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(71) Applicant *(for all designated States except US)*: **Nya Hamlet Pharma AB** [SE/DK]; c/o NatImmune A/S, Fruebjergvej 3, DK-2100 Copenhagen Ø (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **AGGER, Teit** [DK/DK]; Kongevejen 145B, DK-2840 Holte (DK). **BRO, Christoffer** [DK/DK]; Teglværksvej 19, DK-3460 Birkerød (DK).

(74) Agent: **HØIBERG A/S**; St. Kongensgade 59A, DK-1264 Copenhagen K (DK).

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(54) **Title:** METHODS FOR PREPARING CYTOTOXIC COMPLEXES OF EMULSIFIER AND FATTY ACID

(57) **Abstract:** The invention relates to methods for preparing complexes of emulsifiers and fatty acids, which have a cell killing effect, in particular on tumour cells or other malignant cells. The methods involve mixing an emulsifier with a fatty acid using high shear mixing. The emulsifier may be alpha-lactalbumin and the fatty acid may be oleic acid. The complexes obtained according to the methods may be employed in treatment of a variety of disease characterised by the presence of undesired cells, such as various malignant or pre-malignant diseases and infections, in particular viral infections.

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Methods for preparing cytotoxic complexes of emulsifier and fatty acid

All patent and non-patent references cited in the application, or in the present application, are also hereby incorporated by reference in their entirety.

Field of invention

The present invention relates to methods for preparing complexes of emulsifiers and fatty acids, which have a cell killing effect, in particular on tumour cells or other malignant cells. The complexes obtained according to the methods may be employed in treatment of a variety of disease characterised by the presence of undesired cells, such as various malignant or pre-malignant disease and infections, in particular viral infections.

Background of invention

Alpha-lactalbumin (LA) is the major protein in human milk whey. Mature monomeric alpha-lactalbumin consists of 123 amino acid residues (14.2 kDa) in many mammalian species. Human, bovine, equine, caprine, and camelide alpha-lactalbumin all consist of 123 amino acid residues, whereas porcine alpha-lactalbumin consists of 122 amino acids. Human, bovine, caprine and porcine alpha-lactalbumin also comprise a 19 amino acid leader sequence. This 14 KDa protein has been extensively characterised and the crystal structure has been resolved.

It has been shown that complexes of alpha-lactalbumin and a fatty acid or lipid may have cell killing abilities. A fraction from human milk containing an oligomeric complex was described as multimeric alpha-lactalbumin or MAL or HAMLET (human a-lactalbumin made lethal to tumour cells) and was shown to induce in vitro apoptosis selectively in tumour cells, but not in healthy differentiated cells. Danish patent application number PA 2006 01512 describes complexes of alpha-lactalbumin and fatty acid or lipid, which also have cell killing abilities.

Alpha-lactalbumin may undergo conformational switching and may adopt the so called apo state when exposed to low pH, or in the presence of chelators, that release the

strongly bound Ca^{2+} ion. The apo state or molten globule state has native secondary structure, but less well defined tertiary structure than the native state. Similar states of alpha-lactalbumin can also form at neutral pH, upon removal of the tightly bound Ca^{2+} ion, reduction of disulphide bonds or at elevated temperatures (the apo-state).

The apoptotic activity of this folding variant was discovered by serendipity. During a study regarding the effect of human milk on bacterial adherence, it was surprisingly discovered that human milk induced apoptosis in transformed and nontransformed immature cell lines. The apoptotic activity in human milk was isolated and found to be partially unfolded alpha-lactalbumin in an apo-like conformation with native- like secondary structure, but lacking specific tertiary packing of the side chains.

LAC (as used herein) denotes a complex between LA and a fatty acid, said complex having cytotoxic activity. bLAC denotes a complex between bovine LA and a fatty acid, said complex having cytotoxic activity and hLAC denotes a complex between human LA and a fatty acid, said complex having cytotoxic activity. LAC is reported as having therapeutic applications both in the field of antibiotics and cancer therapy in particular, LAC may induce apoptotic cell death in cancer cells and immature cells, but not (or only to a low extent) in mature, healthy cells. These observations suggested that the protein acquires novel biological properties when forming an active complex with a fatty acid or lipid. Thus, reagents such as fatty acids or lipids, such as oleic acid, have been useful in the conversion of LA to LAC.

An active LAC complex has previously been produced by first exposing alpha-lactalbumin in the apo state to a DEAE Trisacyl resin that had been pre-conditioned with oleic acid causing the formation of active complex of alpha-lactalbumin and oleic acid (e.g. Svensson, et al. , (2000) Proc Natl Acad Sci USA, 97,4221-6, WO 03/098223, WO 2005/082406) or by mixing alpha-lactalbumin and oleic acid prior to exposure to an anion exchange resin (Danish patent application PA 2007 00693). Simple mixing of alpha-lactalbumin and oleic acid did not produce active LAC complexes (e.g. Svensson, et al. , (2000) Proc Natl Acad Sci USA, 97,4221-6).

Summary of invention

Thus, it was believed that in order to prepare active complexes of fatty acid and alpha-lactalbumin, it was required to subject alpha-lactalbumin to ion exchange chromatography in the presence of fatty acid. Surprisingly it has now been discovered that the exposure of alpha-lactalbumin and oleic acid to an ion exchange resin is not required for the formation of an active cytotoxic LAC complex. Simple mixing of alpha-lactalbumin and oleic acid did not produce active LAC complexes, but the present invention demonstrates that high shear mixing of the components surprisingly resulted in active LAC complexes.

Thus, it is one objective of the present invention to provide methods for preparing a cytotoxic complex between an emulsifier and a fatty acid, said method comprising the steps of

a. providing an emulsifier in an aqueous solution, wherein said emulsifier is not a fatty acid; and
b. providing a fatty acid; and
c. contacting the emulsifier with the fatty acid; and
d. mixing the emulsifier and the fatty acid using high shear mixing

thereby obtaining a complex between said emulsifier and said fatty acid, wherein said complex has a cytotoxic effect on tumour cells

Whether a complex is cytotoxic may be determined as described herein below in the section “Cytotoxic effect”.

Furthermore, the invention relates to the cytotoxic complexes prepared by the method as well as to such complexes for use as a medicament. In particular, the invention relates to such complexes for use as a medicament for treatment of a clinical disorder selected from the group consisting of viral infections, disorders associated with aberrant cell proliferation, actinic keratosis and disorders associated with angiogenesis.

Description of Drawings

Figure 1 shows the viability of L1210 cells as a dose response to bLAC at a concentration of $1.4x10^6$ cells per ml. The viability was determined by trypan blue

staining and by luminiscense using the the ViaLight Plus kit (Lonza cat. No. LT07-221 or LT07-121). The LC_{50} for bLAC was 0.02-0.05 mg/ml.

Figure 2 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of HSA and/or oleic acid.

Figure 3 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of HSA and oleic acid.

Figure 4 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of HSA and/or oleic acid.

Figure 5 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of bLA and oleic acid.

Figure 6 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with bLA with oleic acid in a 1:15 molar ratio, wherein oleic acid was a 99% pure preparation obtained from Sigma-Aldrich, Denmark or a 65-88% pure preparation obtained from Merck.

Figure 7 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of bovine bLG and oleic acid.

Figure 8 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of bLG and/or oleic acid.

Figure 9 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of MBL and oleic acid.

Figure 10 shows % viability relative to control sample with 0.9% NaCl of L1210 cells at a concentration of $1.4x10^6$ cells per ml when incubated with the indicated preparations of MBL and oleic acid.

Figure 11 shows cell killing activity of three different preparations of complexes prepared from 12.1 mg/mL bLA saturated and mixed under high shear conditions with oleic acid. Water phase and fatty acid phase was separated by a final centrifugation at 150 x g, 3,000 x g or 18,000 x g. Filled circles - bLA saturated with oleic acid (N287-50D); open circles – bLA saturated with oleic acid (N287-55B); triangles – bLA saturated with oleic acid (N287-69C); X – bLAC (N276-77A)

Figure 12 shows cell killing activity of three different preparations of complexes prepared from fatty acid free HSA saturated and mixed under high shear conditions with oleic acid. Water phase and fatty acid phase was separated by a final centrifugation at 150 x g, 3,000 x g or 18,000 x g. Filled circles – HSA saturated with oleic acid (N287-55D), spun at 3000xg, LD_{50} 266 pg/cell; open circles – HSA saturated with oleic acid (N287-50C), spun at 150xg, LD_{50} 105 pg/cell; triangles - HSA saturated with oleic acid (N287-82D), spun at 18,000xg, LD_{50} 203 pg/cell.

Figure 13 shows cell killing activity of two preparations of complexes prepared from bLG saturated and mixed under high shear conditions with oleic acid. Water phase and fatty acid phase was separated by a final centrifugation at first 3,000 x g followed by 18,000 x g. Filled circles – bLG saturated with oleic acid (N287-75C), spun at 3000xg, LD_{50} 43 pg/cell; open circles – bLG saturated with oleic acid (N287-75D), spun at 18,000xg, LD_{50} 87 pg/cell.

Figure 14 shows cell killing by two preparations of complexes of prepared from rhMBL saturated and mixed under high shear conditions with oleic acid. Water phase and fatty acid phase was separated by a final centrifugation at first 3,000 x g followed by 18,000 x g. Filled circles – rhMBL saturated with oleic acid (N287-78D), spun at 3000xg, LD_{50} 76 pg/cell; open circles – rhMBL saturated with oleic acid (N287-78E), spun at 18,000xg, LD_{50} 123 pg/cell.

Figure 15 shows cell killing activity of two preparations of lysozyme saturated and mixed with oleic acid. Water phase and fatty acid phase was separated by a final centrifugation at first 3,000 x g (giving N287-82E) followed by 18,000 x g (giving N287-82F). Filled circles – Lysozyme saturated with oleic acid (N287-82E); open circles – Lysozyme saturated with oleic acid (N287-82F); X – bLAC N276-77A.

Figure 16 shows cell killing activit of two preparations with oleic acid in ethanol mixed into a 3 mg/mL bLA solution in a 1:15 molar ratio (protein vs. oleic acid). One of the preparations was subjected to high shear mixing (on whirleymixer) the other was not. Filled circles – bLA and oleic acid (whirley mixed –high shear), LD_{50} 43 pg/cell; open circles – bLA and oleic acid (tube turned 3 times), LD_{50} 407 pg/cell.

Figure 17 shows cell killing activity of preparations of complexes prepared from oleic acid in ethanol mixed under high shear conditions with a 3 mg/mL bLA solution in different molar ratios. Filled circles – bLA and oleic acid in 1:15 molar ratio, LD_{50} 57 pg/cell; open circles – bLA and oleic acid in 1:10 molar ratio, LD_{50} 61 pg/cell; filled triangles – bLA and oleic acid in 1:5 molar ratio, LD_{50} 135 pg/cell; open triangles – bLA and oleic acid in 1:1 molar ratio, LD_{50} 385 pg/cell.

Figure 18 shows LD_{50} of preparation prepared from bLA saturated with oleic acid incubated at -20°C, 2-8°C and 25°C for the indicated number of weeks and analysed for cell killing activity assuming a constant bLA+bLAC concentration of 8.6 mg/mL.

Figure 19 shows bLA content in a preparation prepared from bLA in 0.9% NaCl solution saturated with oleic acid and mixed under high shear conditionds incubated at -20°C, 2-8°C and 25°C for the indicated number of weeks and analysed for protein (bLA) content by A_{280nm}.

Figure 20 shows LD50 of complexes prepared from bLA in 0.9% NaCl solution with or without 10 mM Tris (pH 8.5) and 8 mM EDTA saturated with oleic acid and mixed under high shear conditions. Water phases (N318-65A+B) were incubated at -20°C and 25°C for the indicated number of weeks and analysed for cell killing activity assuming a constant bLA+bLAC concentration of 8.6 mg/mL in N318-65B and 7.6 mg/mL in N318-65A.

Figure 21 shows the Cell killing activity of preparations resulting from mixing under high shear conditions oleic acid dissolved in ethanol into a 3 mg/mL bLA solution in a 1:15 protein to oleic acid molar ratio with different chelators and buffers. A constant bLA+bLAC concentration of 3 mg/mL was assumed.

Figure 22: Chromatograms of the conversion runs with bLA start material N277-64A (described in Example 22).

Figure 23: Chromatograms of the conversion runs with bLA start material N289-56A (described in Example 22).

Detailed description of the invention

Emulsifier

As used within the present context the term “emulsifier” is meant to cover substances capable of forming and stabilising an emulsion of two liquids which are immiscible. Thus, an emulsifier according to the present invention is capable of stabilising an emulsion of oil and water, preferably an oil in water emulsion.

In particular, it is preferred that the emulsifier comprises at least one hydrophobic and at least one hydrophilic domain, frequently the emulsifier will comprise one hydrophobic and one hydrophilic domain. More preferably, overall the emulsifier is more hydrophilic than hydrophobic, however, the emulsifier should contain at least one hydrophobic domain. Thus, it is preferred that the solubility of the emulsifier is higher in water than in oil.

The emulsifier according to the present invention is not a fatty acid of the formula:

R-COOH,

wherein R is alkyl or alkenyl. In particular, the emulsifier is not any of the fatty acids described in the section “Fatty acids” herein below. In one embodiment, the emulsifier according to the present invention is not a fatty acid.

The emulsifier may be selected from the group consisting non-ionic and ionic emulsifiers, wherein ionic emulsifiers may be anionic, cationic or zwitterionic.

Nonionic emulsifiers may for example be selected from the group consisting of polymeric emulsifiers, such as alkyl poly(ethylene oxide), copolymers of poly(ethylene oxide) and poly(propylene oxide), copolymers of ethylene and vinyl acetate, styrene and butyl acrylates; polyoxyalkylenated alkyl esters, for example with alkyl radical comprising from 10 to 22 carbon atoms; polyalkylene glycols, preferably polyethylene glycols; polyethylene glycol ethers of fatty alcohols; polypropylene glycols; diethylene glycols; polyoxyalkylenated alkyl esters of sorbitan, for example where the alkyl radical comprises from 10 to 22 carbon atoms, such as polyoxyethylene sorbitan monooleate, -laurate (or Tween-20), or –stearate; cellulose derivatives, such as hydroxypropylcellulose, ethylcellulose, methylcellulose or cellulose acetate butyrate mixed ethers; alkyl polyglucosides, such as octyl glucoside or decyl maltoside; fatty alcohols, such as cetyl alcohol or oleyl alcohol; polyalkylene glycol ethers of a fatty alcohols comprising 8 to 30 carbon atoms; and cocamides, such as cocamide MEA, cocamide DEA or cocamide TEA. Examples of other emulsifiers includes Tween, such as Tween 80.

It is however preferred that the emulsifier is ionic. Very preferred emulsifiers to be used with the present invention are polypeptides, which are described in more detail herein below in the section “Polypeptides”. Polypeptides are ionic emulsifiers and dependent on the particular amino acid composition of the polypeptide they may be anionic, cationic or zwitterionic. In one preferred embodiment the polypeptide is anionic at neutral pH, and thus is an anionic emulsifier.

Other useful ionic emulsifiers may for example be the following.

Anionic emulsifiers may for example be emulsifiers comprising one or more groups selected from the group consisting of sulfate, sulfonate or carboxylate groups, preferably carboxylate groups (with the exception of the fatty acids described below). For example the anionic emulsifier may anionic polypeptides as described below. Anionic emulsifiers may also be bile salts, preferably sodium cholate.

Cationic emulsifiers may for example be emulsifiers comprising one or more quaternary ammonium cations or cationic amino groups. For example the cationic emulsifiers may be cationic polypeptides as described below.

Zwitterionic emulsifiers may for example be selected from the group consisting of dodecyl betaine, dodecyl dimethylamine oxide, cocamidopropyl betaine and coco ampho glycinate.

In one embodiment the emulsifier may be one or more phospholipids, for example phospholipids selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and sphingomyelin, preferably the phospholipid is phosphatidylcholine. Hydrogenated phospholipids may also be employed..

The emulsifier may also be a composition comprising phospholipids, such as for example lecithin. Lecithin may for example be egg yolk lecithin or soybean lecithin. In addition to phospholipids, lecithin may also comprises glycolipids and/or triglycerides.

In one embodiment of the invention it is preferred that the emulsifier has a certain minimal size, preferably the emulsifier is at least 0.2 kDa, preferably at least 0.5 kDa, more preferably at least 0.75 kDa, for example at least 1 kDa, such as at least 5 kDa. In embodiments of the invention wherein the emulsifier is a polypeptide it is preferred that the polypeptide is at least 5 kDa, more preferably at least 10 kDa, for example at least 15 kDa, such as in the range of 5 to 500 kDa, for example in the range of 10 to 500 kDa, such as in the range of 15 to 500 kDa, for example in the range of 10 to 400 kDa, such as in the range of 10 to 300 kDa, for example in the range of 10 to 200 kDa, for example in the range of 15 to 400 kDa, such as in the range of 15 to 300 kDa, for example in the range of 15 to 200 kDa, such as in the range of 15 to 100 kDa.

It is preferred that the emulsifier is not cytotoxic to normal, healthy mammalian cells when not in complex with fatty acid. If tested in vitro, then the emulsifier is preferably not cytotoxic to primary, non-malignant mammalian cells in in vitro culture, when not in complex with fatty acid. The assays described herein below for determining cytotoxicity of the complexes of the invention may readily be adapted for determining whether a given emulsifier is cytotoxic to primary, non-malignant cells.

Furthermore, it is preferred that the emulsifier is not cytotoxic in the cell killing assay of Example 3 in the absence of fatty acid at the concentrations used. Thus, in embodiments wherein the emulsifier is Tween 80 it is preferably used at concentrations of less than 0.14% (w/v) and in embodiments wherein the emulsifier is sodium cholate it is preferably used at concentrations lower than 6.7 mM. More preferably, the emulsifier is neither Tween 80 nor sodium cholate.

It is also preferred that the emulsifier is provided in a purified form. In particular, if the emulsifier is from a natural source, it is preferred that the emulsifier is purified from said natural source. Thus, by way of example if the emulsifier is from a natural source for example from a cellular extract, a tissue extract, a body fluid (such as saliva, serum, blood or the like) or milk, then said emulsifier has been isolated from said natural source prior to being contacted with the fatty acid.

Polypeptides

In a very preferred embodiment of the present invention the emulsifier is a polypeptide. A polypeptide is made up of amino acids linked by amide bonds. Polypeptides according to the present invention may comprise any amino acid, however it is preferred that polypeptides only comprises naturally occurring amino acid forming parts of proteins in nature. However, polypeptides within the present invention may also comprise post-translational modifications. Thus, the polypeptides may be covalently linked to other polypeptides, typically by S-S bridges, to phosphate, methyl, saccharides, such as mono-saccharides, oligosaccharide or polysaccharide, which optionally may be branched, lipids and the like. It is preferred that the polypeptide only comprises such post-translational modifications which occur in nature.

The polypeptides may be derived from any suitable species, such as virus, phage, bacteria, archaebacteria, fungi, yeast, plants or animals. Preferably, the polypeptide is derived from an animal, such as insects, protozoans or vertebrates, preferably from a vertebrae, even more preferably from a mammal.

The polypeptide may be an individual polypeptide chain or the polypeptide may be associated with other polypeptides in oligomers. The oligomers may be oligomers of identical polypeptides or of different polypeptides.

The polypeptides may be purified from natural sources, such as milk, serum, tissue extracts, eggs, plant extracts, cell extracts, or the polypeptides may be produced by recombinant methods, for example as described herein below.

In one embodiment of the invention it is preferred that the polypeptide has a theoretical pI, which is not too high. It is therefore preferred that the theroretical pI is at the most 9, preferably at the most 8, more preferably at the most 7, such as in the range of 3 to 9, for example in the range of 3 to 8, such as in the range of 3 to 7, for example in the range of 3 to 6, such as in the range of 4 to 9, such as in the range of 4 to 8, for example in the range of 4 to 7, such as in the range of 4 to 6, such as in the range of 4.5 to 9, for example in the range of 4.5 to 8, such as in the range of 4,5 to 7, for example in the range of 4.5 to 6.

The skilled person will be able to calculate the theoretical pI for a given polypeptide. Theoretical pI for polypeptides are also available in publicly assessable protein databases, such as the Swissprot database. Examples of theoretical pI of a number of polypeptides together with other protein parameters are given in Table 1 below:

Table 1

Polypeptide	**Aliphatic index**	**pI**	**MW**
Human alpha-lactalbumin	100,99	4,83	16225
Bovine alpha-lactalbumin	91,27	4,92	16247
Human MBL	68,87	5,39	26144
Bovine lactoglobulin	106,4	4,93	19883
Human serum albumin	77,09	5,85	69084
Lysozyme G	85,5	9,57	23238
Lysozyme C	81,7	9,36	16239

Accordingly, in embodiments of the inventions, wherein the theoretical pI should not be too high, such as at the most 9, then the polypeptide preferably is not lysozyme.

In one embodiment the overall content of negatively charged amino acids (Asp and Glu) is higher than the overall content of positively charged amino acids (Arg and Lys).

The aliphatic index of the polypeptide according to the present invention is preferably in the range of 50 to 250, such as in the range of 65 to 110.

Herein below a number of different polypeptides are given in order to exemplify the invention. However, other polypeptides may also be useful with the present invention.

Albumin

The polypeptide may for example be a polypeptide belonging to the albumin family, preferably with the proviso that the polypeptide is not alpha-lactalbumin.

For example the polypeptide may be an albumin obtainable from serum, such as serum albumin, such as human serum albumin. For example the polypeptide may be human serum albumin of SEQ ID NO: 5 or a functional homologue thereof sharing at least 75% sequence identity therewith. Functional homologues may be any of the functional homologues described herein below.

The polypeptide of the albumin family may be purified from natural sources, for example from serum or it may be produced by recombinant methods.

Globulin

The polypeptide may for example also be a member of the globulin family, such as globulins from serum or globulin from milk, for example lactoglobulin, such as bovine, caprine, equine, porcine or camelide lactoglobulin, such as bovine, caprine, equine, porcine or camelide beta-lactoglobulin. Thus, for example the polypeptide may be bovine beta-lactoglobulin (bLG) of SEQ ID NO: 4 or a functional homologue thereof sharing at least 75% sequence identity therewith. The functional homologue may be any of the functional homologues described herein below.

The polypeptide of the globulin family may be purified from natural sources, for example from serum or milk, or it may be produced by recombinant methods. In one preferred embodiment, bovine beta-lactoglobulin is purified from bovine milk.

Collagen domain and lectins

The polypeptide may also be a polypeptide comprising a collagen domain. A collagen domain according to the present invention is a domain including the motif -G-X-X- several times, in general at least 5 times, preferably at least 10 times, wherein G is glycine and X is any naturally occurring amino acid.

Polypeptides comprising collagen domains are frequently capable of oligomerisation, thus the polypeptide may be present as an individual polypeptide or as an oligomer of polypeptides.

The polypeptide may also be a lectin, i.e. a polypeptide capable of associating with one or more carbohydrates. Preferably the lectin comprises a carbohydrate binding domain.

The polypeptide may also be a lectin comprising a collagen domain, preferably a polypeptide comprising a carbohydrate binding domain and a collagen domain.

Polypeptides including a collagen domain may for example be ficolins or mannose binding lectin, such as mammalian mannose binding lectin. Thus, for example the polypeptide may be human mannonse binding lectin (hMBL) of SEQ ID NO: 3 or a functional homologue thereof sharing at least 75% sequence identity therewith. Functional homologues may be any of the functional homologues described herein below. Examples of useful functional homologues of MBL are for example described in Danish patent application PA 2006 01555 in the sections “MBL and MBL variants” and “Functional homologues” on pages 7-11. In particular, it is preferred that functional homologues of MBL retain at least some MBL function, preferably the functional homologues of MBL are capable of activating C4 in the presence of MASP-2. A useful functional assay for evaluating MBL function includes the assays, which are described in WO03/033522 in the section “Functionality” on pages 27-28.

