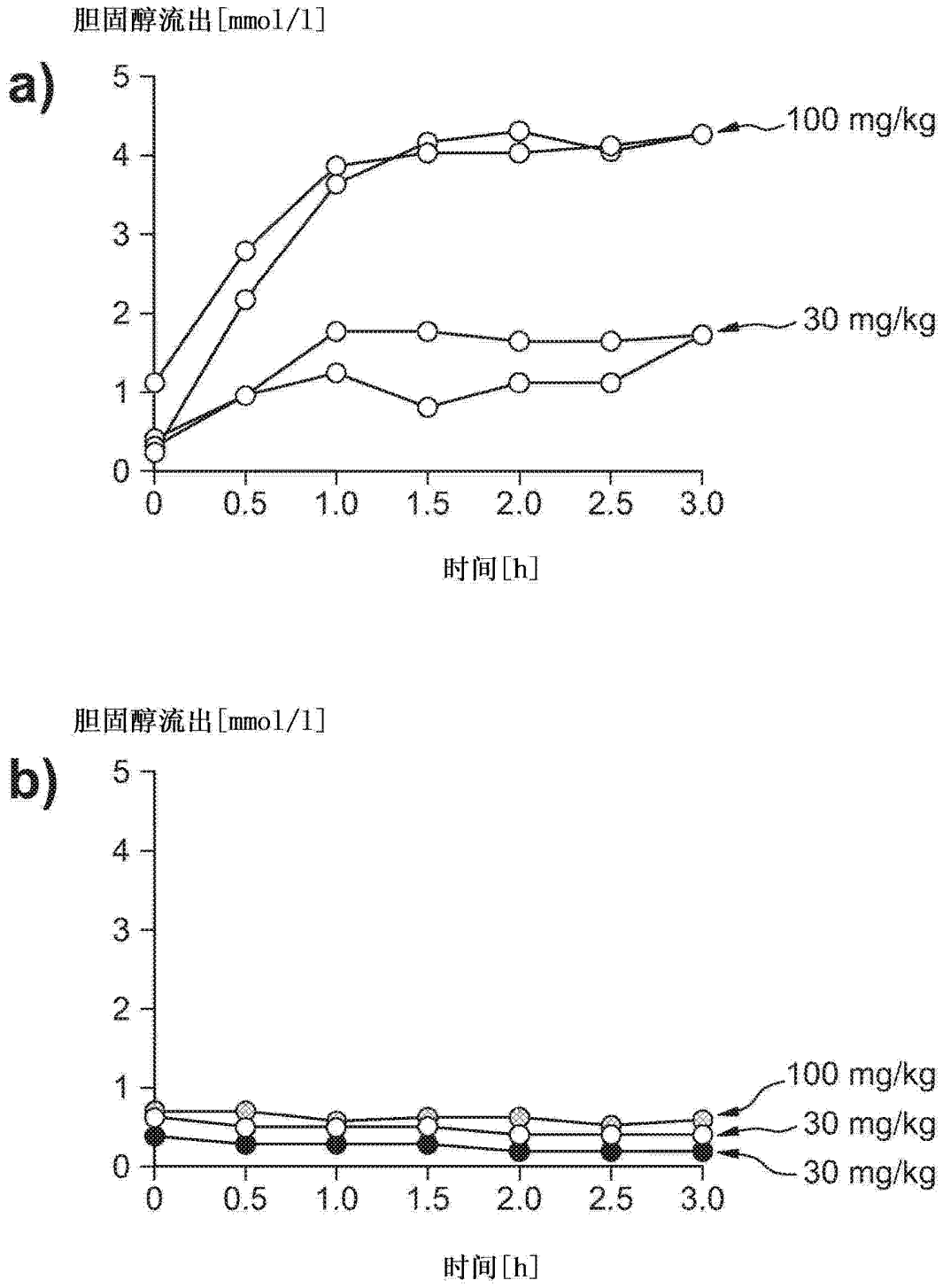

胆固醇流出[mmol/l]
a)
5
4
3
2
1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
100 mg/kg
30 mg/kg
时间[h]
胆固醇流出[mmol/l]
b)
5
4
3
2
1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
100 mg/kg
30 mg/kg
30 mg/kg
时间[h]