In embodiments wherein the polypeptide is MBL or functional homologues thereof, the following applies. 3 individual MBL polypeptides in general associate to form a so-called monomer. These monomers, each consisting of 3 MBL polypeptides may then associate to form oligomers, such as dimers, trimers, tetramers, pentamers, hexamers or even higher oligomers. It is preferred within the present invention that the majority of MBL is present as oligomers comprising at least two monomers, preferably at least 3 monomers, wherein the monomers each consists of 3 MBL polypeptides.

The polypeptide comprising a collagen domain may be purified from natural sources, for example from serum or tissue extracts, or it may be produced by recombinant methods. In one preferred embodiment, human mannose binding protein is produced by recombinant methods involving heterologous expression of MBL in *in vitro* cultured mammalian cells, such as HEK cells and purification from the culture medium by conventional protein purification techniques. Useful methods for purifying MBL include for example the methods described in Examples 1, 2, 4 and 6 of WO03/033522 and in Examples 1, 2 and 2 of WO00/70043.

Fatty acid binding protein family

The polypeptide may for example be a polypeptide belonging to the fatty acid binding protein (FABP) superfamily. The fatty acid-binding protein (FABP) superfamily is constituted by 14-15 kDa soluble proteins which bind with a high affinity either long-chain fatty acids (LCFAs), bile acids (BAs) or retinoids. FABPs are members of the superfamily of lipid-binding proteins (LBP). The primary role of all the FABP family members is regulation of fatty acid uptake and intracellular transport. The structure of all FABPs is similar - the basic motif characterizing these proteins is beta-barrel, and a single ligand (e.g. a fatty acid, cholesterol, or retinoid) is bound in its internal water-filled cavity. FABPs have a tissue-specific distribution pattern.

For example the polypeptide may be a FABP with tissue-specific distribution for example: L-FABP (liver), I-FABP (intestinal), H-FABP (muscle and heart), A-FABP (adipocyte), E-FABP (epidermal), Il-FABP (ileal), B-FABP (brain), M-FABP (myelin) or T-FABP (testis).

The FABP may for example be a mammalian FABP such as human, bovine, caprine, equine, porcine or camelide FABP. Thus for example the polypeptide may be human adipocyte FABP of SEQ ID NO: 6 or a functional homologue thereof sharing at least 75% sequence identity therewith. Functional homologues may be any of the functional homologues described herein below.

The polypeptide of the FABP family may be purified from natural sources, for example from serum or it may be produced by recombinant methods.

Alpha-lactalbumin

In one embodiment of the present invention the polypeptide is a polypeptide other than alpha-lactalbumin.

However, in a preferred embodiment of the invention the polypeptide is alpha-lactalbumin.

Alpha-lactalbumin may be mammalian lactalbumin, such as human, bovine, caprine, equine, porcine or camelide alpha-lactalbumin. For example, alpha-lactalbumin may be bovine alpha-lactalbumin of SEQ ID NO: 2 or human alpha-lactalbumin of SEQ ID NO: 1 or a functional homologue sharing a sequence identity with any of the aforementioned sequences of at least 75%. Functional homologues may be any of the functional homologues described herein below. Examples of useful alpha-lactalbumins as well as functional homologues thereof are for example described Danish patent application PA 2007 00693 in the sections “Alpha-lactalbumin” and “Functional homologues of alpha-lactalbumin” p. 9-21. In particular, it is preferred that functional homologues of alpha-lactalbumin retain at least some alpha-lactalbumin function, preferably the functional homologues of alpha-lactalbumin are capable of forming a biologically active complex with a fatty acid, preferably oleic acid, said complex comprising cytotoxic activity or cell killing activity. A useful functional assay for evaluating alpha-lactalbumin function includes the assay, which is described in Example 7 of PA 2007 00693.

Alpha-lactalbumin may be purified from natural sources, for example from milk, or it may be produced by recombinant methods. In one preferred embodiment, bovine alpha-lactalbumin is purified from milk. In another preferred embodiment, human alpha-lactalbumin is produced by by recombinant methods involving heterologous expression of alpha-lactalbumin in host cells, for example yeast cells and purification from the culture medium by conventional protein purification techniques. Useful methods for purifying alpha-lactalbumin include for example the methods described in Examples 1, 2, 4 and 6 of WO03/033522 and in Examples 1, 2 and 2 of WO00/70043.

It is preferred that purified alpha-lactalbumin is used for the methods of the invention and that alpha-lactalbumin is purified prior to contacting with fatty acid. It is thus preferred that the liquid solution comprising the emulsifier provided in step a) of the method according to the invention comprises purified alpha-lactalbumin. Thus, said liquid solution is preferably essentially devoid of at least one, more preferably at least two, for example of all milk constituents other than water and alpha-lactalbumin, where "essentially devoid of" within the present context means that said components are present at levels below detection level. In particular, said liquid solution is preferably essentially devoid of at least one, preferably at least two, for example of all milk polypeptides other than alpha-lactalbumin. Said milk polypeptide may for example be selected from the group consisting of immunoglobulins and casein.

Functional homologues of polypeptides

A functional homologue of a polypeptide of a given sequence within the present invention is a polypeptide sharing at least some sequence identity with the given sequence and which shares at least one function, preferably, has cytotoxic activity when in complex with oleic acid.

Several methods may be used to determine whether a complex of a functional homologue and oleic acid has cell killing activity. More preferably, a complex of a functional homologue and oleic acid has an LD_{50} of at the most 50 mg/ml, preferably at the most 40 mg/ml, even more preferably at the most 30 mg/ml, yet more preferably at the most 20 mg/ml, even more preferably at the most 10 mg/ml, yet more preferably at the most 5 mg/ml, even more preferably at the most 1 mg/ml, for example at the most

0.75 mg/ml, such as at the most 0.5 mg/ml, when determined as described in Example 3 herein below.

Preferably, evolutionary conservation between polypeptides of different closely related species, e.g. assessed by sequence alignment, can be used to pinpoint the degree of evolutionary pressure on individual residues. Preferably, polypeptide sequences from at least 2, preferably at least 3, more preferably at least four different species where the function of the polypeptide is conserved are compared, for example but not limited to mammals including rodents, monkeys and apes. Conserved residues are more likely to represent essential amino acids that cannot easily be substituted than residues that change between species. For example, such an alignment may be performed using ClustalW from EBML-EBI. It is evident from the above that a reasonable number of modifications or alterations of a polypeptide sequence does not interfere with the activity of a given polypeptide. Thus, preferably, functional homologues of a given polypeptide comprise all residues, which are conserved between at least 4, such as at least 3, for example at least 2 different species. Functional homologues may thus comprise one or more amino acid substitutions at residues, which are not conserved between at least 4, such as at least 3, for example at least 2 different species.

It is preferred that at least some, for example at least 50%, such as all amino acid substitutions are “conservative”. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide.

A person skilled in the art will know how to make and assess ‘conservative’ amino acid substitutions, by which one amino acid is substituted for another with one or more shared chemical and/or physical characteristics. Conservative amino acid substitutions are less likely to affect the functionality of the protein. Amino acids may be grouped according to shared characteristics. A conservative amino acid substitution is a substitution of one amino acid within a predetermined group of amino acids for another amino acid within the same group, wherein the amino acids within a predetermined group exhibit similar or substantially similar characteristics, preferably the groups are

the groups listed below in "Lower levels of similarity", even more preferably the groups are the groups listed below in "High level of similarity".

Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within the groups of amino acids indicated herein below:

Lower levels of similarity:

Polarity:

i) Amino acids having polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys,)

ii) Amino acids having non-polar side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp, Pro, and Met)

Hydrophilic or hydrophobic:

iii) Hydrophobic amino acids (Ala, Cys, Gly, Ile, Leu, Met, Phe, Pro, Trp, Tyr, Val)

iv) Hydrophilic amino acids (Arg, Ser, Thr, Asn, Asp, Gln, Glu, His, Lys)

Charges:

v) Neutral amino acids (Ala, Asn, Cys, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val)

vi) Basic amino acids (Arg, His, Lys)

vii) Acidic amino acids ((asp, Glu)

High level of similarity:

viii) Acidic amino acids and their amides (Gln, Asn, Glu, Asp)

ix) Amino acids having aliphatic side chains (Gly, Ala Val, Leu, Ile)

x) Amino acids having aromatic side chains (Phe, Tyr, Trp)

xi) Amino acids having basic side chains (Lys, Arg, His)

xii) Amino acids having hydroxy side chains (Ser, Thr)

xiii) Amino acids having sulphor-containing side chains (Cys, Met),

More preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

It is clear from the above outline that the same functional homologue or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

Polypeptides of the invention may comprise standard and non-standard amino acids or mixtures of both. It is preferred that the polypeptides only comprise standard amino acids. There are twenty standard naturally occurring amino acids and two special amino acids, selenocysteine and pyrrolysine, as well as a vast number of "nonstandard amino acids" which are not incorporated into protein in vivo. Examples of nonstandard amino acids include the sulfur-containing taurine and the neurotransmitters GABA and dopamine. Other examples are lanthionine, 2-Aminoisobutyric acid, and dehydroalanine. Further non standard amino acids are ornithine and citrulline.

Non-standard amino acids are usually formed through modifications to standard amino acids. For example, taurine can be formed by the decarboxylation of cysteine, while dopamine is synthesized from tyrosine and hydroxyproline is made by a posttranslational modification of proline (common in collagen). Examples of non-naturally occurring amino acids are those listed e.g. in 37 C.F.R. section 1.822(b)(4), all of which are incorporated herein by reference.

Both standard and non standard amino acid residues described herein can be in the “D” or “L" isomeric form, preferably “L” isomeric form.

It is contemplated that a functional equivalent according to the invention may comprise any amino acid including non-standard amino acids. In preferred embodiments a functional equivalent comprises only standard amino acids.

The standard and/or non-standard amino acids may be linked by peptide bonds or by non-peptide bonds, preferably however by peptide bonds. The term peptide also embraces post-translational modifications introduced by chemical or enzyme-catalyzed reactions, as are known in the art. Such post-translational modifications can be introduced prior to partitioning, if desired. Amino acids as specified herein will preferentially be in the L-stereoisomeric form. Amino acid analogs can be employed instead of the 20 naturally-occurring amino acids. Several such analogs are known, including fluorophenylalanine, norleucine, azetidine-2-carboxylic acid, S-aminoethyl cysteine, 4-methyl tryptophan and the like.

A functional homologue within the scope of the present invention is a polypeptide that exhibits at least some sequence identity with a polypeptide of a given sequence, preferably functional homologues have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity with a given polypeptide sequence.

Sequence identity can be calculated using a number of well-known algorithms and applying a number of different gap penalties. The sequence identity is calculated relative to the full-length sequence of the reference polypeptide. Any sequence alignment tool, such as but not limited to FASTA, BLAST, or LALIGN may be used for searching homologues and calculating sequence identity. Moreover, when appropriate any commonly known substitution matrix, such as but not limited to PAM, BLOSSUM or PSSM matrices may be applied with the search algorithm. For example, a PSSM (position specific scoring matrix) may be applied via the PSI-BLAST program. Moreover, sequence alignments may be performed using a range of penalties for gap

opening and extension. For example, the BLAST algorithm may be used with a gap opening penalty in the range 5-12, and a gap extension penalty in the range 1-2.

Functional homologues may in one embodiment further comprise chemical modifications such as ubiquitination, labeling (e.g., with radionucleotides, various enzymes, etc.), pegylation (derivatization with polyethylene glycol), or by insertion (or substitution by chemical synthesis) of amino acids such as ornithine, which do not normally occur in human proteins.

In addition to the peptidyl compounds described herein, sterically similar compounds may be formulated to mimic the key portions of the peptide structure and that such compounds may also be used in the same manner as the peptides of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. For example, esterification and other alkylations may be employed to modify the amino terminus of, e.g., a di-arginine peptide backbone, to mimic a tetra peptide structure. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

Peptides with N-terminal alkylations and C-terminal esterifications are also encompassed within the present invention. Functional equivalents also comprise glycosylated and covalent or aggregative conjugates formed with the same molecules, including dimers or unrelated chemical moieties. Such functional equivalents are prepared by linkage of functionalities to groups which are found in fragment including at any one or both of the N- and C-termini, by means known in the art.

Functional homologues may also be deletion or addition mutants. The addition may be addition of at least one amino acid, an addition of from preferably 2 to 250 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids.

A functional homologue may be a deletion mutant which have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 %

sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity.

Deletion mutants suitably comprise at least 20 or 40 consecutive amino acid and more preferably at least 80 or 100 consecutive amino acids in length.

It is preferred that functional homologues of a given polypeptide comprises at the most 500, more preferably at the most 400, even more preferably at the most 300, yet more preferably at the most 200, such as at the most 175, for example at the most 160, such as at the most 150 amino acids in addition to the sequence of the given polypeptide.

Examples of functional homologues of alpha-lactalbumin

Functional homologues of alpha-lactalbumin may be functional homologues as described above in this section. More specific examples of useful functional homologues are given below.
Preferably, evolutionary conservation between alpha-lactalbumin of different closely related species, e.g. assessed by sequence alignment, can be used to pinpoint the degree of evolutionary pressure on individual residues. Preferably, alpha-lactalbumin sequences are compared between species where alpha-lactalbumin function is conserved, for example but not limited to mammals including rodents, monkeys and apes. Residues under high selective pressure are more likely to represent essential amino acids that cannot easily be substituted whereas residues that change between species may more preferably be substituted. For example, such an alignment may be performed using ClustalW from EBML-EBI comparing porcine alpha-lactalbumin and human alpha-lactalbumin (see Figure 1 A of PCT application WO2008/058547). Figure 1A of PCT application WO2008/058547 shows an alignment of the protein sequences of bovine, human, equine, caprine, bovine, camelide and porcine alpha-lactalbumin wherein identical residues ("*") and residues with conservative (":") and semi-conservative (".") substitutions are marked. It is evident from the above that a reasonable number of modifications or alterations of the bovine or human alpha-lactalbumin sequence does not interfere with the activity of the alpha-lactalbumin molecule according to the invention. Such alpha-lactalbumin molecules are herein referred to as functional equivalents of bovine or human alpha-lactalbumin, and may be

such as variants and fragments of native bovine or human alpha-lactalbumin as described here below.

Functional assays can for example be used in order to determine if alpha-lactalbumin function is conserved. Functional assays for evaluating alpha-lactalbumin function include assay for cytotoxic activity in complex with oleic acid and include, but are not limited to, assays described herein in Example 3 and in Danish patent application PA 2007 0693.

Accordingly, in one embodiment of the invention it is preferred that functional homologues of alpha-lactalbumin comprises a sequence with high sequence identity to SEQ ID NO: 1 or SEQ ID NO:2, wherein none of the conserved residues marked with "*" in figure 1A of PCT application WO2008/058547 are substituted. It is furthermore preferred within this embodiment that the residues marked with ":" in figure 1A of PCT application WO2008/058547are either not substituted or only substituted by conservative substitution, more preferably by substitution with an amino acid with a high level of similarity as defined herein below.

Thus in one embodiment it is preferred that functional homologues of bovine alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 2, wherein residues E1, L3, E7, V8, L15, Y18, V21, S22, V27, Q39, A40, I41, N44, I59, K62, Q65, I85, M90, N102, S112, D116, K122 are either not substituted or substituted only by conservative substitution, more preferably substituted only an amino acid with a high level of similarity as defined herein below.

It is even further preferred within the present invention that functional homologues of alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO:1 or SEQ ID NO: 2, wherein residues marked with "." in figure 1A of PCT application WO2008/058547 are either not substituted or are only substituted by conservative substitutions, such as with amino acids with lower levels or high level of similarity as defined herein below. Accordingly, it is preferred that functional homologues of bovine alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 2, wherein residues D14, K16, G17, G20, P24, S47, N56, D63, D64, N74, V92, and A109 are either not substituted or only substituted by conservative substitutions, such as with amino acids with lower level or high level of similarity as defined herein below.

It is also comprised within the present invention that functional homologues of alpha-lactalbumin may have a sequence with high sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2, wherein the unmarkes residues in Figure 1A of PCT application WO2008/058547 may be substituted with any other amino acid. Thus, functional homolgoues of human alpha-lactalbumin may have a sequence with high sequence identity to SEQ ID NO: 1, wherein residues F9, R10, E11, G19, W25, T29, T30, T33, Q43, D46, T48, N66, P67, H68, S70, I89, K98, V99, L118, and L123 are either not substituted or substituted with any other amino acid.

Functional homologues of alpha-lactalbumin may also be addition mutants as described herein above, in particular addition mutants may be functional homologues comprising at least one additional methionine. Preferably, the addition mutant consists of SEQ ID 1 or SEQ ID 2 extended by one additional Methionine at the N- or C-terminus, preferably at the N-terminus.

Preparation of polypeptides

Polypeptides may be purified from natural sources, which should be selected according to the occurrence of the polypeptide. Non-limiting examples of natural sources includes cell extracts, tissue extracts, plant extracts, body fluids, such as saliva or serum, milk or eggs. Polypeptides may also be recombinantly produced as described in more details herein below and then optionally be purified from host cells expressing the heterologous protein, from host organisms, such as transgenic plants or animals expressing the heterologous polypeptide or from tissue culture medium from host cells expressing the heterologous polypeptides.

Purification of proteins in general involves one or more steps of removal of or separation from contaminating nucleic acids, phages and/or viruses, other proteins and/or other biological macromolecules. The procedure may comprise one or more protein isolation steps. Any suitable protein isolation step may be used with the present invention. The skilled person will in general readily be able to identify useful protein isolation steps for a given polypeptide using routine experimentation.

The protein isolation steps useful with the present invention may be commonly used methods for protein purification including for example chromatographic methods such as for example gas chromatography, liquid chromatography, ion exchange chromatography and/or affinity chromatography; filtration methods such as for example gel filtration and ultrafiltration; precipitation, such as ammonium sulphate precipitation and/or gradient separation such as sucrose gradient separation. The purification may comprise one or more of the aforementioned methods in any combination.

The aforementioned methods are well known to the skilled person and may for example be performed as described in the "Protein Separation Handbook Collection" including the titles "Antibody Purification", "The Recombinant Protein Handbook", "Protein Purification", "Ion Exchange Chromatography", "Affinity Chromatography", "Hydrophobic Interaction Chromatography", "Gel Filtration", "Reversed Phase Chromatography", "Expanded Bed Adsorption" and "Chromatofocusing" prepared by Amersham Biosciences and available from GE.

The polypeptides of the invention may also be recombinantly prepared, in particular functional homologues are preferably produced recombinantly. Useful recombinant production methods includes conventional methods known in the art, such as by expression of heterologuos polypeptide or functional homologues thereof in suitable host cells such as *E. coli*, *S. cerevisiae* or *S. pombe* or insect or mammalian cells suitable for production of recombinant proteins (see below). The skilled person will in general readily be able to identify useful recombinant techniques for the production of recombinant proteins in general.

In one embodiment the polypeptides are produced in a transgene plant or animal. By a transgenic plant or animal in this context is meant a plant or animal which has been genetically modified to contain and express a nucleic acid encoding the given polypeptide or functional homolgue hereof.

In one aspect of the present invention, the polypeptide is produced by host cells comprising a first nucleic acid sequence encoding the given polypeptide or a functional homologue thereof operably associated with a second nucleic acid capable of directing expression in said host cells. The second nucleic acid sequence may thus comprise or even consist of a promoter that will direct the expression of protein of interest in said

cells. A skilled person will be readily capable of identifying useful second nucleic acid sequence for use in a given host cell.

The process of producing recombinant polypeptide or a functional homologue thereof in general comprises the steps of:

-providing a host cell

-preparing a gene expression construct comprising a first nucleic acid sequence encoding a given polypeptide or a functional homologue thereof operably linked to a second nucleic acid sequence capable of directing expression of said protein of interest in the host cell

-transforming the host cell with the construct,

-cultivating the host cell, thereby obtaining expression of the polypeptide or the functional homologue thereof.

The recombinant polypeptide thus produced may be isolated by any conventional method for example by any of the protein purification methods described herein above. The skilled person will be able to identify a suitable protein isolation steps for purifying any protein of interest.

In one embodiment of the invention, the recombinantly produced LA or the functional homologue thereof is excreted by the host cells.

In a preferred embodiment of the invention, the polypeptide is recombinantly produced in vitro in host cells and is isolated from cell lysate, cell extract or from tissue culture supernatant. In a more preferred embodiment polypeptide is produced by host cells that are modified in such a way that they express the polypeptide of interest. In an even more preferred embodiment of the invention said host cells are transformed to produce and excrete the polypeptide.

The gene expression construct may comprise a viral based vector, such as a DNA viral based vector, a RNA viral based vector, or a chimeric viral based vector. Examples of

DNA viruses are cytomegalo virus, Herpex Simplex, Epstein-Barr virus, Simian virus 40, Bovine papillomavirus, Adeno-associated virus, Adenovirus, Vaccinia virus, and Baculo virus. However, the gene expression construct may for example only comprise a plasmid based vector.

In one aspect the invention provides an expression construct encoding a given polypeptide or functional homologues thereof, featured by comprising one or more intron sequences for example from the native gene. Additionally, it may contain a promoter region derived from a viral gene or a eukaryotic gene, including mammalian and insect genes.

The promoter region is preferably selected to be different from the native promoter, and preferably in order to optimize the yield, the promoter region is selected to function most optimally with the vector and host cells in question.

In a preferred embodiment the promoter region is selected from a group comprising Rous sarcoma virus long terminal repeat promoter, and cytomegalovirus immediate-early promoter, and elongation factor-1 alpha promoter.

In another embodiment the promoter region is derived from a gene of a microorganism, such as other viruses, yeasts and bacteria.

In order to obtain a greater yield of recombinant LA or functional homologue thereof, the promoter region may comprise enhancer elements, such as the QBI SP163 element of the 5' end untranslated region of the mouse vascular endothelian growth factor gene

One process for producing recombinant LA according to the invention is characterised in that the host cell culture is may be eukaryotic, for example a mammalian cell culture or a yeast cell culture.

Useful mammalian cells may for example be human embryonal kidney cells (HEK cells), such as the cell lines deposited at the American Type Culture Collection with the numbers CRL-1573 and CRL-10852, chick embryo fibroblast, hamster ovary cells, baby hamster kidney cells, human cervical carcinoma cells, human melanoma cells,

human kidney cells, human umbilical vascular endothelium cells, human brain endothelium cells, human oral cavity tumor cells, monkey kidney cells, mouse fibroblast, mouse kidney cells, mouse connective tissue cells, mouse oligodendritic cells, mouse macrophage, mouse fibroblast, mouse neuroblastoma cells, mouse pre-B cell, mouse B lymphoma cells, mouse plasmacytoma cells, mouse teratocarcinoma cells, rat astrocytoma cells, rat mammary epithelium cells, COS, CHO, BHK, 293, VERO, HeLa, MDCK, WI38, and NIH 3T3 cells.

The host cells may also either be prokaryotic cells or yeast cells. Prokaryotic cells may for example be *E. coli*. Yeast cells may for example be *Saccharomyces, Pichia or Hansenula*.

The aforementioned methods are well known to the skilled person and may for example be performed as described in the Current Protocols in Molecular Biology, 2001, by John Wiley and Sons, Inc. edited by Frederick M. Ausubel et al.

Fatty acids

The present invention regards complexes of emulsifier and fatty acid, wherein the emulsifier may be any of the emulsifiers described above, and the fatty acid may be any of the fatty acids described in this section.

The fatty acid according to the present invention has the general formula:

R-COOH,

wherein R is alkyl or alkenyl, such as C_4 to $C_{80,}$alkyl or alkenyl, preferably C_{10} to C_{30} alkyl or alkenyl, more preferably alkenyl. Preferably, said alkyl or alkenyl are unbranched.

Fatty acids are carboxylic acids, which preferably have a long unbranched aliphatic chain. As the biosynthesis of fatty acids involves acetyl-CoA, in which the acetic unit contains two C-atoms, most natural fatty acids have an even number of C atoms ranging from 4 to 80 C atoms. The aliphatic chain of a fatty acid can be either saturated

or unsaturated. Saturated fatty acids are saturated with hydrogen and thus have no double bonds. Unsaturated fatty acids can be either mono-unsaturated (or MUFAs), having one double bond or poly-unsaturated (PUFAs), having 2 or more double bonds. The fatty acids of the present invention may be a saturated fatty acid or an unsaturated fatty acid, however preferably the fatty acid is an unsaturated fatty acid.

In preferred embodiments of the invention the fatty acid is selected from the group of C4 to C30, for example from C6 to C28, such as from C8 to C26, for example from C10 to C24, such as from C12 to C22, for example from C14 to C20, such as from C16 to C20, for example from the group of C16, C17, C18 and C20, such as from the group of C16, C18 and C20. In one preferred embodiment the fatty acid is selected from the group of C16, C17, C18 and C20. In one particular embodiment of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof, then the fatty acid is preferably a C17 fatty acid, more preferably a C17 fatty acid comprising at least one double bond, which preferably is a cis double bond, more preferably all double bonds are cis double bonds, even more preferably said C17 fatty acid comprises a cis double bond in the 10 position.

Fatty acids are often described using the number of C-atoms of the chain and the number, location and conformation of double bonds. Steric acid, for example, has a chain of 18 C-atoms and no double bonds and can be described as C18:0, oleic acid has a chain of 18 C-atoms and one double bond and can be described as C18:1, linoleic acid has a chain of 18 C-atoms and two double bonds and can be described as C18:2 and so forth.

The double bond is located on the *x*th carbon-carbon bond, counting from the carboxyl terminus. The Latin prefixes *cis* (on this side) and *trans* (across) describe the conformation of the double bonds by describing the orientation of the hydrogen atoms with respect to said double bond. Double bonds in the *cis* conformation are preferred. The position of the double bond is frequently indicated as the last number, following the integer indicating the number of double bonds. Thus, for example oleic acid having an 18 carbon chain with one double bond between carbon 9 and 10 may be described as C18:1:9cis and α-linolenic acid having an 18 carbon chain with three double bonds between carbon 9 and 10, 12 and 13 and 15 and 16, respectively, may be described as

C18:3:9,12,15. Cis or trans may be indicated after the position of the double bond. If there is more than one double bond and they all are of the same conformation, then the term cis or trans may be indicated after indication of the position of all double bonds and thus relates to the conformation of all double bonds. Thus, for example Linoleic acid having an 18 carbon chain with 2 double bonds, which are both cis double bonds between carbons 9 and 10 and 12 and 13, respectively may be described as C18:2:9,12cis

In preferred embodiments of the present invention the fatty acid has in the range of 0 to 6 double bonds, for example in the range of 1 to 5 double bonds, such as the number of double bonds is selected from the group of 1, 2, 3 or 4 double bonds. In more preferred embodiments of the invention the fatty acid has 1 or 3 double bonds. In a most preferred embodiment of the invention the fatty acid has one double bond.

Examples of unsaturated fatty acids that may be used with the invention includes for example:

Oleic acid: $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ or C18:1:9cis
Elaidic acid: $CH_3(CH_2)_7CH==CH(CH_2)_7COOH$ or C18:1:9trans
Linoleic acid: $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$ or C18:2:9,12cis
Alpha-linolenic acid:
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$ or C18:3:9, 12, 15 cis
Gamma linolenic acid: C18:3:6,9,12cis
Arachidonic acid: $CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$ or C20:4:5, 8,11 ,14cis
Eicosapentaenoic acid:
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$ or C20:5: 5,8,11,14,17Cis
Docosahexaenoic acid:
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$ or C22:6: 4,7,10,13,16,19Cis
Erucic acid: $CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$ or C22:1
Vaccenic acid: $CH_3(CH_2)_5CH==CH(CH_2)_9COOH$ or C18:1:11cis
Palmitoleic acid: $CH_3(CH_2)_5CH=CH(CH_2)_7COOH$ or16:1:9cis
Petroselinic acid: $CH_3.(CH_2)_{10}.CH=CH(CH_2)_4.COOH$ or C18:1:6cis

Stearidonic acid: C18:4:6, 9, 12, 15cis
Heptadecenoic Acid: C17:1:10cis
Eicosenoic Acid: $CH_3(CH_2)_7CH=CH(CH_2)_9COOH$ or 20:1:11cis
Gondoic acid or cis-11-EICOSENOIC ACID: $CH_3(CH_2)_7CH=CH(CH_2)_9COOH$ or C29:1:9cis

In an embodiment a mono-saturated fatty acid is complexed with emulsifier. More preferred are mono-saturated fatty acids selected from the group of: C17:1:10cis or trans, C16:1:6cis or trans, C16:1:9cis or trans, C16:1:11cis or trans, C18:1:6cis or trans, C18:1:9cis or trans, C18:1:11cis or trans, C18:1:13cis or trans, C20:1:9 cis or trans, C20:1:11cis and trans, C20:1:13cis or trans.

In a preferred embodiment the mono-saturated fatty acid complexed with emulsifier is in the cis conformation such a fatty acid selected from the group of: C17:1:10cis, C16:1:6cis, C16:1:9cis, C16:1:11cis, C18:1:6cis, C18:1:9cis, C18:1:11cis, C18:1:13cis, C20:1:9cis, C20:1:11cis, C20:1:13cis.

In another preferred embodiment the fatty acid complexed with emulsifier is an unsaturated fatty acid in the cis conformation, preferably selected from the group consisting of C17:1:10cis, C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis.

In another preferred embodiment the fatty acid complexed with emulsifier is selected from the group consisting of C16 to C20 fatty acids comprising in the range of 1 to 5 cis double bonds. Thus, the fatty acid may for example be selected from the group consisting of Vaccenic Acid C18:1:11cis, Linoleic Acid C18:2:9,12cis, Alpha Linolenic Acid C18:3:9,12,15, Palmitoleic Acid C16:1:9cis, Heptadecenoic Acid C17:1:10cis, Gamma Linolenic Acid C18:3:6,9,12cis, Stearidonic acid C18:4:6,9,12,15cis, Eicosenoic Acid C20:1:11cis and Eicosapentaenoic Acid C20:5:5,8,11,14,17cis, such as from the group consisting of Vaccenic Acid C18:1:11cis, Linoleic Acid C18:2:9,12cis, Alpha Linolenic Acid C18:3:9,12,15.

In one preferred embodiment the fatty acid is one or more selected from the group consisting of oleic acid, linoleic acid and vaccenic acid. In another preferred embodiment the fatty acid is Heptadecenoic Acid.

In a very preferred embodiment of the invention the fatty acid complexed with alpha-lactalbumin is an unsaturated C16 or C18 fatty acid, preferably a C18 fatty acid, wherein all double bonds are cis double bonds. In this embodiment the fatty acid may preferably comprise 1, for example 2, such as 3, for example 4 double bonds, wherein all double bonds are cis double bonds. Thus, the fatty acid may for example be selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis and C18:4:6, 9, 12, 15cis, preferably selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:1:6cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis and C18:4:6, 9, 12, 15cis, for example selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:3:6,9,12cis, C18:3:9,12,15cis and C18:2:9,12cis. In another preferred embodiment of the invention the fatty acid complexed with alpha-lactalbumin is an unsaturated C17 fatty acid, preferably C17:1:10cis.

Most preferred fatty acids are according to the invention C17:1:10cis, C18:1:9cis and C18:1:11cis. C18:1:9cis is highly preferred for the complex of the invention.

In an alternative embodiment a polyunsaturated fatty acid is complexed with emulsifier. Preferably a polyunsaturated acid selected from the group of C18:2:9,12cis, C18:3:9,12,15cis, C18:3:6,9,12cis, and C20:4:5,8,11 15cis.

In one embodiment the fatty acid is an artificial fatty acid.

The fatty acid may be purified from a natural source, be prepared by organic synthesis or may be commercially purchased. Fatty acids are for example available from Sigma-Aldrich, Denmark or Merck, Darmstadt, Germany

The fatty acid may be provided as a pure fatty acid or in a mixture with impurities and/or other fatty acids, preferably all impurities are lipophilic. Thus, in one embodiment the fatty acid may have a purity of in the range of 50 to 100%, such as in

the range of 60 to 100%, for example in the range of 65-99%, such as in the range of 65-88%, for example approx. 99%, such as 99%.

Method of producing a complex of emulsifier and fatty acid

The complexes according to the present invention are prepared by a method comprising the steps of:

a. providing an emulsifier in an aqueous solution, wherein said emulsifier is not a fatty acid (preferably the emulsifier is a polypeptide); and
b. providing a fatty acid; and
c. contacting the emulsifier with the fatty acid; and
d. mixing the emulsifier and the fatty acid using high shear mixing

The method of preparing cytotoxic complexes of emulsifier and fatty acid is also referred to as “conversion” or “conversion method” herein.

Within the meaning of the present invention an emulsifier is in complex with fatty acid, when said emulsifier is associated with one or more fatty acids. One method for determining whether an emulsifier is associated with a fatty acid is to determine whether emulsifier and fatty acid may be recovered together during a purification. In particular if fatty acid may be recovered together with emulsifier in the aqueous phase after centrifugation for 10 min at 3000 x g, then the emulsifier is said to be in complex with the fatty acid recovered in the aqueous phase.

The emulsifier may be any of the emulsifiers described herein above, and preferably the emulsifier is a polypeptide. Many emulsifiers are commercially available. If the emulsifier is a polypeptide it may be prepared and provided as described herein above in the section “Methods of preparing polypeptides”.

The emulsifier and the fatty acid may be mixed in any suitable ratio, however preferably the fatty acid is added in molar excess over emulsifier, such as polypeptide. Molar excess means that there are more moles of a fatty acid than there is of emulsifier. The molar excess of a fatty acid over emulsifier added during preparation, may be that for

one mole emulsifier in the range of 1.5 moles and 50 moles of a fatty acid are added, such as for one mole emulsifier in the range of 2 moles and 45 moles of a fatty acid are added, for example in the range of 3 moles and 40 moles of a fatty acid are added. Thus, in a preferred embodiment the concentration of fatty acid, such as oleic acid is in the range of 0.01 mM to 200 mM, such as in the range of 0.05 mM to 100 mM, for example in the range of 0.1 mM to 50 mM, such as in the range of 0.2 mM to 25 mM, for example in the range of 0.5 mM to 10 mM, such as in the range of 1mM to 5mM, such as around 2 mM.

In one embodiment of the invention the method furthermore comprises a step of adding an alcohol. Preferably said alcohol is added to the fatty acid, more preferably said alcohol is added to said fatty acid. Thus the method may for example comprise the steps of

a. providing an emulsifier in an aqueous solution, wherein said emulsifier is not a fatty acid; and
b. providing a fatty acid; and
c. providing an alcohol; and
d. contacting the fatty acid with the alcohol, thereby obtaining a mixture of fatty acid and alcohol; and
e. contacting the emulsifier with the mixture of fatty acid and alcohol; and
f. mixing the emulsifier, the fatty acid and the alcohol using high shear mixing

The alcohol may be any alcohol, preferably, the alcohol has the general formulae:

R-OH, wherein R is alkyl or alkenyl, even more preferably C_{2-20} alkyl or alkenyl; yet more preferably alkyl, even more preferably C_{2-20} alkyl, for example C_{2-10} alkyl, such as C_{2-5} alkyl, for example ethanol.

Preferably the alcohol is added in excess of the fatty acid. When both alcohol and fatty acid are liquids it is preferred that ratio on a volume basis between alcohol and fatty acid is at least 2:1, preferably at least 5:1, for example at least 10:1, such as in the range of 2 -100:1, for example in the range of 5-100:1, such as in the range of 5 -50:1, for example in the range of 5-20:1, such as in the range of 10 -100:1, for example in

the range of 10-50:1, such as in the range of 10-20:1, for example in the range of 10-15:1.

In embodiments of the invention wherein alcohol is added it is preferred that the fatty acid is added in molar excess over emulsifier, such as polypeptide. The molar excess of a fatty acid over emulsifier added during preparation, may be that for one mole emulsifier in the range of 1.5 moles to 50 moles of a fatty acid are added, such as for one mole emulsifier in the range of 2 moles to 45 moles of a fatty acid are added, for example in the range of 3 moles to 40 moles of a fatty acid are added, such as for one mole emulsifier in the range of 5 moles to 30 moles of a fatty acid are added, for example in the range of 10 moles to 20 moles of a fatty acid are added.

Typically, the alcohol/fatty acid mixture is added to emulsifier in aqueous solution so that for each 20 µl of pure fatty acid in the range of 1 to 100 ml, preferably in the range of 5 to 50 ml, more preferably in the range of 10 to 30, even more preferably in the range of 15 to 25 ml, such as around 20 ml, for example 20 ml emulsifier in aqueous solution is added. The concentration of the emulsifier in the aqueous solution is adjusted in order to obtain the above-mentioned molar ratio between fatty acid and emulsifier.

In other embodiments of the invention no alcohol is added during the conversion method. In these embodiments it is preferred that fatty acid at a purity as described herein above in the section “Fatty acid” is mixed with emulsifier in aqueous solution using high shear mixing.

The concentration of the emulsifier in the aqueous solution is in general in the range of 0.1 to 50 mg/ml, more preferably in the range of 0.1 to 25 mg/ml, yet more preferably in the range of 0.1 to 20 mg/ml, for example in the range of 0.1 to 15 mg/ml, such as in the range of 1 to 50 mg/ml, for example in the range of 1 to 25 mg/ml, such as in the range of 1 to 20 mg/ml, for example in the range of 1 to 15 mg/ml, such as in the range of 5 to 50 mg/ml, for example in the range of 5 to 25 mg/ml, such as in the range of 5 to 20 mg/ml, for example in the range of 5 to 15 mg/ml, such as in the range of 10 to 50 mg/ml, for example in the range of 10 to 25 mg/ml, such as in the range of 10 to 20 mg/ml, for example in the range of 10 to 15 mg/ml. This is in particular the case for embodiments wherein no alcohol is added.

Mixing the emulsifier and the fatty acid using high shear mixing may be performed using any suitable high shear mixing method known to the skilled person.

Examples includes high speed mixing in a stirred tank, preferably with baffles, by vigorous stirring with an impeller capable of producing turbulent flow, such as one or more rushton turbines, mixing by use of a motionless mixer, containing a number of mixing elements successively dividing and combining the fluid stream mixing in a tank by jet mixing with high flow, mixing using a propeller-like mixer, vortex mixing or subjecting the liquid to a quick flow through a narrow opening or a capillary.

Depending on the particular method of mixing, high shear may be determined using a variety of different parameters.

In one embodiment it is preferred that "high shear mixing" is mixing with a shear at least equivalent to the shear obtained by vortex mixing using an IKA MS2 Minishaker (IKA Works Inc., Wilmington, NC 28405, USA) vortex mixer with at least 1000 rpm, preferably at least 1500 rpm, even more preferably at least 2000 rpm, more preferably at least 2200 rpm, yet more preferably at least 2500 rpm, such as in the range of 1000 to 2500 rpm, for example in the range of 1000 to 2500 rpm, such as in the range of 1000 to 2500 rpm, for example in the range of 2000 to 2500 rpm, such as 2500 rpm in the range of 1000 to 10,000 rpm, for example in the range of 1000 to 5000 rpm for at least 5 sec., preferably at least 10 sec., such as in the range of 5 sec. to 1 hour, for example in the range of 5 sec. to 10 min, such as in the range of 5 sec. to 1 min, for example in the range of 5 sec. to 30 sec, such as in the range of 5 sec. to 20 sec., for example in the range of 10 sec. to 1 hour, such as in the range of 10 sec. to 10 min., such as in the range of 10 sec. to 1 min, for example in the range of 10 sec. to 30 sec, such as in the range of 10 sec. to 20 sec., for example for 10 sec.

In a very preferred embodiment of the invention "high shear mixing" is vortex mixing using a vortex mixer at at least 1000 rpm, preferably at least 1500 rpm, even more preferably at least 2000 rpm, more preferably at least 2200 rpm, yet more preferably at least 2500 rpm, such as in the range of 1000 to 10,000 rpm, for example in the range of 1000 to 5000 rpm, such as in the range of 1000 to 3000 rpm, for example in the range of 2000 to 10,000 rpm, such as in the range of 2000 to 5000 rpm, for example in

the range of 2000 to 3000 rpm, for example in the range of 2000 to 3000, such as in the range of 2200 to 2500, such as in the range of 2500 to 10,000 rpm, for example in the range of 2500 to 5000 rpm, such as in the range of 2500 to 3000 rpm, for example in the range of 2400 to 2600 rpm, such as 2500 rpm in the range of 1000 to 10,000 rpm, for example in the range of 1000 to 5000 rpm for at least 5 sec., preferably at least 10 sec., such as in the range of 5 sec. to 1 hour, for example in the range of 5 sec. to 10 min, such as in the range of 5 sec. to 1 min, for example in the range of 5 sec. to 30 sec, such as in the range of 5 sec. to 20 sec., for example in the range of 10 sec. to 1 hour, such as in the range of 10 sec. to 10 min., such as in the range of 10 sec. to 1 min, for example in the range of 10 sec. to 30 sec, such as in the range of 10 sec. to 20 sec., such as 15 to 25 sec., for example for 10 sec, such as 20 sec. Vortex mixing is sometimes also referred to as whirley mixing and vortex mixer may also be referred to as whirley mixers. Any suitable vortex mixer may be used with the methods of the invention for example an IKA MS2 Minishaker (IKA Works Inc., Wilmington, NC 28405, USA).

In some embodiments the “high shear mixing” may be performed by subjecting the liquid to a quick flow through a capillary. Preferably, said quick flow has a laminar flow rate of at least 1 m/s, preferably at least 5 m/s, such as at least 10 m/s, for example in the range of 1 to 50 m/s, such as in the range of 5 to 25 m/s, for example in the range pf 5 to 15 m/s, such as around 10 m/s. The inner diameter of the capillary is preferably at the most 500 µm, preferably at the most 250 µm, more preferably at the most 180 µm, such as in the range of 50 to 250 µm, for example in the range of 100 to 200 µm, such as in the range of 150 to 180 µm. Examples of methods for subjecting a liquid to a quick flow through a capillary are described in Jaspe and Hagen, 2006, Biophysical Journal, 91: 3415-3424.

In one embodiment of the invention high shear mixing may be performed using an impeller mixer in which the mixture is centrifuged against the walls of the mixer chamber. The speed of the mixer will depend upon the size and capacity of the mixer. The mixing may for example be carried out at a speed of at least 5 ms^{-1}, such as between 30 and 80 ms^{-1}.

In one embodiment the high shear mixing may be performed using a propeller-like mixer. For propeller-like mixers the mixing efficiency may be approximated by the

turnover rate, where the turnover rate is the stir rate (rev/sec.) times the turnover volume (ml/rev)) divided by the aqueous volume measured in turnovers/sec. It is preferred that the mixing efficiency be greater than about 0.10 turnovers/sec, and preferably greater than 0.5 turnovers/sec and most preferably greater than 1 turnover/sec.

In one embodiment the high shear mixing may be achieved using a pump comprising a stator and a rotor. The shear rate may then be defined by the equation shear rate = V x 1000/W where shear rate is expressed in units of sec', V is the circumferential linear velocity of the rotor, in meters per second, and W is the gap width defined by the stator and the rotor, in millimeters. Preferred shear rates are indicated below. In one embodiment, the stator and the rotor define a gap width of about 0.01 to about 1 millimeter. Within this range, the gap width may preferably be at least about 0.05 millimeter, more preferably at least about 0.10 millimeter. Also within this range, the gap width may preferably be up to about 0.5 millimeter, more preferably up to about 0.25 millimeter. In another embodiment, the rotor has a circumferential linear velocity of about 1 to about 100 meters per second. Within this range, the velocity may preferably be at least about 5 meters per second, more preferably at least about 10 meters per second. Also within this range, the velocity may preferably be up to about 60 meters per second, more preferably up to about 40 meters per second. Apparatus suitable in this particular embodiment is described, for example, in European Patent No. 135,697 B1 to Schreiber or commercially available as, for example, the Siefer Trigonal SM 180 centrifugal pump from Wilhelm Siefer GmbH & Co., Velbert, Germany.

In one embodiment the “high shear mixing” is mixing at a shear rate of at least 10,000 sec^{-1}, preferably at least 20,000 sec^{-1}, such as at least 50,000 sec^{-1}, for example at least 100,000 sec^{-1}, such as in the range of 10^4 to 10^8 sec^{-1}, for example in the range of 10^4 to 10^7 sec^{-1}, such as in the range of 10^5 to 10^8 sec^{-1}, for example in the range of 10^5 to 10^7 sec^{-1}.

The exact shear rate during a given mixing procedure may be difficult to determine and also the shear rate may not be completely homogenous throughout a sample. Thus preferably the above-mentioned shear rates indicate an approximate average shear rate during mixing.

For embodiments of the invention wherein the high shear mixing is performed by subjecting the liquid to a quick flow through a capillary the shear rate may be determined as follows:

Average shear rate= $\frac{8Q}{3\pi R^3}$

wherein Q is the laminar flow rate and R is the inner radius of the capillary. Details regarding methods for determining the shear rate of laminar flow through a capillary may for example be found in Jaspe and Hagen, 2006, Biophysical Journal, 91:3415-3424.

Following the mixing step the conversion may optionally contain a step of centrifugation. The cytotoxic complexes are contained within the aqueous phase and thus following centrifugation the aqueous phase is then preferably recovered. In particular, in embodiments of the invention wherein no alcohol is added during the conversion, then it is preferred that the method comprises a step of centrifugation in order to remove excess fatty acid.

The centrifugation is preferably performed at no more than 20,000 x g, such as no more than 18,000 x g, for example at in the range of 100 x g to 20,000 x g, for example in the range of 150 x g to 18,000 x g for in the range of 2 to 20 min., preferably for in the range of 5 to 10 min. It is preferred that only one round of centrifugation is performed.

Ratio

When used herein “approximately” means a given value +/- 10%.

The ratio between the emulsifier (i.e. any of the emulsifiers described herein above in the sections “Emulsifier” and “Polypeptide”) and the fatty acid (i.e. any of the fatty acids described herein above in the section “Fatty acids”) in the complex may be any useful ratio. Thus, by way of example, the ratio between alpha-lactalbumin and oleic acid in complex may be any useful ratio. It is generally preferred that the molar ratio of fatty

acid to emulsifier in the complex is at least 1:1, however preferably it is higher as described below. Thus, the ratios indicated in this section relates to the ratio in the complex and not to the ratios of the mixture before complex formation.

In certain embodiments and in particular in embodiments wherein the emulsifier is alpha-lactalbumin, then it is preferred that the molar ratio of fatty acid to emulsifier is at least 2:1, preferably at least 3:1, even more preferably at least 4:1, yet more preferably at least 5:1, such as at least 6:1, for example at least 7:1, such as in the range of 2:1 to 30:1, for example in the range of 3:1 to 30:1, such as in the range of 4:1 to 30:1, for example in the range of 5:1 to 30:1 , such as in the range of 2:1 to 25:1, for example in the range of 2:1 to 20:1 , such as in the range of 2:1 to 15:1, for example in the range of 2:1 to 13:1 , such as in the range of 3:1 to 25:1, for example in the range of 3:1 to 20:1 , such as in the range of 3:1 to 15:1, for example in the range of 3:1 to 13:1, such as in the range of 4:1 to 25:1, for example in the range of 4:1 to 20:1 , such as in the range of 4:1 to 15:1, for example in the range of 4:1 to 13:1, such as in the range of 5:1 to 25:1, for example in the range of 5:1 to 20:1 , such as in the range of 5:1 to 15:1, for example in the range of 5:1 to 13:1 such as in the range of 6:1 to 25:1, for example in the range of 6:1 to 20:1 , such as in the range of 6:1 to 15:1, for example in the range of 6:1 to 13:1, preferably in the range of 7:1 to 12:1.

In some embodiments wherein the emulsifier is alpha-lactalbumin, then it is preferred that the molar ratio of fatty acid to emulsifier is at least 2:1:, for example at least 3:1, such as at least 4:1, for example at least 5:1, such as at least 6:1, for example at least 10:1, such as at least 11:1, for example at least 12:1, such as at least 13:1, for example at least 14:1, such as at least 15:1, for example at least 16:1, such as at least 17:1, for example at least 18:1, such as at least 19:1, for example at least 20:1.