图 23

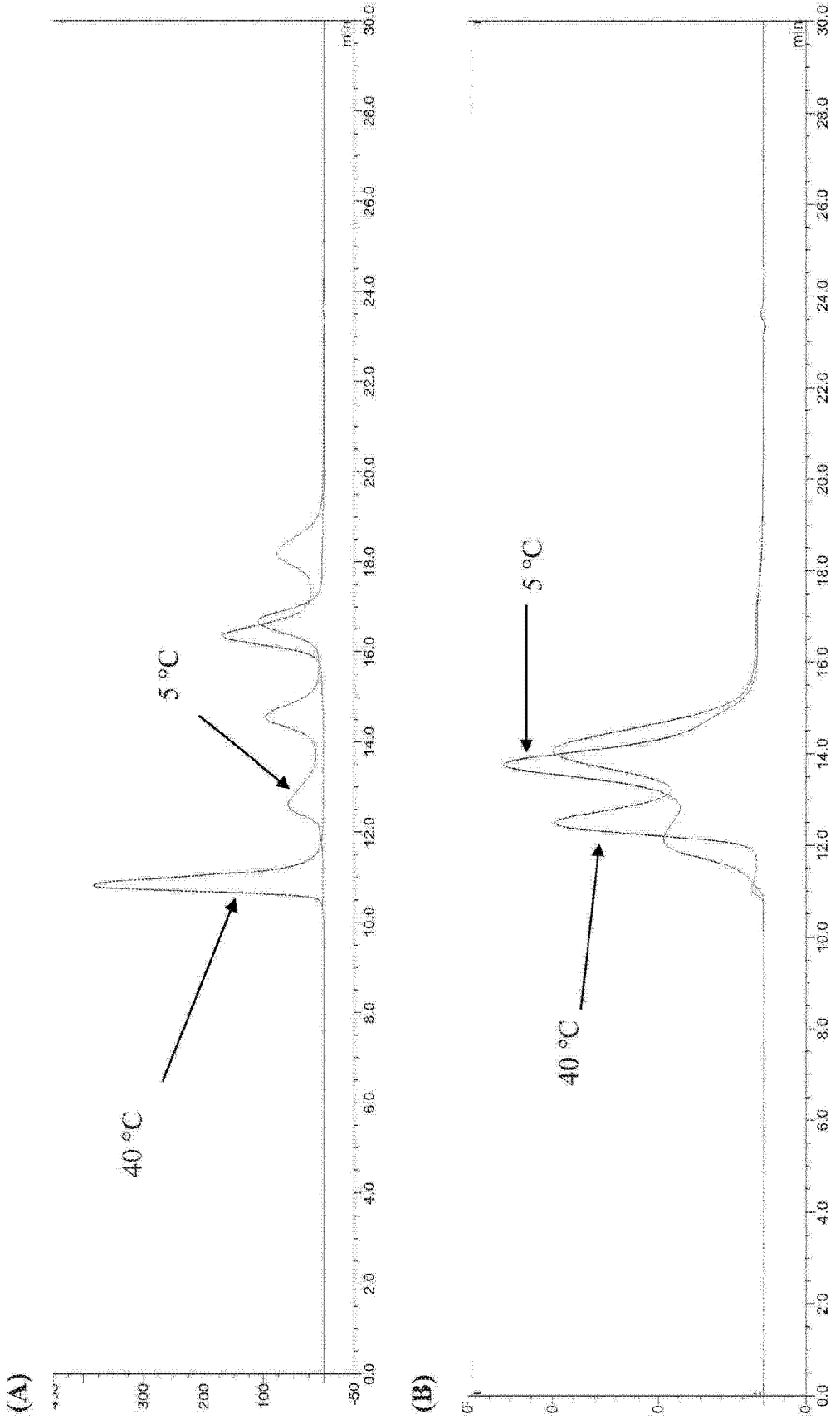


图 24