In some embodiments wherein the emulsifier is alpha-lactalbumin, then it is preferred that the molar ratio of fatty acid to emulsifier is approximately 2:1:, for example approximately 3:1, such as approximately 4:1, for example approximately 5:1, such as approximately 6:1, for example approximately 10:1, such as approximately 11:1, for example approximately 12:1, such as approximately 13:1, for example approximately 14:1, such as approximately 15:1, for example approximately 16:1, such as approximately 17:1, for example approximately 18:1, such as approximately 19:1, for example approximately 20:1.

It is very preferred that the ratio of fatty acid to emulsifier is at least 2:1, however it is even more preferred that the ratio of fatty acid to emulsifier is at least 4.5:1, preferably at least 5:1.

"At least X:1" as used herein is meant to cover any ratio wherein X is the indicated number or a higher number. Thus, by way of example at least 2:1, covers for example also 5:1.

Ratio before mixing

In order to achieve above-mentioned ratios it is preferred that the ratio of fatty acid to emulsifier before mixing is higher than the ratios described above in the section "Ratio". Thus, the ratio before mixing is preferably at least 1.5, such as at least 1.6 times, for example at least 1.7 times, such as at least 1.8 times, for example at least 1.9 times, such as at least 2 times higher than the desired ratio in the complex.

Thus it is preferred that the ratio of fatty acid to emulsifier before mixing is at least 8:1, preferably at least 10:1, for example at least 15:1.

Complexes of emulsifier and fatty acid

The complex according to the invention may be any combination of any of the emulsifiers mentioned herein above in the sections "Emulsifier" and "Polypeptide" and any of the fatty acids described herein above in the section "Fatty acid".

Below a non-limiting list of examples of specific complexes is given:

Complex of Human serum albumin and oleic acid
Complex of Human serum albumin and linoleic acid
Complex of Human serum albumin and vaccenic acid
Complex of Human serum albumin and heptadecenoic acid
Complex of human MBL and oleic acid

Complex of human MBL and linoleic acid
Complex of human MBL and vaccenic acid
Complex of human MBL and heptadecenoic acid Complex of bovine beta lactoglobulin and oleic acid
Complex of bovine beta lactoglobulin and linoleic acid
Complex of bovine beta lactoglobulin and vaccenic acid
Complex of bovine beta lactoglobulin and heptadecenoic acid
Complex of phosphatidylcholin and oleic acid
Complex of phosphatidylcholin and linoleic acid
Complex of phosphatidylcholin and vaccenic acid
Complex of phosphatidylcholin and heptadecenoic acid

Cytotoxic effect

The complexes obtained by the methods according to the present invention preferably have a cytotoxic effect on tumour cells. The terms “Cytotoxic effect” and “cytotoxic activity” are used interchangeably herein.

In addition to being cytotoxic to tumour cells the complexes obtained according to the methods of the invention may also be cytotoxic to other cells, in particular cells with aberrant proliferation, for example enhanced proliferation. The complexes may also be cytotoxic to infected cells, such as cells infected by virus, bacteria or mycoplasma. Cytotocity to such cells may be determined as described in Example 3 herein below except that the cells to be tested are used in place of L1210 cells.

In one embodiment of the invention it is preferred that the complexes have a low cytotoxicity or are not cytotoxic to healthy cells, such as non-malignant, non-infected cells, preferably the complexes have a low cytotoxicity or are not cytotoxic to healthy cells *in vivo*.

The cytotoxic effect may be determined by a number of different *in vivo* or *in vitro* assays. *In vitro* assays in general comprise contacting tumour cells cultivated *in vitro* with the complex and determining the rate of living cells. On that basis an LC_{50} may be calculated (see more below). It is preferred that the complexes of the invention has at least some cytotoxic effect using such an in vitro assay. In vivo assays in general

comprises contacting an animal suffering from cancer, for example a mouse suffering from a cancer, which may for example have been induced or which is a xenograft, with the complex and determining tumour cell mass. Preferably, the complexes of the invention are capable of reducing tumour cell mass significantly *in vivo*, such as a reduction to less than 80%, such as less than 50%.

In vitro assays for cytotoxic effect in general comprises:

a) cultivating tumour cells in vitro
b) adding in different containers various predetermined concentrations of complex to a predetermined number of said cells
c) incubating said cells with said complex for a predetermined amount of time
d) determining the rate of surviving cells
e) determining the concentration of complex capable of killing 50% of the cells, thereby determining the LC_{50}.

The concentration of complex is herein given as the concentration of emulsifier unless otherwise stated. Similarly, the LC_{50} is the concentration given as the concentration of emulsifier capable of killing 50% of cells. Thus, by way of example the LC_{50} of a solution comprising alpha-lactalbumin complexed with oleic acid, may be given as the alpha-lactalbumin concentration in mg/mL in the solution required to kill 50% of the cells in the assay.

Cytotoxic activity may also be measured as LD_{50}. In vitro assays for cytotoxic effect determined as LD_{50} in general comprises:

a) cultivating tumour cells in vitro
b) adding in different containers various predetermined amount of complex to a predetermined number of said cells,
c) incubating said cells with said complex for a predetermined amount of time
d) determining the rate of surviving cells
e) determining the amount of complex capable of killing 50% of the cells, thereby determining the LD_{50}.

The amount of complex is also referred to as dose and is herein given as the amount of emulsifier per cell , i.e. weight of emulsifier per cell (in general measured in pg/cell). Similarly, the LD_{50} is the dose given as the dose of emulsifier capable of killing 50% of cells. Thus, by way of example the LD_{50} of a solution comprising alpha-lactalbumin complexed with oleic acid, may be given as the dose of alpha-lactalbumin in pg/cell in the solution required to kill 50% of the cells in the assay.

The concentration or dose of complex capable of killing 50 % of a given cell population is calculated and is called LC_{50} and LD_{50}, respectively. The rate of surviving cells may be determined using any suitable assay known to the skilled person, for example using tryphan blue staining (tryphan blue only stains dead cells, but not living cells) as described in Example 3 herein below or by determining cellular ATP levels. Cellular ATP levels is a measure of cell viability. ATP level may be determined by a number of different assays, for example assays exploiting that luciferase may generate a luminescent signal from luceferin in the presence of ATP, for example as described in Example 3 herein below.

The tumour cells may be any tumour cells, such as human or murine tumour cells derived from any tumour or otherwise malignant cells. The cells may be directly obtained from a patient or they may be an established cell lined. In a preferred embodiment the cell line is L1210.

The predetermined number of cells may be selected individually, however in a preferred embodiment it is preferred that the concentration of cells is in the range of 0.1 to 10 $x10^6$, preferably in the range of 1 to 2 $x\ 10^6$, for example about 1.4 $x\ 10^6$, such as 1.4 $x\ 10^6$.

The complex may be incubated with the cells for any suitable amount of time, in general for in the range of 30 min. to 5 hours, such as in the range of 30 min to 2 hours, such as in the range of 45 min to 75 min. for example for about 1 hour.

Prior to adding complex and during the time cells are incubated with complex, the cells should otherwise be maintained at standard conditions for in vitro cell culture. It is however preferred that the cells are kept in the absence of serum during incubation with complex.

One suitable method for determining to cytotoxicity of complexes to tumour cells is the "cell killing assay" described in Danish patent application PA 2007 00693 in Example 7.

However, it is preferred that the cell killing assay described herein in example 3 is employed.

Thus, it is preferred that the complex according to the invention has an LC_{50} of at the most 50 mg/ml, preferably at the most 40 mg/ml, even more preferably at the most 30 mg/ml, yet more preferably at the most 20 mg/ml, even more preferably at the most 10 mg/ml, yet more preferably at the most 5 mg/ml, even more preferably at the most 1 mg/ml, for example at the most 0.75 mg/ml, such as at the most 0.5 mg/ml with respect to L1210 cells at a concentration of 1.4×10^6 cells/mL in *in vitro* culture.

More preferably, the complex according to the invention has an LC_{50} of at the most 50 mg/ml, preferably at the most 40 mg/ml, even more preferably at the most 30 mg/ml, yet more preferably at the most 20 mg/ml, even more preferably at the most 10 mg/ml, yet more preferably at the most 5 mg/ml, even more preferably at the most 1 mg/ml, for example at the most 0.75 mg/ml, such as at the most 0.5 mg/ml, when determined as described in Example 3 herein below.

It is also very preferred, that the complex according to the invention has an LD_{50} of at the most 400 pg/cell, preferably at the most 300 pg/cell, more preferably at the most 200 pg/cell, even more preferably at the most 150 pg/cell, yet more preferably at the most 100 pg/cell, even more preferably at the most 90 pg/cell, yet more preferably at the most 80 pg/cell, even more preferably at the most 70 pg/cell, yet more preferably at the most 60 pg/cell, for example at the most 55 pg/cell, in particular when determined as described in Example 3 herein below.

In addition to being cytotoxic to tumour cells the complexes of the invention may also be cytotoxic to other cells, in particular cells with aberrant proliferation, for example enhanced proliferation. The complexes may also be cytotoxic to infected cells, such as cells infected by virus, bacteria or mycoplasma. Cytotocity to such cells may be

determined as described in Example 3 herein below except that the cells to be tested are used in place of L1210 cells.

In one embodiment of the invention it is preferred that the complexes have a low cytotoxicity or are not cytotoxic to healthy cells, such as non-malignant, non-infected cells, preferably in vivo.

Treatment

The complexes obtained by the methods according to the invention may be used for the manufacture of a medicament for a clinical disorder wherein selective cytotoxicity is desirable. The clinical disorder may be selected from the group consisting of respiratory tract infections, cancer and warts or for the inhibition of angiogenesis. In a preferred embodiment the cancer is bladder cancer. In another preferred embodiment the warts are caused by papiloma infection.

Infection of the respiratory tract

In one embodiment of the invention the complexes may be used in the treatment of Infections of the respiratory tract, e.g., meningitis, otitis and sinusitis, which are caused by bacteria which enter via the nasopharynx.

Viral infections of the respiratory tract may be caused by such as adenovirus, influcenza viruses, respiratory cyncytial virus (RSV), parainfluenza, Phinoviruses and coronaviruses.

In an embodiment the composition according to the invention is for the treatment of infections of the respiratory tract. The medicament according to the invention may be inhaled in the form of a mist into the upper respiratory airways.

Treatment of tumors

In one embodiment of the invention tumors of both the benign or malignant type may further be treated using the complexes according to the invention.

Wart

A wart is generally a small, rough tumour, typically on hands and feet, that resembles a cauliflower. Warts are common, and are caused by a viral infection, specifically by the human papillomavirus (HPV). They typically disappear after a few months but can last for years and can recur.

A range of different types of wart have been identified, which differ in shape and site affected, including:

Common wart (verruca vulgaris): a raised wart with roughened surface, most common on hands and knees.

Flat wart (verruca plana): a small, smooth flattened wart, tan or flesh coloured, which can occur in large numbers; most common on the face, neck, hands, wrists and knees.

Filiform or digitate wart: a thread- or finger-like wart, most common on the face, especially near the eyelids and lips.

Plantar wart (verruca, verruca pedis): a hard sometimes painful lump, often with multiple black specks in the centre; usually only found on pressure points on the soles of the feet.

Mosaic wart: a group of tightly clustered plantar-type warts, commonly on the hands or soles of the feet.

Genital wart (venereal wart, condyloma acuminatum, verruca acuminata): wart affecting the genital areas.

In a preferred embodiment the complexes according to the invention is for the treatment of warts, which is preferably treated by topical application of a medicament according to the invention.

Papillomas

Papilloma refers to a benign epithelial tumor, which may or may not be caused by Human papillomavirus. Alternative causes are such as Choroid plexus papilloma (CPP).

Two types of papilloma often associated with HPV are squamous cell papilloma and transitional cell papilloma (also known as "bladder papilloma".)

In a preferred embodiment the LAC composition according to the invention is for the treatment of papillomas, which is preferably treated by topical application of a medicament comprising the complexes according to the invention.

Cancer

Cancerous diseases are scientifically designated neoplasia or neoplasms and may be benign or malignant. Cancers are classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. The following general categories are applied:

Carcinoma: malignant tumors derived from epithelial cells. This group includes the most common cancers, comprising the common forms of breast, prostate, lung and colon cancer.

Lymphoma and Leukemia: malignant tumors derived from blood and bone marrow cells.

Sarcoma: malignant tumors derived from connective tissue, or mesenchymal cells

Mesothelioma: tumors derived from the mesothelial cells lining the peritoneum and the pleura.

Glioma: tumors derived from glia, the most common type of brain cell

Germinoma: tumors derived from germ cells, normally found in the testicle and ovary.

Choriocarcinoma: malignant tumors derived from the placenta.

In a preferred embodiment the complexes according to the invention is for the treatment of cancer.

Medicaments, as defined herein below, for treatment of cancer are according to the invention preferably applied directly to the tumour.

Mucosal tumors

The conditions found at mucosal surfaces can be quite unique in terms of properties such as pH. and the like. Mucosal surfaces are found inter alia in the nasal passages, in the mouth, throat, oesophagus, lung, stomach, colon, vagina and bladder.

Particular mucosal surfaces that may be treated with in accordance with the invention include throat, lung, colon and bladder surfaces which tumours.

Bladder cancer

Bladder cancer refers to any of several types of malignant growths of the urinary bladder. The most common type of bladder cancer begins in cells lining the inside of the bladder and is called urothelial cell or transitional cell carcinoma (UCC or TCC).

In a more preferred embodiment the complexes according to the invention is for the treatment of bladder cancer.

Glioblastome

A glioma is a type of primary central nervous system (CNS) tumor that arises from glial cells. The most common site of involvement of a glioma is the brain, but they can also affect the spinal cord, or any other part of the CNS, such as the optic nerves.

In a more preferred embodiment the complexes according to the invention is for the treatment of glioma/glioblastome.

Angiogenesis.

Tumour angiogenesis is the proliferation of a network of blood vessels that penetrates in to cancerous growths, supplying nutrients and oxygen and removing waste products. The process of angiogenesis is initiated when tumor cells release molecules signalling to the normal host tissue, activating genes and proteins to encourage growth of new blood vessels. A series of natural inhibitors of angiogenesis have been identified, and are believed to prevent and/or inhibit the growth and spread of cancer cells.

The complexes of the invention may also be used for inhibiting angiogenesis for example in treatment and/or inhibition of cancer.

Actinic keratosis

Actinic keratosis (AK) is a UV light–induced lesion of the skin that may progress to invasive squamous cell carcinoma. Actinic keratosis is a scaly bump that forms on the skin surface ranging from barely perceptible rough spots of skin to elevated, hyperkeratotic plaques several centimeters in diameter. Most often, they appear as multiple discrete, flat or elevated, keratotic lesions. Lesions typically have an erythematous base covered by scale (hyperkeratosis). They are usually 3-10 mm in

diameter and gradually enlarge into broader, more elevated lesions. Actinic keratosis (AK) is also referred to as solar keratosis, sun spots, or precancerous spots. With time, actinic keratoses may develop into invasive squamous cell carcinoma and Actinic keratosis is thus a precursor of skin cancer. The most aggressive form of keratosis, actinic cheilitis, appears on the lips and can evolve into squamous cell carcinoma.

The complexes of the present invention may also be used in the treatment of actinic keratosis. For that particular purpose, the complexes are preferably formulated for topical application directly to the diseased site.

Pharmaceutical composition

The present invention provides pharmaceutical compositions comprising the complexes of emulsifier and fatty acid obtained by the conversion methods described herein.

In one aspect the present invention relates to a pharmaceutical composition. The pharmaceutical composition may be formulated in a number of different manners, depending on the purpose for the particular pharmaceutical composition.

For example the pharmaceutical composition may be formulated in a manner so it is useful for a particular administration form. Preferred administration forms are described herein below.

In one embodiment the pharmaceutical composition is formulated so it is a liquid. For example the composition may be a solution or a suspension comprising the complexes. Said liquid may be suitable for parenteral administration, for example for injection or infusion.

The liquid may be any useful liquid, however it is frequently preferred that the liquid is an aqueous liquid. For many purposes, in particular when the liquid should be used for parenteral administration, it is furthermore preferred that the liquid is sterile. Sterility may be conferred by any conventional method, for example filtration, irradiation or

heating. Furthermore, it is preferred that the liquid has been subjected to a virus reduction step, in particular if the liquid is formulated for parenteral administration.

Virus reduction may for example be performed by nanofiltration or virus filtering over a suitable filter, such as a Planova filter consisting of several layers. The Planova filter may be any suitable size for example 75N, 35N, 20N or 15N or filters of different size may be used, for example Planova 20N.Virus reduction may also comprise a step of prefiltering with another filter, for example using a filter with a pore size of the the range of 0.01 to 1 µm, such as in the range of 0.05 to 0.5 µm, for example around 0.1 µm. Virus reductions may also include an acidic treatment step.

The pharmaceutical composition may be packaged in single dosage units, which may be more convenient for the user. Hence, pharmaceutical compositions for bolus injections may be packages in dosage units of for example at the most 10 ml, preferably at the most 8 ml, more preferably at the most 6 ml, such as at the most 5 ml, for example at the most 4 ml, such as at the most 3 ml, for example around 2.2 ml.

The pharmaceutical composition may be packaged in any suitable container. In one example a single dosage of the pharmaceutical composition may be packaged in injection syringes or in a container useful for infusion.

In another embodiment of the present invention the pharmaceutical composition is a dry composition. The dry composition may be used as such, but for most purposes the composition is a dry composition for storage only. Prior to use the dry composition may be dissolved or suspended in a suitable liquid composition, for example sterile water.

It is also comprised within the invention that the pharmaceutical composition may be applied topically to the site of the site, for example in the form of a lotion, a crème, an ointment, a spray, such as an aerosol spray or a nasal spray, rectal or vaginal suppositories, drops, such as eye drops or nasal drops, a patch, an occlusive dressing or the like.

The pharmaceutical compositions containing the complexes of the invention may be prepared by any conventional technique, e.g. as described in Remington: The Science

and Practice of Pharmacy 1995, edited by E. W. Martin, Mack Publishing Company, 19th edition, Easton, Pa.

The terms "medicament" and "pharmaceutical composition" are used interchangeably herein.

The pharmaceutically acceptable additives may be any conventionally used pharmaceutically acceptable additive, which should be selected according to the specific formulation, intended administration route etc. For example the pharmaceutically acceptable additives may be any of the additives mentioned in Nema et al, 1997. Furthermore, the pharmaceutically acceptable additive may be any accepted additive from FDA´s "inactive ingredients list", which for example is available on the internet address http://www.fda.gov/cder/drug/iig/default.htm.

In some embodiments of the present invention it is desirable that the pharmaceutical composition comprises an isotonic agent. In particular when the pharmaceutical composition is prepared for administration by injection or infusion it is often desirable that an isotonic agent is added.

Accordingly, the composition may comprise at least one pharmaceutically acceptable additive which is an isotonic agent.

The pharmaceutical composition may be isotonic, hypotonic or hypertonic. However it is often preferred that a pharmaceutical composition for infusion or injection is essentially isotonic, when it is administrated. Hence, for storage the pharmaceutical composition may preferably be isotonic or hypertonic. If the pharmaceutical composition is hypertonic for storage, it may be diluted to become an isotonic solution prior to administration.

The isotonic agent may be an ionic isotonic agent such as a salt or a non-ionic isotonic agent such as a carbohydrate.

Examples of ionic isotonic agents include but are not limited to NaCl, $CaCl_2$, KCl and $MgCl_2$. Examples of non-ionic isotonic agents include but are not limited to mannitol and glycerol.

It is also contained within the present invention that at least one pharmaceutically acceptable additive is a buffer. For some purposes, for example, when the pharmaceutical composition is meant for infusion or injection, it is often desirable that the composition comprises a buffer, which is capable of buffering a solution to a pH in the range of 4 to 10, such as 5 to 9, for example 6 to 8.

However, in other embodiments of the invention the pharmaceutical composition may comprise no buffer at all or only micromolar amounts of buffer.

The buffer may for example be selected from the group consisting of TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate and triethanolamine buffer.

In a preferred embodiment the buffer is TRIS. TRIS buffer is known under various other names for example tromethamine including tromethamine USP, THAM, Trizma, Trisamine, Tris amino and trometamol. The designation TRIS covers all the aforementioned designations.

The buffer may furthermore for example be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. For example the buffer may be selected from the group consisting of monobasic acids such as acetic, benzoic, gluconic, glyceric and lactic, dibasic acids such as aconitic, adipic, ascorbic, carbonic, glutamic, malic, succinic and tartaric, polybasic acids such as citric and phosphoric and bases such as ammonia, diethanolamine, glycine, triethanolamine, and TRIS.

The pharmaceutical compositions may comprise at least one pharmaceutically acceptable additive which is a stabiliser. The stabiliser may for example be a detergent, a polymer, a polyhydric alcohol, a metal ion, a reducing agent, a chelating agent, a sugar or a protein, however any other suitable stabiliser may also be used with the present invention.

For example the stabiliser may be selected from the group consisting of poloxamers, Tween-20, Tween-40, Tween-60, Tween-80, Brij, metal ions, amino acids, polyethylene glycol, Triton, EDTA, ascorbic acid, Triton X-100, NP40 or CHAPS.

The pharmaceutical composition according to the invention may also comprise one or more cryoprotectant agents. In particular, when the composition comprises freeze-dried protein or the composition should be stored frozen it may be desirable to add a cryoprotecting agent to the pharmaceutical composition.

The cryoprotectant agent may be any useful cryoprotectant agent, for example the cryoprotectant agent may be selected from the group consisting of dextran, glycerin, polyethylenglycol, sucrose, trehalose and mannitol.

Accordingly, the pharmaceutically acceptable additives may comprise one or more selected from the group consisting of isotonic salt, hypertonic salt, buffer and stabilisers. Furthermore, the pharmaceutically acceptable additives may comprise one or more selected from the group consisting of isotonic agents, buffer, stabilisers and cryoprotectant agents. For example, the pharmaceutically acceptable additives comprise glucosemonohydrate, glycine, NaCl and polyethyleneglycol 3350.

Administration

The pharmaceutical composition may be prepared so it is suitable for one or more particular administration methods. Furthermore, the method of treatment described herein may involve different administration methods.

In general any administration method, wherein the complexes may be administered to an individual in a manner so that active complex may reach the site of disease may be employed with the present invention.

For example, the pharmaceutical compositions of the invention may be administered parenterally, that is by intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration.

In general, for injection and infusion the pharmaceutical composition should be a sterile liquid, which preferably also has been subjected to a virus reduction step.

Injection may be injection to any preferred site, for example injection may be selected from the group consisting of intravenous, subcutaneous, intra-arterial, intra-muscular and intra-peritonal injection. Infusion is generally intra-venous infusion. Injection may also be directly to site of the disease. This may in particular be applicable when treating a cancer which is a solid tumour.

Furthermore, the route of administration may be topical administration to for example a mucosal membrane or to the skin. The mucosal membrane to which the pharmaceutical preparation of the invention is administered may be any mucosal membrane of the mammal to which the biologically active substance is to be given, e.g. in the nose, vagina, eye, mouth, genital tract, lungs, gastrointestinal tract, or rectum.

Topical administration to the skin may for example be in the form of a lotion, cream, ointment, drops, transdermal patch or the like.

The pharmaceutical compositions according to the present invention may be administered once or more than once, for example they may be administered in the range of 2 to 5 times, such as 5 to 10 times, for example 10 to 20 times, such as 20 to 50 times, for example 50 to 100 times, such as more than 100 times.

The dosage of complex to be administered depends on the individual to be treated as well as on the clinical condition and the mode of administration. In general, in the range of 0.5 µg to 50 mg, such as in the range of 1 µg to 20 mg, for example in the range 1 µg to 2 mg, such as in the range of 1 µg to 100 µg may be administered per administration to a given individual, such as a human being. The amount indicated is in respect to emulsifier.

The individual to be treated may be any individual in need thereof, preferably a human being. The treatment may be prophylactic, ameliorating or curative, preferably ameliorating or curative, more preferably at least ameliorating.

Stability

In one embodiment the invention relates to a method of preparing a stable, pharmaceutical composition comprising a complex between an emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) and a fatty acid (e.g. oleic acid), said complex having cytotoxic activity, said method comprising the steps of

a. providing an emulsifier in an aqueous solution, wherein said emulsifier is not a fatty acid; and
b. providing a fatty acid; and
c. contacting the emulsifier with the fatty acid; and
d. forming a complex between the emulsifier and the fatty acid;
e. thereby obtaining a solution comprising a complex between said emulsifier and said fatty acid, wherein said solution has a starting cytotoxic activity with a starting LD_{50} in respect of dose of emulsifier; and
f. formulating said complex in a liquid solution having a pH of at least 6.5,

thereby obtaining a pharmaceutical composition comprising a complex between an emulsifier and a fatty acid, said composition retaining cytotoxic activity after storage, wherein the 2 weeks storage LD_{50} in respect of dose of emulsifier after storage for 2 weeks at 25°C is at the most 3 times higher than the starting LD_{50}.

The method may in addition comprise addition of a chelator, which for example may be added to the aqueous solution step a), to the solution in step e) or to the liquid solution in step f). The chelator may be any compound capable of binding to divalent cations through more than one coordination site. The chelator may for example be selected from the group consisting of EDTA, EGTA, BAPTA, porphyrins (such as heme, porphine or porphine substitued with one or more entities) and citrate, preferably from the group consisting of EDTA and citrate.

As indicated above the starting LD_{50} and the storage LD_{50} are both determined in respect of dose of emulsifier (e.g. alpha-lactalbumin). After storage some of the initial cytotoxic complex may have disintegrated and accordingly some emulsifier may be present as part of a complex with fatty acid, whereas some emulsifier may not be in complex with fatty acid. Emulsifier not in complex with fatty acid preferably does not have cytotoxic acitivty (see herein above in the section “Emulsifier”) and accordingly, the LD_{50} in respect of dose of emulsifier will be higher, if some of the emulsifier is

present in an inactive not complexed form. This is certainly the case, when the emulsifier is alpha-lactalbumin.

It is desirable to prepare a pharmaceutical composition, wherein the LD_{50} is retained at a low level even after storage. By the term "stable" in this context is meant that the composition retains cytotoxic activity after storage.

Thus preferably, the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 2 weeks storage at 25ºC is preferably at the most 3 times, for example at the most 2.7 times, more preferably at the most 2.5 times, for example at the most 2.2 times, yet more preferably at the most 2 times, yet more preferably at the most 1.9 times, even more preferably at the most 1.8 times, yet more preferably at the most 1.7 times, even more preferably at the most 1.6 times, even more preferably at the most 1.5, yet more preferably at the most 1.4 times, for example at the most 1.3 times, such as at the most 1.2 times higher than the starting LD_{50}.

It is also preferred that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 3 weeks storage at 25ºC is preferably at the most 3 times, for example at the most 2.7 times, more preferably at the most 2.5 times, for example at the most 2.2 times, yet more preferably at the most 2 times, yet more preferably at the most 1.9 times, even more preferably at the most 1.8 times, yet more preferably at the most 1.7 times, even more preferably at the most 1.6 times, even more preferably at the most 1.5, yet more preferably at the most 1.4 times, for example at the most 1.3 times, such as at the most 1.2 times higher than the starting LD_{50}.

It is also very much preferred that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 4 weeks storage at 25ºC is preferably at the most 4 times, more preferably at the most 3.5 times, yet more preferably at the most 3 times, yet more preferably at the most 2.8 times, even more preferably at the most 2.6 times, yet more preferably at the most 2.4 times, even more preferably at the most 2.3 times, even more preferably at the most 2.2, for example at the most 2 times higher than the starting LD_{50}.

Accordingly, it is preferred - in particular in embodiments of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof - that the LD_{50} in

respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 2 weeks storage at 25ºC preferably is at the most 400 pg/cell, preferably at the most 300 pg/cell, more preferably at the most 200 pg/cell, even more preferably at the most 150 pg/cell, yet more preferably at the most 100 pg/cell, even more preferably at the most 90 pg/cell, yet more preferably at the most 80 pg/cell, even more preferably at the most 70 pg/cell, such as at the most 60 pg/cell, for example at the most 55 pg/cell, in particular when determined as described in Example 3 herein below.

It is thus very preferred, -in particular in embodiments of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof - that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 2 weeks storage at 25ºC preferably is at the most 100 pg/cell, preferably at the most 80 pg/cell when determined as described in Example 3 herein below.

It is also preferred - in particular in embodiments of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof - that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 3 weeks storage at 25ºC preferably is at the most 400 pg/cell, preferably at the most 300 pg/cell, more preferably at the most 200 pg/cell, even more preferably at the most 150 pg/cell, yet more preferably at the most 100 pg/cell, even more preferably at the most 90 pg/cell, yet more preferably at the most 80 pg/cell, even more preferably at the most 70 pg/cell, such as at the most 60 pg/cell, for example at the most 55 pg/cell, in particular when determined as described in Example 3 herein below.

It is also preferred - in particular in embodiments of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof - that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) after 4 weeks storage at 25ºC preferably is at the most 400 pg/cell, preferably at the most 300 pg/cell, more preferably at the most 200 pg/cell, even more preferably at the most 150 pg/cell, yet more preferably at the most 100 pg/cell, particularly when determined as described in Example 3 herein below.

It is thus very preferred - in particular in embodiments of the invention wherein the emulsifier is alpha-lactalbumin or a functional homologue thereof - that the LD_{50} in respect of dose of emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof)

after 2 weeks storage at 25ºC preferably is at the most 100 pg/cell, when determined as described in Example 3 herein below.

It is also preferred that the complexes of the stable pharmaceutical compositions retain the molar ratio between fatty acid and emulsifier. Thus, if the starting ratio of fatty acid to emulsifier (e.g. alpha-lactalbumin or a functional homologue thereof) is Y:1, then preferably, the ratio after 2 weeks storage at 25ºC preferably is at least 0.4xY:1, preferably at least 0.5xY:1, even more preferably at least 0.6xY:1, yet more preferably at least 0.7xY:1, even more preferably at least 0.8xY:1.

One preferred method of preparing stable pharmaceutical compositions according to the invention involves a step of formulating said complex in a liquid solution having a pH of at least 6.5. Thus, the pH of the final pharmaceutical composition should be at least 6.5. The pH may be adjusted to at least 6.5 at any step and it can thus be done before or after mixing emulsifier and fatty acid. If the pH is adjusted at an early step it is important that it is kept at the high pH of at least 6.5 or it must be adjusted again at the end of the procedure.

Preferably, the pH is at least 6.5, more preferably at least 7, such as at least 7.1, for example at least 7.2, such as at least 7.3, for example at least 7.4, such as at least 7.5, for example at least 7.6, such as at least 7.7, for example at least 7.8, such as at least 7.9, for example at least 8, preferably at least 8.1, for example at least 8.2. It is also preferred that the pH is not too high and thus preferably the pH is in the range of 6.5 to 11, such as in the range of 6.5 to 10.5, for example in the range of 6.5 to 10, such as in the range of 6.5 to 9.5, for example in the range of 6.5 to 9, such as in the range of 7 to 11, such as in the range of 7 to 10.5, for example in the range of 7 to 10, such as in the range of 7 to 9.5, for example in the range of 7 to 9, such as in the range of 7.5 to 11, such as in the range of 7.5 to 10.5, for example in the range of 7.5 to 10, such as in the range of 7.5 to 9.5, for example in the range of 7.5 to 9, such as in the range of 8 to 11, such as in the range of 8 to 10.5, for example in the range of 8 to 10, such as in the range of 8 to 9.5, for example in the range of 8 to 9, such as in the range of 7.1 to 7.9, for example in the range of 8.1 to 8.9, such as in the range of 8.2 to 8.8, for example in the range of 8.3 to 8.7, such as in the range of 9.1 to 9.9.

In one embodiment of the invention, the pH is any of the aforementioned with the proviso that the pH is not 8, 9 or 10.

In general the pH is adjusted with the aid of a buffer, which for example may be added to the liquid solution in step f) of above-mentioned method, to the solution in step e) of the method above or to the aqueous solution comprising the emulsifier in step a) of the method above.

The buffer may be any buffer suitable of buffering to the desired pH such as a buffer selected from the group consisting of TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate and triethanolamine buffer.

In a preferred embodiment the buffer is TRIS. TRIS buffer is known under various other names for example tromethamine including tromethamine USP, THAM, Trizma, Trisamine, Tris amino and trometamol. The designation TRIS covers all the aforementioned designations.

Examples

Example 1

Preparation of protein and oleic acid complexes in the presence of EtOH

20 µl 99% pure oleic acid (obtainable from Sigma-Aldrich, St. Louis, USA; cat. No. O1008) or Larodan AB (Malmø, Sweden, cat.no. 10-1801) is mixed with 250 µl ethanol. The mixture is added to 20 ml protein solution, wherein the protein concentration in the solution is adjusted to obtain a molar ratio of protein to oleic acid of 1:15. When specifically indicated the protein concentration may also be adjusted to obtain another molar ratio. For proteins which are oligomers of several individual polypeptides, the molar ratio above is given in respect of the individual polypeptides and not in respect of the number of oligomers.

The protein/oleic acid/ethanol mixture is subjected to high shear mixing by vigorous vortex mixing at 2500 rpm for 10 s.

The resulting mixture is ready for use for example in the cell killing assay as described herein below in Example 3.

Example 2

Preparation of protein and oleic acid complexes saturated with oleic acid

250 µl 99% pure oleic acid (obtainable from Sigma-Aldrich, St. Louis, USA; cat. No. O1008) or Larodan AB (Malmø, Sweden, cat.no. 10-1801) is mixed with 2500 µl protein solution (concentration typically in the range of 10-15 mg/mL). The mixture is subjected to high shear mixing by vigorous vortex mixing at 2500 rpm for 20 sec.

The mixture is incubated for 10 min. at room temperature and is then centrifuged in the range of 150 x g to 18,000 x g.

The aqueous phase is retrieved and ready to use for example in the cell killing assay as described herein below in Example 3.

Example 3

Cell killing assay

L1210 cells are in general kept in RPMI 1640 medium with 1% sodium pyruvate and 1% non-essential amino acids supplemented with 5% fetal calf serum at 37ºC and 5% CO_2. In general, cells from day 2 after last sub-culturing having a viability of at least 90% as determined by tryphan blue exclusion were gently spun down (10 minutes at 200 x g) and washed in PBS buffer followed by another spin (10 minutes at 200 x g). The cells were then re-suspended in growth medium without serum at a cell concentration of $2 \cdot 10^6$/mL, which was verified by a cell count.

A 2-fold dilution series (from 2x and up to to 2128x) of a sample prepared as described for example in Example 1 or Example 2 is made in 0.9% NaCl. 20 µl of each dilution is added to a well of a 96 well plate. In general a control is also made comprising only 0.9% NaCl.

50 µl cell suspension in serum free RPMI 1640 medium with 1% sodium pyruvate and 1% non-essential amino acids containing 100,000 L1210 cells is added to each well and the plate is incubated for 1 hour at 37°C and 5% CO_2.

30 µl medium comprising 33.3% fetal bovine serum is added to each well and the plate is incubated for 1 hour at 37°C and 5% CO_2, followed by incubation for 10 min. at room temperature.

The cell viability is assayed using the ViaLight Plus kit from Lonza (formerly Cambrex Bioscience), cat. No. LT07-221 or LT07-121 according to manufacturers instructions. This kit contains lyophilized AMR (ATP Monitoring Reagent) PLUS, cell lysis reagent and assay buffer.
50 µl cell lysis buffer is added per well and the plate is incubated for 10 min. at room temperature.

100 µl AMR PLUs reagent is added to each well and the plate is incubated for 5 minutes, after which luminiscense is determined by reading the plate in a luminometer (e.g. BMG Lumistar Optima) with an integrated reading time of 1 second.

Another method is use of the Cytotoxicity Bioassay kit from Lonza and a BMG Lumistar Optima luminometer as described in the Vialight Plus manual.

Another method for determining cell viability is by tryphan blue staining. As the results obtained with the ViaLight Plus kit correlates with the results of Tryphan blue staining (see figure 1) it is generally not required to determine cell viability using both test.

Tryphan blue staining may be performed as follows:

Aseptically withdraw a sample from a cell culture and dilute in tryphan blue solution (0.4 %) as necessary (typically 1:2).
Mix the solution and count the cells in a hemacytometer within 3 minutes – after that time the living cells will start to take up the dye.
The viable cells will be slightly opalescent, round and pale with a darker outline. The nonviable cells will be dark blue.

In general the results are displayed as % viability compared to the viability of cells treated with 0.9% NaCl.

LD_{50} and LC_{50} values were calculated by evaluating the fit for protein dose in µL per well versus the luminescence using a four parameter logistic fit (given by $Y=Y_0+A/[1+(X/X_0)^B]$) in SigmaPlot Ver. 10 for Windows. X_0 corresponds to the LD_{50} expressed as µL/well. Using the cell concentration of the applied cells and the protein (*e.g.* bLA+bLAC) sample concentration LD_{50} can be expressed as pg/cell and the LC_{50} can be calculated. In cases where the dilution series did not cover the cell killing curve fully, Y_0 was forced to zero in the fit.

In the following examples 'concentration in the assay' and in LC_{50} is defined as the concentration of the protein (*e.g.* bLA) which the L1210 cells encounter during the first one hour of incubation (before addition of medium with serum).

Example 4

<u>bLAC and hLAC</u>

Biologically active complexes of bovine alpha-lactalbumin and oleic acid (bLAC) and human alpha-lactalbumin and oleic acid (hLAC) were prepared as described previously in Example 1 of Danish Patent application PA 2006 01512.

The samples were subjected to the cell killing assay described in Example 3 herein above. Figure 1 shows the results for bLAC and also demonstrates the comparability of the tryphan blue method and the ViaLight method. The LC_{50} of bLAC was in the range of 0.02 to 0.5 mg/ml, whereas the LC50 for hLAC was in the range of 0.05 to 0.11 mg/ml.

Example 5

<u>Human serum albumin and oleic acid complexes</u>

Essentially fatty acid-free human serum albumin (HSA) was obtained from Sigma (A3782). In general HAS was dissolved in 0.9% NaCl to a concentration of 14 mg/ml.

Complexes of HSA and oleic acid were prepared as described in Example 2. The protein concentration in the protein solution was approx. 14 mg/ml and the

centrifugation was performed at 150 x g for 5 min, 3000 x g for 10 min. or at 3000 x g for 10 min. followed by centrifugation at 18,000 x g for 6 min.

The samples thus obtained were used for a cell killing assay performed as described in Example 3. Several controls were included:

A control comprising 4 mg/ml HSA in the absence of oleic acid.

A control sample comprising 0.9% NaCl saturated with oleic acid.

As is apparent from figure 2, pure HSA or pure oleic acid in 0.9% NaCl had negligible effect on viability, whereas the HSA/oleic acid complexes prepared according to Example 2 as described above (centrifugation at 150 x g for 5 min) killed almost all cells (see sample designated 4 mg/ml HSA saturated with oleic acid). Complexes prepared as described in Example 1 killed approx. half of the cells.

As shown in figure 3, the LC_{50} for HSA/oleic acid complexes prepared as described in Example 2 was 0.15 mg/ml for samples centrifuged at 150 x g for 5 min and 0.38 mg/ml for samples centrifuged at 3000 x g for 10 min.

Figure 4 shows that centrifugation at 3000 x g for 10 min yielded more active complex than centrifugation at first 3000 x g for 10 min followed by 6 min at 18,000 x g.

Example 6

Bovine alpha-lactalbumin and oleic acid

Bovine alpha-lactalbumin (bLA) was purified from bovine milk as described in Example 1 of Danish Patent application PA 2006 01512 and in Example 1 of PCT application WO2008/058547.

Complexes of bovine alpha-lactalbumin (bLA) and oleic acid were prepared as described in Example 2. The protein concentration in the protein solution was 12 mg/mL and the centrifugation was performed at 150 x g for 5 min, at 3000 x g for 10 min or at 18,000 x g for 10 min.

The samples thus obtained were used in a cell killing assay as described in Example 3. As disclosed in figure 5, the LC_{50} was 0.06 mg/ml for samples centrifuged at 150 x g, 0.24 mg/ml for samples centrifuged at 3000 x g and 0.06 mg/ml for samples centrifuged at 18,000 x g.

Complexes of bLA and oleic acid were also prepared as described in Example 1. Two different oleic acid preparations were used, namely pure oleic acid from Sigma (99% oleic acid) and EP quality oleic acid from Merck (65-88% oleic acid). These samples were also subjected to the cell killing assay described in Example 3. As disclosed in figure 6A the LC_{50} of the complexes prepared with oleic acid obtained from Sigma was 0.16 mg/ml. Figure 6B disclosed that the LC_{50} of the complexes prepared with oleic acid from Merck was 0.05 mg/ml.

Example 7

Bovine beta-Lactoglobulin and oleic acid

Bovine beta-Lactoglobulin (bLG) was obtained from Sigma (L3908). In general beta-Lactoglobulin was dissolved in 0.9% NaCl at a concentration of 10 mg/ml.

Complexes of bLG and oleic acid were prepared as described in Example 2. The protein concentration in the protein solution was 10 mg/mL and the centrifugation was performed at 3000 x g for 10 min or at 18,000 x g for 10 min.

These samples were subjected to the cell killing assay as described in Example 3. As shown in figure 7, the LC_{50} for bLG/oleic acid complexes prepared as described in Example 2 was 0.06 mg/ml for samples centrifuged at 3000 x g for 10 min and 0.12 mg/ml for samples centrifuged at 18,000 x g for 10 min.

Complexes of bLG and oleic acid (obtained from Sigma Aldrich, Denmark) were also prepared as described in Example 1. More specifically 1.09 mg/ml bLG was mixed using high shear mixing with 0.9 mM oleic acid in the presence of 0.4% (v/v) ethanol.

As controls a sample comprising 0.9 mM oleic acid and 0.4% (v/v) ethanol and another sample comprising 19 mg/ml bLG were prepared.

The sample and the controls were used in the cell killing assay as described in Example 3 and figure 8 displays the results. As is apparent the controls only had very limited impact on viability, whereas the complex of bLG and oleic acid killed almost all cells.

Example 8

Mannose binding lectin and oleic acid

Recombinant human mannose binding lectin (rhMBL) was prepared as described in Example 1 of WO03/033522. The rhMBL thus obtained is mainly oligomeric, i.e. rhMBL comprises oligomers of individual rhMBL polypeptides.

rhMBL is generally used at a concentration of 10 mg/ml in 10 mM Tris and 140 mM NaCl (pH 7.2).

Complexes of rhMBL and oleic acid were prepared as described in Example 2. The protein concentration in the protein solution was 10 mg/mL and the centrifugation was performed at 3000 x g for 10 min or at 18,000 x g for 10 min.

These samples were subjected to the cell killing assay as described in Example 3. As shown in figure 9, the LC_{50} for rhMBL/oleic acid complexes prepared as described in Example 2 was 0.10 mg/ml for samples centrifuged at 3000 x g for 10 min and 0.17 mg/ml for samples centrifuged at 18,000 x g for 10 min.

Complexes of rhMBL and oleic acid were also prepared essentially as described in Example 1 except that the molar ratio between rhMBL polypeptides (i.e. not oligomers) and oleic acid was 1:24.

The sample was used in the cell killing assay as described in the Example 3 and figure 10 displays the results. The LC_{50} was 0.50 mg/ml.

Table 1

Table one summarises the results obtained in Examples 3 to 8.

Protein	LC50 (mg/mL)	
	Saturated with oleic acid	Oleic acid dissolved with EtOH
bLA	0.06; 0.24; 0.06	0.16; 0.05
HSA	0.15; 0.38;	
bLG	0.12	
rhMBL	0.17	0.50

Example 9

Ratio of oleic acid to alpha-lactalbumin in an active bLAC complex

Preparation of CMP from milk

The starting material CMP (Concentrated Milk Plasma) was obtained from Arla Foods. CMP was prepared by filtration/ultrafiltration from skimmed milk at Arla foods. CMP preparation is done to remove casein from other milk proteins, primarily bLA and bLG. Prior storage, CMP was sterile filtrated and stored frozen. For this bLAC batch, a total of 900 mL CMP was used as starting material prior the next step: Acid treatment.

Acid treatment

An acid treatment was performed on the CMP to destroy potential virus.
The CMP was split in two parts. On one part (450 mL), acid treatment was done by adding 1M HCl to obtain pH=3.0, followed by gentle stirring for 3 hours at room temperature. At the end of treatment, the pH of the preparation was adjusted to pH=6.5 using 1M NaOH. On the other part 450 mL, acid treatment was done by adding 1M glycine pH. The pH was 3.18 after glycine addition and further adjusted with HCl to obtain pH=3.0, followed by gentle stirring for 3 hours at room temperature. At the end of treatment, the pH of the preparation was adjusted to pH=6.5 using 1M NaOH.

bLG depletion

The acid treated CMP (presently pH 6.5) was added to an AIEC resin (Q Sepharose XL) equilibrated at pH 8.5. bLA was expected to be recovered in the flow through fraction due to the competition with bLG on the resin.

As the next step (HIC purification) is also known to remove bLA from bLG, it was decided to proceed to the next purification step using the AIEC Recovery. Chromatography conditions were as follows: Column area 5.3 cm^2; Bed height 10 cm; Flow 40 cm/hr (13 mL/min); Run temperature 20-25°C; Application buffer Tris (10 mM) NaCl (100 mM) pH=8.5; Elution buffer: Tris (10 mM) NaCl (1 M) pH=8.5; Regeneration buffer: NaOH (1 M).

Recovery: Estimated bLA recovery was 500-600 mg/cm^2 (SE-HPLC, MET168).

bLA purification

The purification of bLA primarily to remove bLG was further done by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6FF high-sub.

Prior chromatography, the recovery from the previous AIEC step containing bLA and bLG was conditioned with EDTA, which resulted in a partially unfolded bLA, allowing its binding to the hydrophobic matrix. bLA was eluted from the matrix by addition of Ca^{2+}, which released bLA in its native state. bLG was removed during the washing of the column.

Initially, 346 mL of AIEC1 recovery was conditioned with EDTA and added to the column in two cycles. However, loads were diminished to 152 mL before conditioning in the succeeding two cycles.

In all four runs, and independent on volume load, approximately 600 mg bLA was recovered.

Chromatography conditions were as follows: Column area 19.6 cm^2; Bed height 15 cm; Flow 40 cm/hr (13 mL/min); Run temperature 20-25°C; Application buffer Tris (50 mM), EDTA (1 mM), $(NH4)_2SO_4$ (0.5 M), pH=7.5; Elution buffer: Tris (50 mM), $CaCl_2$ (1 mM) pH=7.5; Regeneration buffer: NaOH (1 M).

Recovery: Estimated bLA load was 50 - 120 mg/cm^2 (SE-HPLC, MET168) and estimated bLA yield was 30 mg/cm^2 (SE-HPLC, MET168).

Conversion of bLA to bLAC

Prior conversion, bLA recovered from the hydrophobic exchange runs was pooled and sterile filtered through 0.2 µm (Minikleenpak 20 filter from Pall). The pool of bLA was divided in two portions stored at +4ºC prior the conversion step.

EDTA and oleic acid were added to one part of bLA pool. The molar ratio between EDTA and bLA and oleic acid and bLA was respectively 15 and 30. After mixing for 30 min., the conditioned bLA sample was applied to the anion exchange column of Q Sepharose XL. The unconverted bLA was removed during the first step gradient of 40% B-buffer, and bLAC was eluted in the second step of the salt gradient consisting of 70% B-buffer (0.7 M NaCl).

The two conversion runs were performed identically and the chromatography conditions were as follows: Column area 19.6 cm^2; Bed height 10 cm; Flow 40 cm/hr (13 mL/min); Run temperature 20-25°C; Application buffer Tris (50 mM) NaCl (0.1 M) pH=8.5; Elution buffer: Tris (50 mM), NaCl (1 M) pH=8.5; Regeneration buffers: Acetic acid 1 M, NaOH (0.5 M), ethanol 20 and 70%.

Between the two runs the column was regenerated with a sequence of acetic acid, NaOH, ethanol 20% and ethanol 70%. Between acid and base CIP's, the column was reequilibrated with Tris (100 mM) pH 8.5.

After conversion, bLAC recovered from the conversion runs was pooled and sterile filtered through 0.2 µm (Minikleenpak 20 filter from Pall). The pool of bLAC was stored at +4ºC prior the next step (Nanofiltration).

Recovery: Estimated bLA load was 57 mg/cm^2 (SE-HPLC, MET168) and estimated bLAC yield was 37 mg/cm^2 (SE-HPLC, MET168).

The bLAC complexes thus obtained did have cell killing activity towards L1210 cells in an assay performed as described int Example 3. The LC_{50} was 0.06 mg/mL.

Ratio of lipid to alpha-lacatalbumin

The test sample (typically 250-300 µL) was taken, and 10.0 µL diluted nonadecanoic acid (19:0, 12.5 mg/mL in chloroform) was added as internal standard. The solution was then evaporated to dryness with nitrogen, and subsequently the fatty acids were converted into their methyl esters by addition of acidified methanol and heating for 1 hour at 85°C.

After heat treatment the solution was cooled to room temperature and 1 mL hexane was added. The solution is vortexed and centrifuged. The hexane phase was isolated and again evaporated to dryness with nitrogen.

The precipitate was rediluted in 200 µL hexane and the fatty acid methyl esters analysed by GC, using a Varian 3400 System equipped with a split/splitless injector and flame ionization detector (FID).

Injection volume was 3.0 µL at 240°C, split ratio is 1:20, and applied column was a FFAP column (Zebron, 30 m x 0.32 mm x 0.25 µm).

Oven program: 140 °C for 1 min, increasing to 240°C (by 8°C per min), and finally 240 °C for 10 min. FID temperature is 250°C.

Lipid content (mass ratio) was calculated in relation to the concentration of the internal standard.

To obtain the total content of lipid, areas of the fatty acid peaks were summed and lipid content is calculated in relation to the content of the internal standard

Results:

bLAC (N276-77A), protein:oleic acid 1:12, LC_{50} = 0.06 mg/mL

Example 10

The bLA used in this and the following examples was purified from skimmed milk or from whole milk and essentially as described in Example 1 of PCT application WO2008/058547. One preparation purified from whole milk had a bLA concentration of

12.1 mg/mL, whereas purified from skimmed milk had a concentration of 8.6 mg/mL bLA. Both of these were not calcium depleted, but were formulated in a 0.9% NaCl solution without calcium or buffer.

In this and the following examples complexes of protein and fatty acid were prepared by either of the following two methods:

Method A: In the first method a surplus of at least 99% pure oleic acid (obtainable from Sigma-Aldrich, St. Louise, USA cat.no. O1008 or Larodan AB, Malmø, Sweden, cat.no. 10-1801) was added to the protein solution (*e.g.* 100 µL oleic acid to 1000 µL protein solution) in a plastic tube. Then the two phases were mixed on a whirleymixer (MS2 Minishaker, IKA, Staufen, Germany) for approximately 20 seconds on setting 2200 min^{-1} to allow the protein solution to absorb oleic acid. Mixing was followed by approximately 5 -10 minutes incubation at 20-25°C, and then the water and fatty acid phases were separated by centrifugation at first 3000 x g for 10 minutes and then secondly the water phase was centrifuged again at 18,000 x g for 10 minutes to remove all non- emulsified oleic acid. In some instances the resulting water phase was sterile-filtrated on a sterile Pall Acrodisc® Syringe filter with 0.8/0.2 µm Supor membrane.

Method B: At least 99% pure oleic acid (obtainable from Sigma-Aldrich, St. Louise, USA cat.no. O1008 or Larodan AB, Malmø, Sweden, cat.no. 10-1801) was first dissolved in 96% ethanol (20 µL oleic acid per 250 µL 96% ethanol) and then added to the protein solution to the intended protein oleic acid molar ratio. Usually, 14 µL oleic acid-ethanol mix was added per 1000 µL protein solution - more was never added. The resulting solution was then mixed on a whirleymixer (MS2 Minishaker, IKA, Staufen, Germany) for approximately 20 seconds on setting 2200 min^{-1}. In some instances the resulting water phase was sterile-filtrated on a sterile Pall Acrodisc® Syringe filter with 0.8/0.2 µm Supor membrane.

Example 11

Different proteins and oleic acid

Complexes of various proteins and oleic acid were prepared essentially as described in Example 10, methods A and B unless otherwise stated. The proteins are as identicated and were obtained as described in Examples 4-8 and 10, respectively.

The cell killing activity of the complexes thus prepared is shown in Table 2.

Table 2

Overview of cell killing activity of the complexes prepared by high shear mixing five different proteins with oleic acid by two different methods.

Protein	**Protein solution saturated with oleic acid**[c], (water phase tested after separation by centrifugation)		**Oleic acid dissolved in ethanol and mixed with the protein solution in a 1:15 molar ratio**[d] (protein:oleic acid).	
	LD_{50} (pg/cell)	**LC_{50}**(mg/mL)[a]	**LD_{50}** (pg/cell)	**LC_{50}** (mg/mL)[a]
bLA	14 - 83	0.02 - 0.12	43 - 110	0.05 – 0.16
HSA	203	0.29	~ 2800	~ 4
bLG	87	0.12	<700[e]	<1.0
rhMBL	123	0.17	368[b]	0.50[b]
Lysozyme	>1980	>2.9	>600	>0.86

[a]LC_{50} at a L1210 cell concentration of $1.4 \cdot 10^6$7mL.
[b]Protein and oleic acid mixed in 1:24 molar ratio!
[c]Prepared as described in Example 10, method A
[d]Prepared as described in Example 10, method B
[e]exact LD_{50} was not determined

Example 12

<u>bLA mixed with oleic acid</u>

bLA at a concentration of 12.1 mg/mL in 0.9% NaCl prepared as described in Example 10 was in three separate assay runs saturated with oleic acid and mixed under high shear conditions. The ratio of bLA to oleic acid before mixing was 1:518 (N287-69C) or 1:368 for the rest of the preparations. After mixing the mixtures were centrifuged at either 150 x g, 3000 x g or 18000 x g for 10 minutes to separate and collect the water phase (see also Example 10, method A for more details). The results of the subsequent test of cell killing activity performed as described in Example 3 are shown in figure 11. Other data on the activity of bLA saturated with oleic acid and high shear mixed as described in Example 10 are shown in table 3. bLA alone has been tested in the highest concentration shown in figure 11 and no effect on cell viability was observed).

The final content of oleic acid in the complexes of preparation N287-69C prepared according to method A of Example 10 (bLA saturated with oleic acid) was determined to 4.4 mM giving a molar ratio of protein to oleic acid of 1:5. In another preparation (N277-30A) of bLAC the concentration has been measured to 7.7 mM giving a 1:13 molar ratio.

Table 3
Overview of cell killing activity of complexes of bLA solution and oleic acid prepared according to method A of Example 10

Preparation ID	LD_{50} (pg/cell)	LC_{50} (mg/mL)[a]
N287-69C	42	0.06
N312-31A	83	0.12
N312-31B[b]	19	0.03
N312-53A	14	0.02
N312-53B[b]	23	0.03
N318-06B	37	0.05
N318-65A[c]	76	0.11
N318-65B	74	0.11

[a]LC_{50} at a L1210 cell concentration of $1.4 \cdot 10^6$7mL.
[b]Oleic acid of lower purity than 99% was used (obtainable from Merck).
[c]with 8 mM EDTA and 10 mM Tris-buffer (pH 8.5).

When mixing oleic acid dissolved in 96% ethanol into 3 mg/mL bLA in 0.9% NaCl in a molar ratio of 1:15 (protein vs. oleic acid) (as described in Example 10, method B) the subsequent test of cell killing activity showed that this method also results in an active preparation (see table 4). The content of oleic acid in preparation N287-78F with bLA and oleic acid mixed in a 1:15 molar ratio (assuming that Merck oleic acid consists of 100 % oleic acid – which is not the case) was determined to be 1.05 mM resulting in a 1:5 molar ratio of protein vs. oleic acid.

Table 4.
Overview of cell killing activity of preparations resulting from mixing oleic acid dissolved in ethanol into a bLA solution in a 1:15 protein to oleic acid molar ratio (as described in Method B of Example 10).

Preparation ID	LD_{50} (pg/cell)	LC_{50} (mg/mL)[a]
N287-75E	110	0.16
N287-78F[b]	41	0.05
N287-86C	57	0.08
N295-09A	43	0.06
N312-12A	36	0.05

[a]LC_{50} at a L1210 cell concentration of $1.4 \cdot 10^6$7mL.

[b]Oleic acid with lower purity than 99% was used (obtainable from Merck).

Example 13

HSA mixed with oleic acid

HSA was obtained as described above in Example 5.

Fatty acid free HSA at a concentration of 14 mg/mL in 0.9% NaCl was in three separate assay runs saturated with oleic acid, mixed and centrifuged at either 150 x g, 3000 x g or 18000 x g to separate and collect the water phase (according to method A of Example 10). The results of the subsequent test of cell killing activity are shown in figure 12. HSA alone has been tested in the highest concentration shown in figure 12 and no effect on cell viability was observed.

The content of oleic acid in the complexes of preparation N287-50C prepared from HSA saturated with oleic acid was measured to 57.9 mM giving a molar ratio of protein to oleic acid of 1:276.

When mixing oleic acid dissolved in 96% ethanol into 4 mg/mL HSA in 0.9% NaCl in a molar ratio of 1:15 (protein vs. oleic acid) according to method B of Example 10 the subsequent test of cell killing activity showed that this method results in complexes with low cell killing activity estimated at $LD_{50} \approx 2800$ pg/cell ($LC_{50} \approx 4$ mg/mL) (see Table 2).

Example 14

bLG mixed with oleic acid

bLG was obtained as described in Example 7.

bLG at a concentration of 10 mg/mL in 0.9% NaCl was saturated with oleic acid, mixed and centrifuged at first 3000 x g and then 18000 x g to separate and collect the water phase (see details in method A of Example 10). The results of the subsequent test of cell killing activity are shown in figure 13. bLG alone has been tested in the highest concentration shown in the figure, and no effect on cell viability was observed.

When mixing oleic acid dissolved in 96% ethanol into 3.8 mg/mL bLG in 0.9% NaCl in a molar ratio of 1:15 (protein vs. oleic acid) according to method B of Example 10 the subsequent test of cell killing activity showed that this method results in a fully active preparation at 700 pg/cell (1.0 mg/mL), but LD_{50} was not determined.

Example 15

rhMBL mixed with oleic acid

rhMBL was obtained as described herein above in Example 8.

rhMBL at a concentration of 10 mg/mL in 10 mM Tris and 140 mM NaCl (pH pH=7.2) was saturated with oleic acid, mixed and centrifuged at first 3000 x g and then 18000 x g to separate and collect the water phase (according to method A of Example 10). The results of the subsequent test of cell killing activity are shown in figure 14. rhMBL alone has been tested in the highest concentration shown in the figure, and no effect on cell viability was observed.

When mixing oleic acid dissolved in 96% ethanol with 10 mg/mL rhMBL in 10 mM Tris and 140 mM NaCl (pH 7.2) in a molar ratio of 1:24 (protein vs. oleic acid) (essentially as described in Method B of Example 10) the subsequent test of cell killing activity showed that this method resulted in an active preparation with LD_{50}= 368 pg/cell (LC_{50}= 0.50 mg/mL) (see Table 2).

Example 16

Lysozyme mixed with oleic acid

Lysozyme was obtained from Sigma (cat.no. L7651) and was originated from chicken egg white.

Lysozyme at a concentration of 10 mg/mL in 0.9% NaCl was saturated with oleic acid, mixed and centrifuged at first 3000 x g and then 18000 x g to separate and collect the water phase (according to method A of Example 10). The results of the subsequent test of cell killing activity are shown in figure 15. Lysozyme alone has been tested in the highest concentration shown in the figure, and no effect on cell viability was observed.

When mixing oleic acid dissolved in 96% ethanol into 3 mg/mL lysozyme in 0.9% NaCl in a molar ratio of 1:15 (protein vs. oleic acid) as described in Method B of Example 10 the preparation showed an LD_{50} > 600 pg/cell (LC_{50} > 0.86 mg/mL).

Example 17

No protein but oleic acid

In general oleic acid mixed with a 0.9% NaCl solution yielded no cell killing activity. However, in a few cases (3 out of at least 9 tests) some activity was observed when

oleic acid was added to the 0.9% NaCl dissolved in ethanol and applied undiluted to L1210 cells. These results were not found to be reproducible.

Example 18

Effect of high shear mixing

This example demonstrates the effect of high shear mixing. Complexes of bLA and oleic acid were prepared essentially as described in Method B of Example 10 except that mixing was done either as described in Method B of Example 10 (high shear mixing) or by inverting the container 3 times (not high shear mixing). The bLA was prepared as described in Example 12.

The results are illustrated in figure 16 and show that a 9-fold more active preparation was obtained after high shear mixing.

Example 19

bLA + oleic acid mixed in different molar ratios

Complexes were prepared essentially as described in Method B of Example 10 except that different ratios of oleic acid to protein were tested. Oleic acid dissolved in ethanol was mixed with a 3 mg/mL bLA solution in different molar ratios, and the cell killing activities of the resulting preparations were determined (see figure 17). Molar ratios of 1:15 and 1:10 of bLA to oleic acid during mixing resulted in preparations with similar cell killing activities, while a 1:5 ratio resulted in decreased activity and a 1:1 ratio resulted in a preparation with very low activity.

Example 20

bLA mixed with oleic acid – stability and effect of chelator and buffer

The stability of complexes of bLA and oleic acid under different conditions was determined. “Stability” in the context of the present example refers to the complexes retaining their biological activity, preferably their cell killing activity during storage.

Various complexes prepared from bLA (prepared as described in Example 10) saturated with oleic acid were prepared essentially as described in Example 10, method A. Thus, an 8.6 mg/mL bLA solution was saturated with oleic acid and mixed under high shear conditions. The water phase was sterile filtered (the sterile filtration

did not result in changed cell killing activity), and the preparation incubated at -20°C, 2-8°C and 25°C. Cell killing activities and bLA+bLAC concentrations by A_{280nm} were determined after 0, 2 and 4 weeks of incubation at the three incubation conditions. More specifically, the protein content was determined by A_{280nm} nanodrop spectrometry. Both at 2-8°C and 25°C the cell killing activity was LD_{50} >200 pg/cell after two weeks, while the activity in the aliquots stored at -20°C had decreased 3-fold (LD_{50} had increased 3-fold) after 4 weeks (see figure 18). The loss of activity was not caused by decreased protein concentration (see figure 19). The aliquots incubated at 2-8°C and 25°C showed lower A_{280nm} compared to that stored at -20°C – maybe due to loss of dissolved oleic acid.

A stability study was performed with complexes prepared essentially as described in method A, Example 10 from a bLA solution with or without 10 mM Tris and 8 mM EDTA was saturated with oleic acid and subjected to high shear mixing. The isolated water phases (N318-65A and N318-65B) were sterile filtrated and aliquots incubated at -20°C and 25°C. The cell killing activities of the two preparations were determined before and after different periods of incubation at the two temperatures (see figure 20). From the accelerated stability samples at 25°C it was concluded that the preparation with 10 mM Tris and 8 mM EDTA still retained its cell killing activity after at least 4 weeks.

The content of oleic acid was measured in the two preparations after 8 weeks at 25°C. It was found that N318-65A (containing EDTA and Tris-buffer) contained 1.5 mM oleic acid resulting in a protein to oleic acid molar ratio of 1:3 (assuming a bLA+bLAC concentration of 7.6 mg/mL). N318-65B contained only 35 µM oleic acid.

To further study the effects of chelator (which binds divalent cations, such as Ca^{2+}) and buffer a 3-level full factorial design with 2 factors with no replicates was set up. The two factors were chelator and buffer. The three levels for chelator were 3.25 mM EDTA, 3.25 mM citrate and no chelator, while those for buffer were addition of Tris buffer (pH 8.5), acetate buffer (pH 5.0) or no buffer. All nine possible combinations were prepared by mixing oleic acid dissolved in ethanol into a 3 mg/mL bLA solution essentially as described in Method B of Example 10 except that the bLA solution contained the two factors at the desired level. As illustrated in figure 21 the preparations with Tris buffer had the highest cell killing activity just after preparation.

Aliquots of all nine preparations were placed at -20°C and 25°C and analysed for cell killing activity after 2 weeks at 25°C. Only the three preparations with Tris buffer (pH 8.5 in stock solution) still had detectable cell killing activity (see table 5), and therefore, only these three preparations were measured again after 4-5 weeks at 25°C.

Table 5

Cell killing activity of preparations resulting from mixing oleic acid dissolved in ethanol into a 3 mg/mL bLA solution in a 1:15 protein to oleic acid molar ratio with different chelators and buffers.

Chelator / Buffer	Item no.	Cell killing LD_{50} (pg/cell)[a]		
		0 weeks	2 weeks at 25°C	4-5 weeks at 25°C
None / none	N326-22B	131	>200	-
Citrate / none	N326-12E	116	>200	-
EDTA / none	N326-22C	83	>200	-
None / Tris (pH 8.5)	N326-22D	52	70	95
Citrate / Tris (pH 8.5)	N326-22E	38	45	62
EDTA / Tris (pH 8.5)	N326-12D	22	27	49
None / Acetate (pH 5.0)	N326-22F	>200	>200	-
Citrate / Acetate (pH 5.0)	N326-12C	74	>200	-
EDTA / Acetate (pH 5.0)	N326-12B	70	>200	-

[a]Assuming a constant bLA concentration of 3 mg/mL in all preparations.

Oligomerisation determined by SEC analysis and oleic acid content determined by GC were also measured on the samples (see table 6 and 7). The three preparations with Tris buffer, which retained their cell killing activity at 25°C showed a considerable amount of oligomerisation after 5-6 weeks at 25°C.

Table 6

SEC analysis of preparations resulting from mixing oleic acid dissolved in ethanol into a 3 mg/mL bLA solution in a 1:15 protein to oleic acid molar ratio with different chelators and buffers. Samples were stored at 25°C until analysis.

Chelator / Buffer	Item no.	**bLA monomer** (mg/mL)			**bLA oligomer** (mg/mL)		
		0 weeks[b]	2 weeks	5-6 weeks	0 weeks[b]	2 weeks	5-6 weeks
None / none	N326-22B	2.84	2.80	-	0.00	0.00	-
Citrate / none	N326-12E	2.78	2.86	-	0.00	0.00	-
EDTA / none	N326-22C	2.82	2.87	-	0.00	0.02	-
None / Tris (pH 8.5)	N326-	2.75	2.52	1.72	0.00	0.02[a]	0.44[a]

	22D						
Citrate / Tris (pH 8.5)	N326-22E	2.77	2.56	1.78	0.00	0.00	0.27[a]
EDTA / Tris (pH 8.5)	N326-12D	2.78	2.09	0.33	0.00	0.11[a]	1.84[a]
None / Acetate (pH 5.0)	N326-22F	2.73	2.78	-	0.00	0.02	-
Citrate / Acetate (pH 5.0)	N326-12C	2.44	2.56	-	0.00	0.00	-
EDTA / Acetate (pH 5.0)	N326-12B	2.67	2.72	-	0.00	0.00	-

[a]Trimer and tetramer.
[b]Some preparations had been stored for 1 week at -20°C before analysis.

Table 7

Content of oleic acid according to GC analysis of preparations resulting from mixing oleic acid dissolved in ethanol into a 3 mg/mL bLA solution in a 1:15 protein to oleic acid molar ratio with different chelators and buffers.

Chelator / Buffer	**Item no.**	**Molar ratio bLA vs. oleic acid[a]**	
		0 weeks (5-6 weeks at -20°C)	**3-4 weeks at 25°C**
None / none	N326-22B	1:2	Oleic acid below 0.01 mg/mL[b]
Citrate / none	N326-12E	1:1	Oleic acid below 0.01 mg/mL
EDTA / none	N326-22C	1:3	1:0.5
None / Tris (pH 8.5)	N326-22D	1:10	1:4
Citrate / Tris (pH 8.5)	N326-22E	1:10	1:10
EDTA / Tris (pH 8.5)	N326-12D	1:8	1:7
None / Acetate (pH 5.0)	N326-22F	Oleic acid below 0.01 mg/mL	Oleic acid below 0.01 mg/mL
Citrate / Acetate (pH 5.0)	N326-12C	1:2	Oleic acid below 0.01 mg/mL
EDTA / Acetate (pH 5.0)	N326-12B	1:3	Oleic acid below 0.01 mg/mL

[a]Assuming a constant bLA concentration of 3 mg/mL in all preparations.
[b]Detection limit for oleic acid was 0.01 mg/mL by the GC method.

To identify and make a relative quantification of bLA vs. bLAC in the samples a Q-sepharose anion exchange chromatography column was used. The column separates bLA and bLAC, and for the nine samples peaks were identified at retention volumes corresponding to bLA and bLAC reference samples. The obtained data were converted to the results presented in table 8.

Table 8

Analysis of relative content of bLA vs. bLAC by anion exchange chromatography in preparations resulting from mixing oleic acid dissolved in ethanol into a 3 mg/mL bLA solution in a 1:15 protein to oleic acid molar ratio with different chelators and buffers.

Chelator / Buffer	Item no.	0 weeks	2 weeks	4-5 weeks at 25°C	
				bLA	bLAC
None / none	N326-22B	-	-	100%	0%
Citrate / none	N326-12E	-	-	100%	0%
EDTA / none	N326-22C	-	-	100%	0%
None / Tris (pH 8.5)	N326-22D	-	-	83%	17%
Citrate / Tris (pH 8.5)	N326-22E	-	-	46%	54%
EDTA / Tris (pH 8.5)	N326-12D	-	-	12%	88%
None / Acetate (pH 5.0)	N326-22F	-	-	100%	0%
Citrate / Acetate (pH 5.0)	N326-12C	-	-	100%	0%
EDTA / Acetate (pH 5.0)	N326-12B	-	-	100%	0%

The pH in bLA mixed with oleic acid under high shear conditions was checked by making a fresh preparation of bLA in 0.9% NaCl mixed with oleic acid in a 1:15 molar ratio and measuring pH 6.4. After 9 weeks at -20°C preparation N326-12D, containing bLA mixed with oleic acid after addition of Tris buffer (pH 8.5) and EDTA, was measured to have a pH of 8.2, which may be assumed to be very close to the pH just after mixing.

To study the effect of pH on stability 3 mg/mL bLA was mixed under high shear conditions with oleic acid in a 1:15 molar ratio in the presence of 3.25 mM EDTA and 10 mM acetate (pH 5.0) essentially as described in Method B in Example 10. After mixing of the preparations the pH was increased to 8.5 using NaOH in one preparation while another similar sample remained at the mixing pH. Aliquots of both were stored at -20°C and 25°C, and analysed for cell killing activity (table 9) and oleic acid content (table 10). Content of oleic acid was analysed on samples stored for 2 weeks at -20°C and assumed to be similar to the content just after production.

Table 9

Cell killing activity of preparations of bLA + oleic acid mixed in presence of 10 mM acetate (pH 5.0) and 3.25 mM EDTA followed by changing the pH to 8.5 or leaving the pH unchanged.

Storage pH	Item no.	LD_{50} (pg/cell)[a]	
		0 weeks	3 weeks at 25°C
5.0	N326-90C	93	> 200
8.5	N326-90B	51	28

[a]Assuming a constant bLA concentration of 3 mg/mL in all preparations.

Table 10

Content of oleic acid according to GC analysis in preparations of bLA + oleic acid mixed in presence of 10 mM acetate (pH 5.0) and 3.25 mM EDTA followed by changing the pH to 8.5 or leaving the pH unchanged.

Storage pH	Item no.	Molar ratio bLA vs. oleic acid[a]	
		2 weeks -20°C	2 weeks 25°C
5.0	N326-90C	1:3	Oleic acid below 0.01 mg/mL[b]
8.5	N326-90B	1:9	1:9

[a]Assuming a constant bLA concentration of 3 mg/mL in all preparations.
[b]Detection limit for oleic acid was 0.01 mg/mL by GC method.

Example 21

Heating bLA and oleic acid

The effect of heating preparations of bLA and oleic acid to 40°C or 70°C for 10 minutes just after the mixing process compared to no heating was tested with no chelator or buffer in the preparations. The preparations produced had lower cell killing activity compared to that obtained normally, but apparently heating helped produce cell killing activity. Thus, the preparation heated to 70ºC had a lower LD_{50}, than the preparation heating to 40ºC, which in turn had a lower LD_{50} than the unheated preparation (room temperature). As observed previously when not including a buffer the preparations were not stable at 25°C. SEC analysis showed no oligomerisation after 2 weeks at 25°C. For the analysis of oleic acid content and SEC analysis it was assumed that the results obtained from analysing samples stored for 2 weeks at -20°C were close to those that would have been obtained if analysing just after production.

Example 22

Conversion of bLA to bLAC with different fatty acids

Conversion of bLA to complex with fatty acids

Two batches of bLA were used for the conversion.

Table 16: Batches of bLA used for conversion

bLA batch N277-64A	7x11.7 mL aliquot stored at -20ºC prepared from bLA batch N278-50C produced at NatImmune. bLA concentration: 4.01 mg/mL (SEC-HPLC) with bLA reference N262-06A
bLA batch N289-56A (=N289-78B)*	6x18 mL aliquot stored at -20ºC prepared from bLA batch N177-76B produced at Biovian. bLA concentration: 2.5 mg/mL (SEC-HPLC) with bLA reference N286-28A

*From the same bLA batch (N177-76B), but from different bags of this batch.

The following fatty acids were purchased from Larodan (Sweden) for the conversion of bLA to complex.

Table 17: Fatty acids used for conversion

Name	No. Of C:Double bond	Configuration	Larodan no.	Purity
Palmitoleic Acid	16:1(n-7)	Cis	10-1601-30	>99%
Heptadecenoic Acid	17:1(n-7)	Cis	10-1701-30	>99%
Oleic acid	18:1(n-9)	Cis	10-1801-17	>99%
Elaidic acid	18:1(n-9)	Trans	10-1810-13	>99%
Vaccenic acid	18:1(n-7)	Cis	10-1812-30	>99%

Linoleic acid	18:2(n-6)	Cis	10-1802-13	>99%
Alpha Linolenic Acid	18:3(n-3)	Cis	10-1803-30	>99%
Gamma Linolenic Acid	18:3(n-6)	Cis	10-1830-30	>99%
Stearidonic acid	18:4(n-3)	Cis	10-1840-4	>97%
Gondoic acid	20:1(n-9)	Cis	10-2001-30	>99%
Eicosapentaenoic Acid	20:5(n-3)	Cis	10-2005-30	>99%

The conversion of bLA to a complex with the different fatty acid was performed as previously described in Examples 4 and 5 of Danish Patent application PA 200700693 and Examples 4 and 5 of PCT application PCT/DK2008/050105.

On the day of conversion, a solution was prepared with the respective fatty acid according to the following Table:

Table 18: Preparation of fatty acid solution

	Melting point (ºC)	Volume (µL) (~mg)	Ethanol (mL)	Equilibration buffer (mL)	Total volume (mL)	F (m
Oleic acid	13	20	0.25	20	20.27	3
Vaccenic acid	39	20	0.25	20	20.27	3
Elaidic acid	44	20	0.25	20	20.27	3
Linoleic acid	-5	20	0.25	20	20.27	3
Alfa Linolenic acid	-11	20	0.25	20	20.27	3
Palmitoleic acid	0.5	18	0.25	20	20.27	3
Eicosapentaenoic acid		21.5	0.25	20	20.27	3
Stearidonic acid	-57	19.5	0.25	20	20.27	3
Heptadecenoic acid		19	0.25	20	20.27	3
Gamma Linolenic acid	?	20	0.25	20	20.27	3
Gondoic acid	25->32	22	0.25	20	20.27	3

bLA, EDTA and fatty acid were mixed with the following ratio:

Table 19: Sample conditioning prior conversion

Mix ID	Sample ID	Fatty acid	Molar ratio EDTA/bLA	Molar ratio Oleic acid/bLA	AIEC load mL	AIEC load
LAC-078	N277-64C	Oleic acid	15	30	43	43
LAC-080	N277-74C	Vaccenic acid	15	30	42	42
LAC-082	N277-77D	Linoleic acid	15	30	42	42
LAC-083	N277-80B	alfa Linolenic acid	15	30	42	42
LAC-125	N289-56C	Palmitoleic acid	19	37	54	34*
LAC-126	N289-59B	Eicosapentaenoic acid	19	37	54	34*
LAC-127	N289-62B	Heptadecenoic acid	19	37	54	34*
LAC-128	N289-64B	gamma Linolenic acid	19	37	54	34*
LAC-132	N289-67B	Gondoic acid	19	37	54	34*
LAC-134	N289-70B	Stearidonic acid	19	37	54	34*
LAC-140	N289-78D	Oleic acid	19	37	54	34*

*Sample conditioning with bLA N277-64A

**Sample conditioning with bLA N289-56A/N289-78B

Conversion with Elaidic acid was not performed, as the conditioned sample made from this fatty acid was not soluble. Elaidic acid is the fatty acid with the highest melting point among those tested and the double bond is a trans double bond.

Conversion with oleic acid was done twice with the two different bLA start materials. The molar ratio between EDTA and bLA and fatty acid and bLA should have been 15 and 30 respectively. Other molar ratios were applied as the bLA reference for the SE-HPLC analysis was changed. With the new reference the bLA concentration is approximately 15% lower. The sample conditioning calculations were done on basis of the concentration measured.

Each conditioned sample was applied on a 0.78 cm^2 column (Tricorn 10/100) newly packed and CIP'ed with Q sepharose XL (10 cm bed height). The load of bLA during the purifications was approximately 54 mg/cm^2 (LAC-078 to LAC-083) and 44 mg/cm^2 (LAC-125 to LAC-140).

bLAC recovery

In every purification the whole bLAC recovery was stored frozen, except for one small sample (1 mL) that was desalted against milliQ-H2O using a NAP-10 column (GE Healthcare). The desalted sample was used for cell killing assay and GC analysis.

bLAC recovery characterization:

Yield

The yields of the conversion runs were determined by size exclusion HPLC (SE-HPLC) run according to standard methods.

Potency

The potency of the converted bLA was determined by cell killing abilities. Cell killing was run according to Example 8. The potency of bLAC determined by cell killing assay is given in pg bLAC per cell (LD50). Before testing in cell killing assay, the bLAC solutions were desalted against milli-Q H2O using NAP-10 desalting column (GE HealthCare).

Identity

The presence of complex bLA and fatty acid was determined by histone binding assay. The histone binding assays was run according to Example 7. Only complex between bLA and fatty acid have histone biding abilities.

GC-Analysis

The amount of lipid and the lipid composition of the bLAC samples were determined by Net-Food lab (Finland) according to traditional methods: After esterification by the Boron trifluoride-methanol method, the fatty acids methyl esters (FAME) in the bLAC samples were analyzed by Gas Chromatography. Lipid content was calculated in relation to the concentration of the internal standard. The method is based on the European Pharmacopoeia (5.6) protocol 2.4.22 (Composition of Fatty Acids by Gas Chromatography, Method C).

RESULTS

Conversion runs

The chromatograms of the conversion of bLAC with the different fatty acids can be seen in Figure 22 and 23.

When bLA N289-56A was used as start material the first eluted peak corresponding to non converted bLA was lower. This can be due to the higher molar ratio between bLA and EDTA and bLA and oleic acid, and also the lower amount of bLA loaded on the column. With these considerations, oleic acid and gondoic acid showed the same elution profile, while with all the other fatty acids the first peak is increased. When stearidonic acid was used for conversion, no peak corresponding to a complex between fatty acid and bLA was obtained. Here all protein eluted in the 1st step gradient.

The yield of the conversions runs based on the SE-HPLC analysis can be seen in next Table:

Table 20: Yield of the conversion runs

Prod. ID	Fatty acid	Recovery	bLAC (mg/mL)	Volume (mL)	bLA load (mg/cm^2)	Yield (mg/cm^2)	Yield (%)	Rank Oleic acid=1
		Start material N267-64A						
LAC-078	Oleic acid	N277-64H	4.1	6.5	54	34	63%	1(def)
		N277-65A*	2.9					
LAC-080	Vaccenic acid	N277-74H	4.9	6.5	54	41	76%	1.20
		N277-74I*	3.3					
LAC-082	Linoleic acid	N277-78E	4.4	7	54	39	73%	1.16
		N277-78F*	2.8					
LAC-083	alfa Linolenic acid	N277-80G	4.1	7.5	54	39	73%	1.16
		N277-80H*	2.7					

New start material N289-56A/78B and new sample conditioning

LAC-125	Palmitoleic acid	N289-57B	3.6	6.5	44	30	68%	0.83
		N289-57C*	2.3					
LAC-126	Eicosapentaenoic acid	N289-59G	2.6	6.5	44	22	49%	0.60
		N289-59H*	1.8					
LAC-127	Heptadecenoic acid	N289-63A	4.0	6.5	44	33	76%	0.93
		N289-63B*	2.6					
LAC-128	gamma Linolenic acid	N289-64G	2.8	6.5	44	23	53%	0.65
		N289-64H*	1.6					
LAC-132	Gondoic acid	N289-68B	5.9	5.5	44	42	95%	1.16
		N289-68C*	3.8					
LAC-134	Stearidonic acid	N289-70F	3.3		44	0**	0%	0.00
LAC-140	Oleic acid	N289-79D	5.1	5.5	44	36	82%	1(def)

*Item no. after desalting

**No converted peak recovered

The yield for the two conversion runs with oleic acid was rather different. Conversion was then performed with different sample conditioning and different bLA start material as discussed earlier. The lower load and different sample conditioning for LAC-140 might explain the better yield obtained.

In Table 21, the yield of conversion with oleic acid was compared to the yield obtained with the other fatty acids for similar sample conditioning, sample load and bLA start material.

Table 21: Yield ranking compared to oleic acid (18:1(n-9))

Fatty acid (From + to -)		Rank (from Table 19)
1. Vaccenic acid	**18:1(n-7)**	**1.20**
2. Gondoic acid	**20:1(n-9)**	**1.16**
3. Linoleic acid	**18:2(n-6)**	**1.16**
4. Alfa-Linolenic acid	**18:3(n-3)**	**1.16**
5. OLEIC ACID	**18:1(n-9)**	**1(def)**
6. Heptadecenoic acid	**17:1(n-7)**	**0.93**
7. Palmitoleic acid	**16:1(n-7)**	**0.83**
8. Gamma-Linolenic acid	**18:3(n-6)**	**0.65**
9. Eicosapentaenoic acid	**20:5(n-3)**	**0.60**
10. Stearidonic acid	**18:4(n-3)**	**0**

Lipid composition by GC analysis

The lipid composition and lipid content of the bLA in complex with the different fatty acids obtained was determined by GC analysis.

As shown in Table 22, the fatty acid used for the conversion was detected in all the corresponding samples analysed by Gas chromatography, except for the sample converted with gamma Linolenic acid. For this sample the amount of fatty acid was below the detection limit of the analysis. As the conversion performed normally for this fatty acid, the sample was sent for retest by GC analysis. Stearidonic acid was not

detected in the sample recovered from the conversion run with this fatty acid. In this case no converted bLA peak was obtained, and the sample corresponded to non-converted bLA was sent to GC analysis.

Table 22: Lipid composition by GC analysis

		Fatty acids identified by Gas chromatography												
		myristic acid	palmitic acid	Palmitoleic acid	Heptadecenoic acid	Stearic acid	Vaccenic acid	Oleic acid	Linoleic acid	□ Linolenic acid	□ Linolenic acid	Stearidonic acid	Gondoic acid	Eicosapentaenoic acid
Sample ID	Fatty acid used for conversion	14:0	16:0	16:1 (n-7)	17:1 (n-7)	18:0	18:1 (n-7)	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	18:3 (n-6)	18:4 (n-6)	20:1 (n-9)	20:5 (n-3)
N277-65A	Oleic acid	0	0	0	0	0	0	**98**	0	0	0	0	0	0
N277-74I	Vaccenic acid	0	0	0	0	0	**95**	0	0	0	0	0	0	0
N277-78F	Linoleic acid	0	0	0	0	0	0	0	**100**	0	0	0	0	0
N277-80H	Alfa-Linolenic acid	0	0	0	0	0	0	0	0	**100**	0	0	0	0
N289-57C	Palmitoleic acid	0	0	**100**	0	0	0	0	0	0	0	0	0	0
N289-59H	Eicosapentaenoic acid	0	0	0	0	0	0	0	0	0	0	0	0	**100**
N289-63B	Heptadecenoic acid	0	0	0	**100**	0	0	0	0	0	0	0	0	0
N289-64H	Gamma- Linolenic acid	Below detection limit												
N289-68C	Gondoic acid	0	0	0	0	0	0	0	0	0	0	0	**92**	0
N289-70F	Stearidonic acid	Only non-converted product tested; below detection limit												
N289-79D	Oleic acid	Not done												

The molar lipid/bLA ratios in the samples were calculated from the results of the GC analysis. The calculations are shown in Table 23.

Table 23: Molar ratio lipid/bLA

SampleID -	Fatty acid	Lipid (mg/mL)	Lipid (µmol/mL)	bLA (µmol/mL)	Molar ratio Lipid/bLA
N277-65A	Oleic acid	1.0	3.7	0.2	18
N277-74I	Vaccenic acid	0.9	3.2	0.2	14
N277-78F	Linoleic acid	0.7	2.6	0.2	13
N277-80H	Alfa-Linolenic acid	0.6	2.2	0.2	12
N289-57C	Palmitoleic acid	0.3	1	0.2	6
N289-59H	Eicosapentaenoic acid	0.1	0.4	0.1	3
N289-63B	Heptadecenoic acid	0.6	2.1	0.2	11
N289-64H	Gamma-Linolenic acid	Below detection limit	no data	0.1	no data
N289-68C	Gondoic acid	1.2	4.4	0.3	16
N289-70F	Stearidonic acid	<0.01			
N289-79D	Oleic acid	Not measured			

The molar ratio between bLA and lipid was found highest when oleic acid was used to conversion. Similar molar ratios to oleic acid were obtained for fatty acids giving the highest yield of conversion, i.e. gondoic acid, vaccenic and linoleic acid. Then the molar ratio lipid/bLA decrease for the two fatty acids were the yields of the conversion was the lowest, i.e. Palmitoleic acid and Eicosapentaenoic acid.

The lipid/bLA ratio in the sample prepared using gamma-Linolenic acid could not be measured.

Identity by Histone assay

The histone binding abilities of the bLAC samples converted with the different fatty acids were compared by histone binding assay. For each plate, a bLAC reference converted with oleic was run. From the binding curve EC50 was determined for each sample based on the SE-HPLC concentration. The EC50 were normalized to the EC50 of the bLAC reference applied in the plate. The results are shown in Table 24.

Table 24: EC50 by histone binding assay

Sample ID	**Plate ID:**	**Fatty acid used**	**EC50**	**Binding (%) to**

		for conversion	(µg/mL)	ref.
N277-65A	N281-20A	Oleic acid	9	107%
N277-30A	N281-20A	Oleic acid (reference)	9.6	100%(def)
N277-74I	N281-22A	Vaccenic acid	7.4	142%
N277-78F	N281-22A	Linoleic acid	19.1	55%
N277-80H	N281-22A	Alfa-Linolenic acid	71.6	15%
N277-30A	N281-22A	Oleic acid (reference)	10.5	100%(def)
N289-57C	N291-80B	Palmitoleic acid	17.5	35%
N289-59H	N291-80B	Eicosapentaenoic acid	41.3	15%
N289-63B	N291-80B	Heptadecenoic acid	10.1	61%
N277-30A	N291-80B	Oleic acid (reference)	6.2	100%(def)
N289-64H	N291-80C	Gamma- Linolenic acid	41.5	13%
N289-68C	N291-80C	Gondoic acid	3.1	174%
N277-30A	N291-80C	Oleic acid (reference)	5.4	100%(def)
N289-70H	N296-04B	Stearidonic acid	No binding	No binding
N277-30A	N296-04B	Oleic acid (reference)	5.6	100%(def)

Table 25: EC50 ranking by histone binding assay

bLA and Fatty acid histone binding ranking		**Histione binding capacity**	**Molar ratio lipid/bLA**
1. Gondoic acid	**20:1(n-9)**	**174%**	**16**
2. Vaccenic acid	**18:1(n-7)**	**142%**	**14**
3. Oleic acid	**18:1(n-9)**	**107%**	**18**
4. Heptadecenoic acid	**17:1(n-7)**	**61%**	**11**
5. Linoleic acid	**18:2(n-6)**	**55%**	**13**
6. Palmitoleic acid	**16:1(n-7)**	**35%**	**6**
7. alfa- Linolenic acid	**18:3(n-3)**	**15%**	**12**

7. Eicosapentaenoic acid	**20:5(n-3)**	**15%**	**3**
9. gamma- Linolenic acid	**18:3(n-6)**	**13%**	**no data**
10. Stearidonic acid	**18:4(n-3)**	**No binding**	**no data**

As shown in Table 25, it seems that there is a good correlation between the molar ratio lipid/bLA and histone binding of the bLA-lipid complex. The higher the molar ratio, the higher binding is obtained, with the exception of the product obtained when the conversion was performed with gamma Linolenic acid (which again behaves differently to the other fatty acids tested).

Potency by cell killing assay

The cell killing ability of the bLA in complex with the different fatty acids was verified. From the cell killing analysis, LD50 was determined for each bLA-fatty acid complex. The results are shown in Table 26:

Table 26: LD50 of bLAC converted with different fatty acids

SampleID	PlateID:	Fatty acid used for conversion	SE-HPLC Conc (mg/mL)	LD50 (pL/cell)	LD50 (pg/cell)	Binding to ref
N277-65A*	N281-24B	Oleic acid	2.94	6.33	19	100% (def)
N277-74I	N281-24B	Vaccenic acid	3.26	6.55	21	90%
N277-78F	N281-24C	Linoleic acid	2.76	6.34	17	112%
N277-80H	N281-24C	Alfa Linolenic acid	2.68	7.92	21	90%
N289-57C	N291-76A	Palmitoleic acid	2.32	13.28	31	126%
N277-30A	N291-76A	Oleic acid (reference)	7.55	5.14	39	100%(d
N289-59H	N291-84A	Eicosapentaenoic acid	1.84	37.44	69	48%
N277-30A	N291-84A	Oleic acid (reference)	7.55	4.33	33	100%(d
N289-63B	N291-84B	Heptadecenoic acid	2.56	8.5	22	145%
N277-30A	N291-84B	Oleic acid (reference)	7.55	4.2	32	100%(d
N289-64H	N291-90A	Gamma Linolenic acid	1.56	26.75	42	90%

N277-30A	N291-90A	Oleic acid (reference)	7.55	5.01	38	100%(d
N289-68C	N291-90B	Gondoic acid	3.83	34.3	131	25%
N277-30A	N291-90B	Oleic acid (reference)	7.55	4.41	33	100%(d
N289-70H		Stearidonic acid			Not tested	

*Also used as reference in the cell killing assay

From the results presented in Table 26, it can be seen that most of the bLA converted with the fatty acid tested have similar LD50 to the bLA converted with oleic acid with the exception of the complex obtained with eicosapentaenoic acid and gondoic acid, which are the fatty acids with the longest carbon chains.

Table 27: LD50 ranking by cell killing assay

bLA and Fatty acid			**Molar ratio lipid/bLA**
1. Heptadecenoic acid	**17:1(n-7)**	**145%**	11
2. Palmitoleic acid	**16:1(n-7)**	**126%**	6
3. Linoleic acid	**18:2(n-6)**	**112%**	13
4. Oleic acid	**18:1(n-9)**	**100% (def)**	18
5. Alfa Linolenic acid	**18:3(n-3)**	**90%**	12
5. Vaccenic acid	**18:1(n-7)**	**90%**	14
5. Gamma Linolenic acid	**18:3(n-6)**	**90%**	no data
6. Eicosapentaenoic acid	**20:5(n-3)**	**48%**	3
7. Gondoic acid	**20:1(n-9)**	**25%**	16
8. Stearidonic acid*	**18:4(n-3)**	**Not tested**	No data

*Not tested.

Claims

1. A method for preparing a cytotoxic complex between an emulsifier and a fatty acid, said method comprising the steps of
    e. providing an emulsifier in an aqueous solution, wherein said emulsifier is not a fatty acid; and
    f. providing a fatty acid; and
    g. contacting the emulsifier with the fatty acid; and
    h. mixing the emulsifier and the fatty acid using high shear mixing

    thereby obtaining a complex between said emulsifier and said fatty acid, wherein said complex has a cytotoxic effect on tumour cells

2. The method according to claim 1, wherein the emulsifier is a protein or a polypeptide.

3. The method according to claim 1, wherein the emulsifier is a polypeptide with a calculated pI lower than 9, for example in the range of 4 to 7, such as in the range of 4.5-6.

4. The method according to any of the preceding claims, wherein the emulsifier is a polypeptide which can adopt apo – or holo- form.

5. The method according to any of the preceding claims, wherein the emulsifier is a polypeptide which is a member of the albumin family.

6. The method according to claim 5, wherein the polypeptide is selected from the group consisting of alpha-lactalbumin and serum albumin.

7. The method according to claim 5, wherein the polypeptide is human alpha-lactalbumin of SEQ ID 1 or bovine alpha-lactalbumin of SEQ ID NO: 2 or a functional homologue thereof with at least 75% sequence identity.

8. The method according to claim 5, wherein the polypeptide is human serum albumin.

9. The method according to any of the claims 1 to 4, wherein the emulsifier is a polypeptide, which is a member of the globulin family.

10. The method according to claim 9, wherein the polypeptide is lactoglobulin, such as bovine lactoglobulin.

11. The method according to any of the claims 1 to 4, wherein the emulsifier is a polypeptide or a multimer of polypeptides, said polypeptide comprising a collagen domain.

12. The method according to any of the claims 1 to 4, wherein the emulsifier is a polypeptide or a multimer of polypeptides, said polypeptide being a member of the lectin family.

13. The method according to any of claims 11 to 12, wherein the polypeptide or multimer of polypeptides is MBL

14. The method according to any of the preceding claims, wherein the size of the emulsifier is in the range of 5-500 kDa, such as in the range of 15 to 100 kDa.

15. The method according to claim 1, wherein the emulsifier is a phospholipid or a mixture of phospholipids.

16. The method according to claim 17, wherein at least one phospholipid is phosphatidylcholine.

17. The method according to any of the preceding claims, wherein the complex has an LC_{50} of at the most 50 mg/ml, preferably at the most 1 mg/ml with respect to L1210 cells at a concentration of 1.4 $x10^6$ cells/mL in *in vitro* culture.

18. The method according any one of claims 1 to 16, wherein the LD_{50} in respect of in vitro cultured L1210 cells is at the most 400 pg/cell, preferably at the most 200, even more preferably at the most 150, yet more preferably at the most 100, even more preferably at the most 60.

19. The method according to any of the preceding claims, wherein the fatty acid is selected from the group of C_{14} to C_{20} fatty acids.

20. The method according to any of the preceding claims, wherein the fatty acid has in the range of 1 to 4 double bonds.

21. The method according to any of the preceding claims, wherein the fatty acid is a C_{16} or C_{18} fatty acid containing in the range of 1 to 4 cis double bonds.

22. The method according to any of the preceding claims, wherein the fatty acid is selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis and C18:4:6, 9, 12, 15cis.

23. The method according to any of the preceding claims, wherein the fatty acid is oleic acid, heptadecenoic acid, linoleic acid or vaccenic acid.

24. The method according to any of the preceding claims, wherein the mixing is performed using a vortex mixer.

25. The method according to claim 24, wherein the mixing is performed using a vortex mixer at at least 2000 rpm.

26. The method according to any of claims 24 and 25, wherein the mixing is done on a vortex mixer for at least 5 sec.

27. The method according to any of claims 1 to 23, wherein the mixing is performed using an impeller mixer.

28. A complex prepared by the method according to any of the preceding claims.

29. The complex according to claim 28 for use as a medicament.

30. A complex according to claim 29 for use as a medicament for treatment of a clinical disorder selected from the group consisting of viral infections, disorders associated with aberrant cell proliferation and disorders associated with angiogenesis.

31. The complex according to claim 30, wherein the clinical disorder is selected from the group consisting of respiratory tract infections, cancer, actinic keratosis and warts.

32. The complex according to claim 30, wherein the clinical condition is bladder cancer.

33. The complex according to claim 30, wherein the clinical condition is a cancer of the brain, such as gliobastoma.

34. The complex according to claim 30, wherein the clinical condition is papiloma.

35. Use of a complex according to claim 28 for the preparation of a medicament for treatment of a clinical disorder selected from the group consisting of viral infections, disorders associated with aberrant cell proliferation and disorders associated with angiogenesis.

36. A method for treatment of a clinical disorder selected from the group consisting of viral infections, disorders associated with aberrant cell proliferation and disorders associated with angiogenesis comprising administering the complex according to claim 28 to an individual in need thereof.

Fig. 1

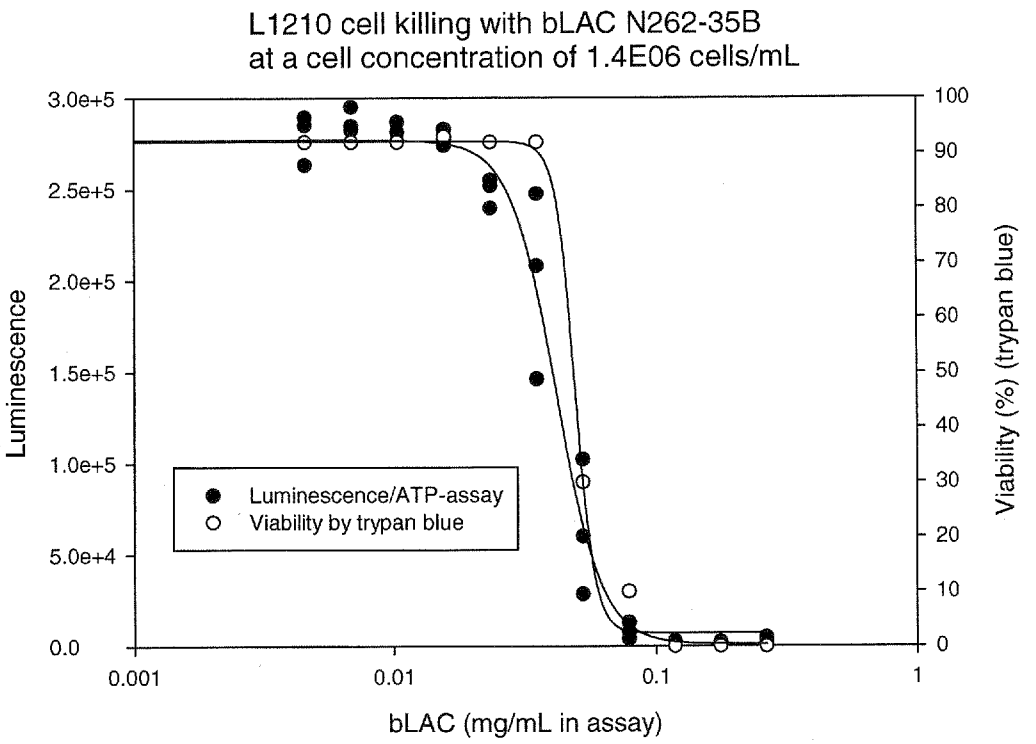

L1210 cell killing with bLAC N262-35B
at a cell concentration of 1.4E06 cells/mL
Luminescence
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
Viability (%) (trypan blue)
100
90
80
70
60
50
40
30
20
10
0
Luminescence/ATP-assay
Viability by trypan blue
0.001
0.01
0.1
1
bLAC (mg/mL in assay)

Fig. 2

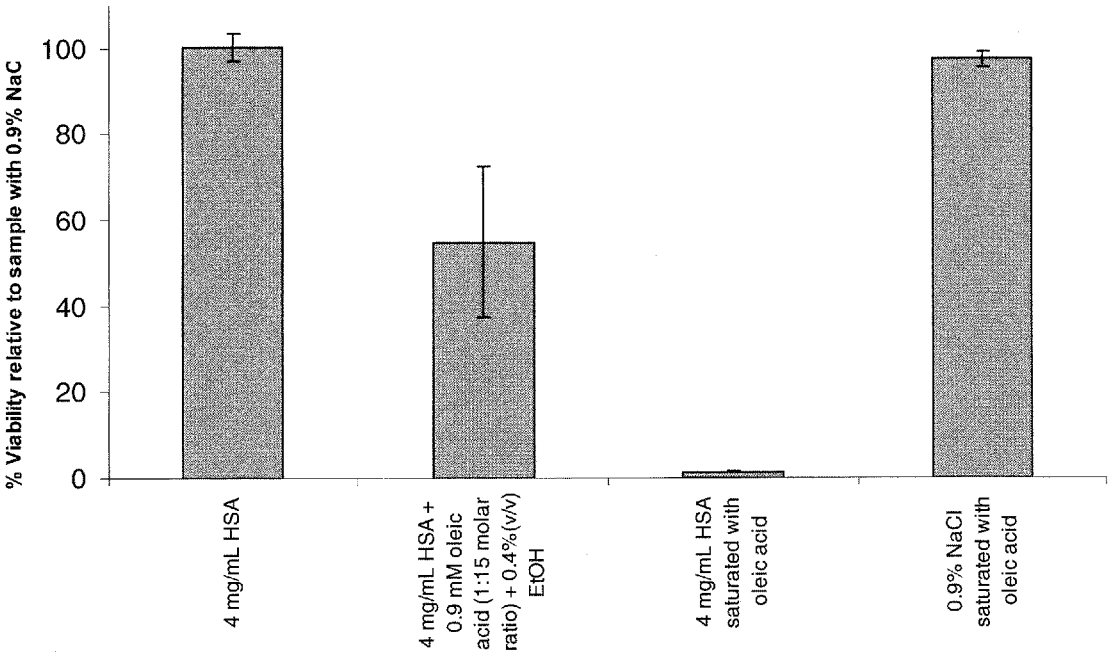

% Viability relative to sample with 0.9% NaC
100
80
60
40
20
0
4 mg/mL HSA
4 mg/mL HSA + 0.9 mM oleic acid (1:15 molar ratio) + 0.4%(v/v) EtOH
4 mg/mL HSA saturated with oleic acid
0.9% NaCl saturated with oleic acid

Fig. 3

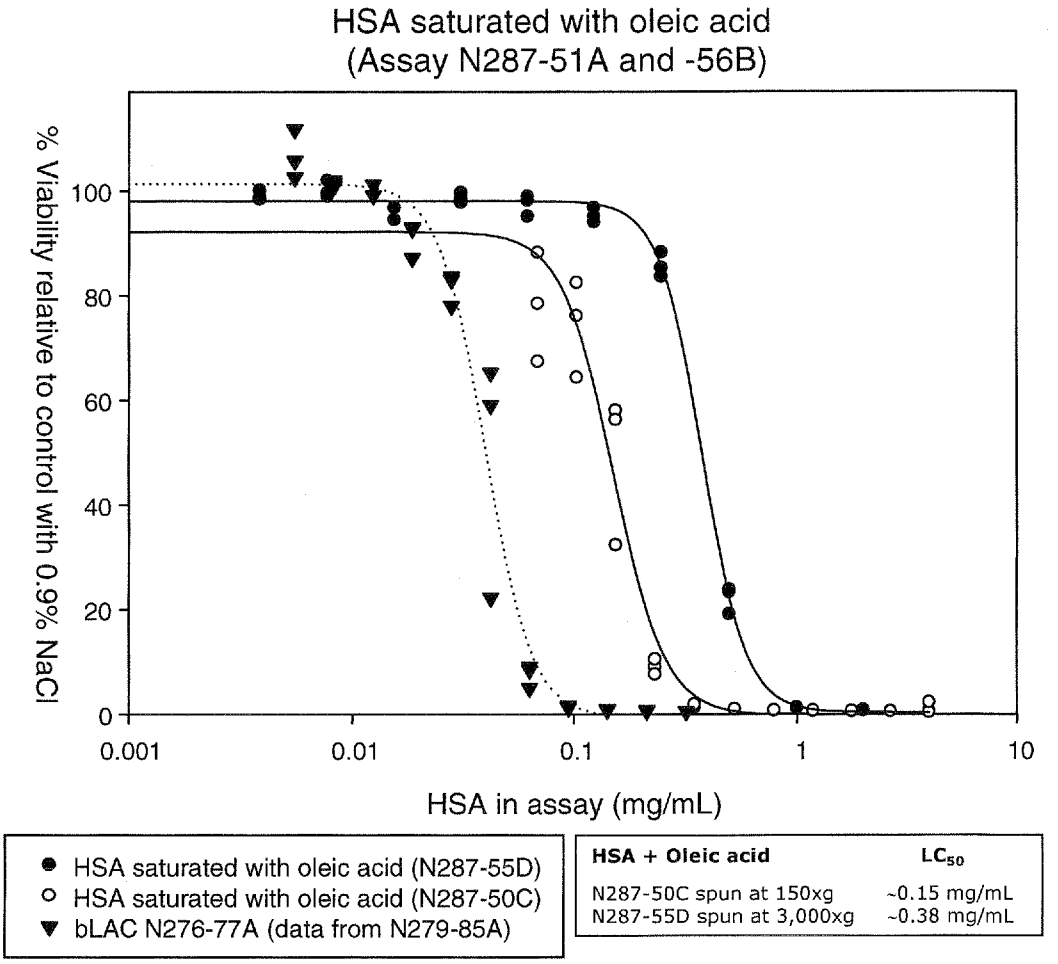

HSA saturated with oleic acid
(Assay N287-51A and -56B)
% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
HSA in assay (mg/mL)
HSA saturated with oleic acid (N287-55D)
HSA saturated with oleic acid (N287-50C)
bLAC N276-77A (data from N279-85A)
HSA + Oleic acid
LC50
N287-50C spun at 150xg
~0.15 mg/mL
N287-55D spun at 3,000xg
~0.38 mg/mL

Fig. 4

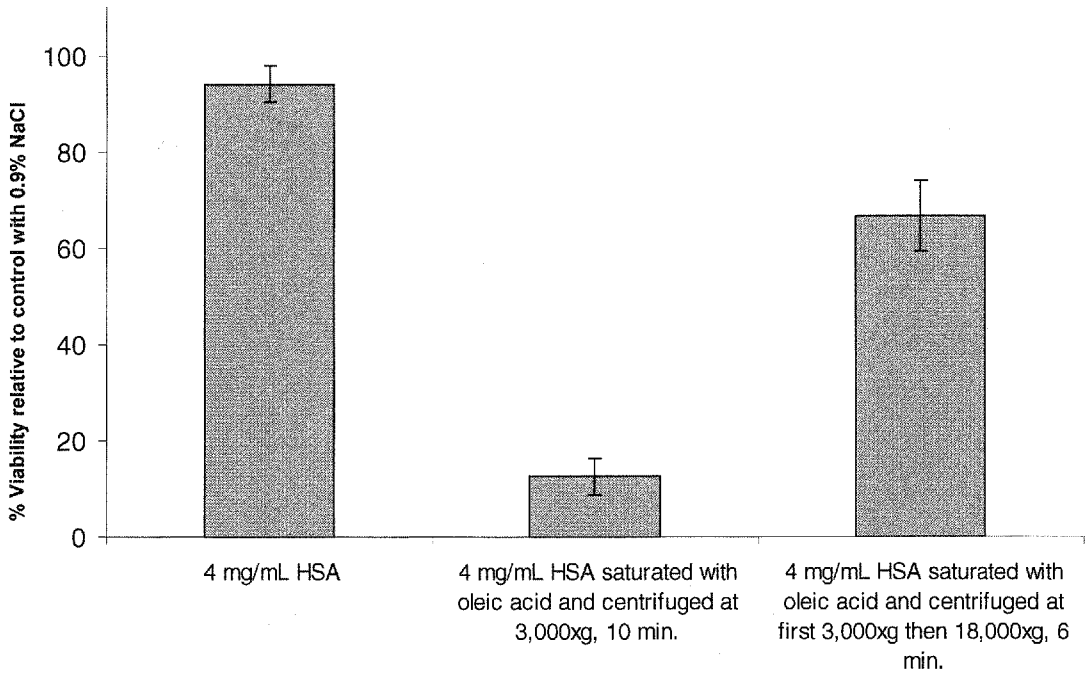
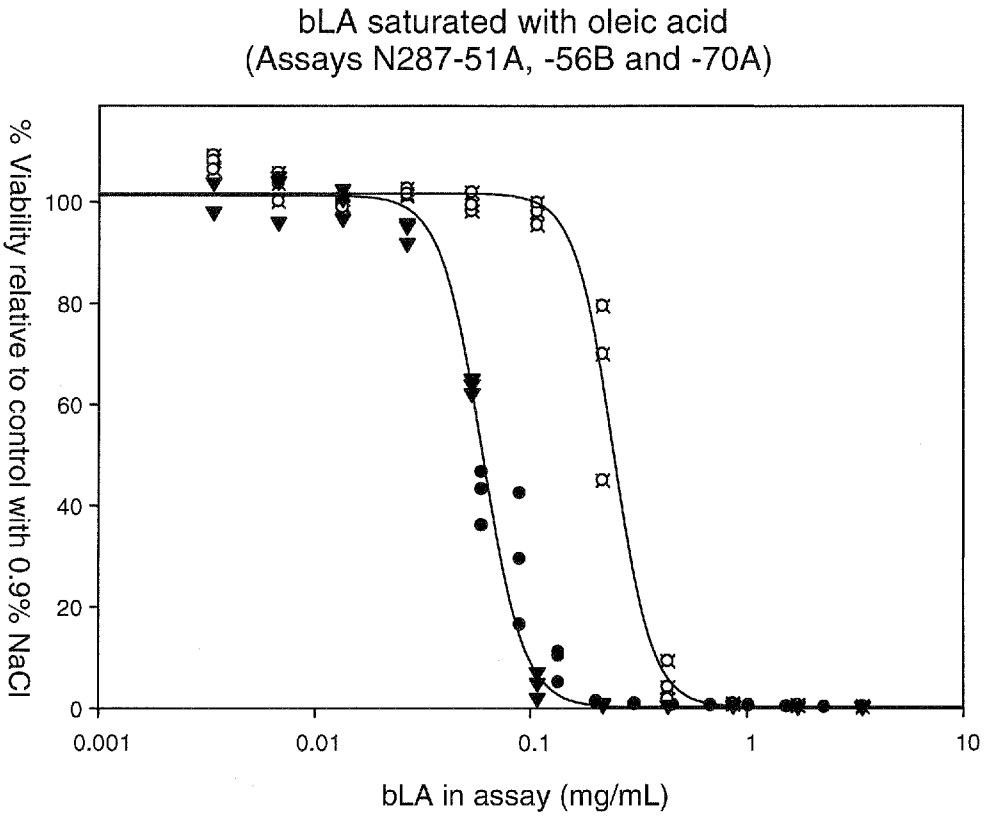
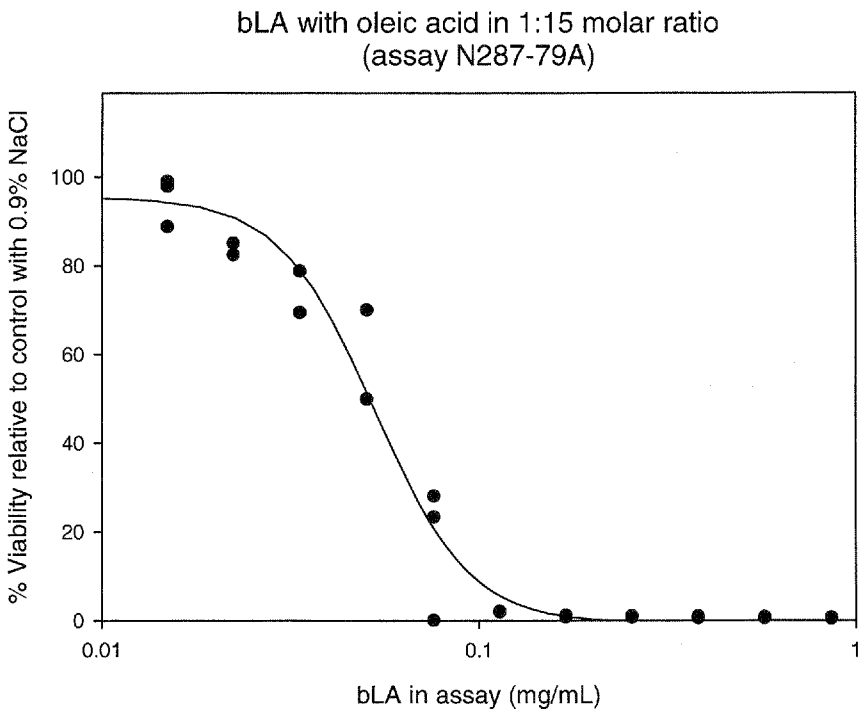

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
4 mg/mL HSA
4 mg/mL HSA saturated with oleic acid and centrifuged at 3,000xg, 10 min.
4 mg/mL HSA saturated with oleic acid and centrifuged at first 3,000xg then 18,000xg, 6 min.

Fig. 5



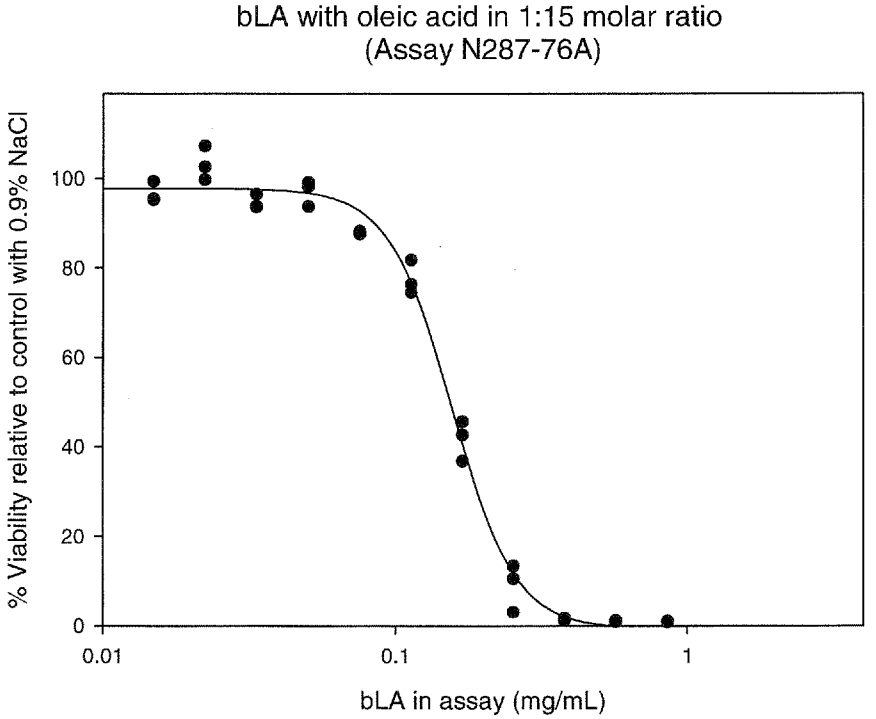
bLA + Oleic acid	LC_{50}
N287-50D spun at 150xg	~0.06 mg/mL
N287-55B spun at 3,000xg	~0.24 mg/mL
N287-69C spun at 18,000xg	~0.06 mg/mL

Fig. 6A



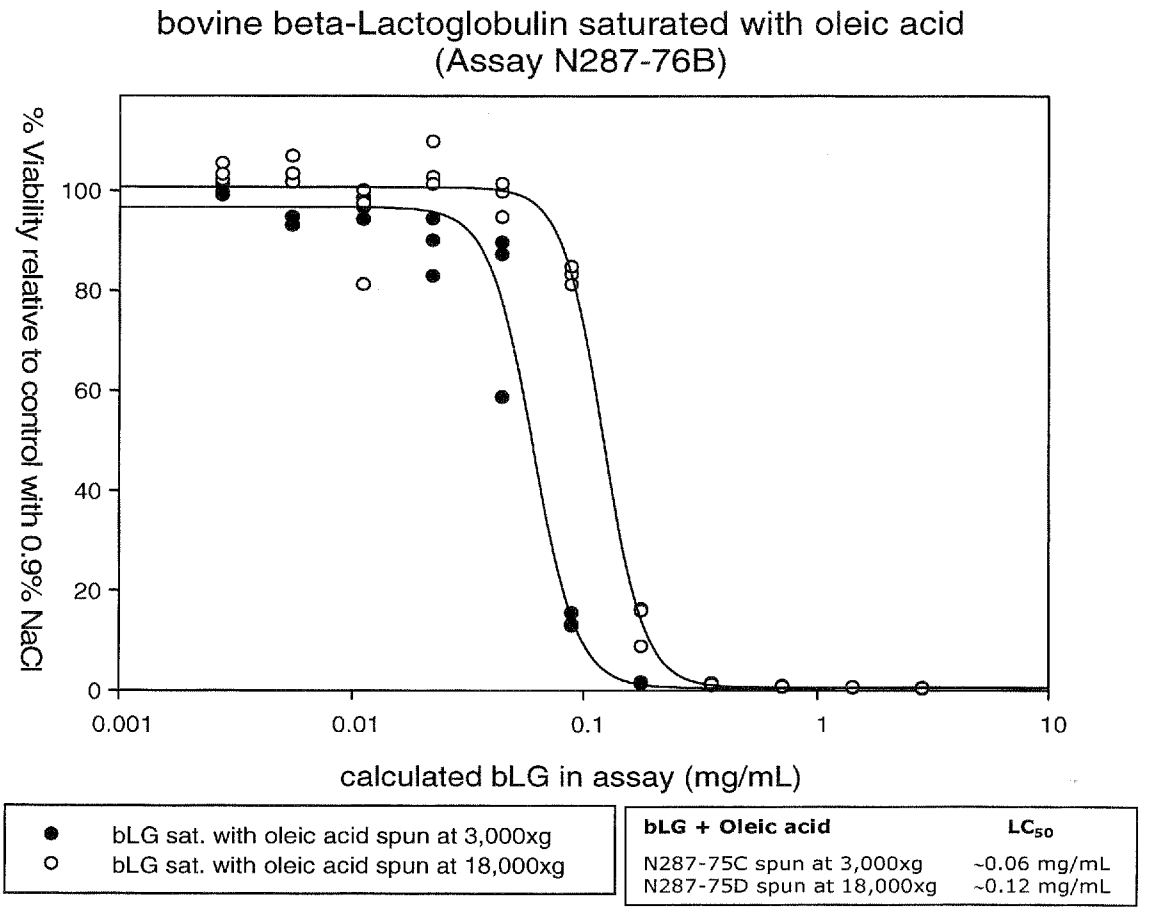
LC_{50}: ~0.16 mg/mL
Sigma oleic acid

Fig. 6B



LC_{50}: ~0.05 mg/mL
Merck oleic acid

Fig. 7



bLG + Oleic acid	LC_{50}
N287-75C spun at 3,000xg	~0.06 mg/mL
N287-75D spun at 18,000xg	~0.12 mg/mL

Fig. 8

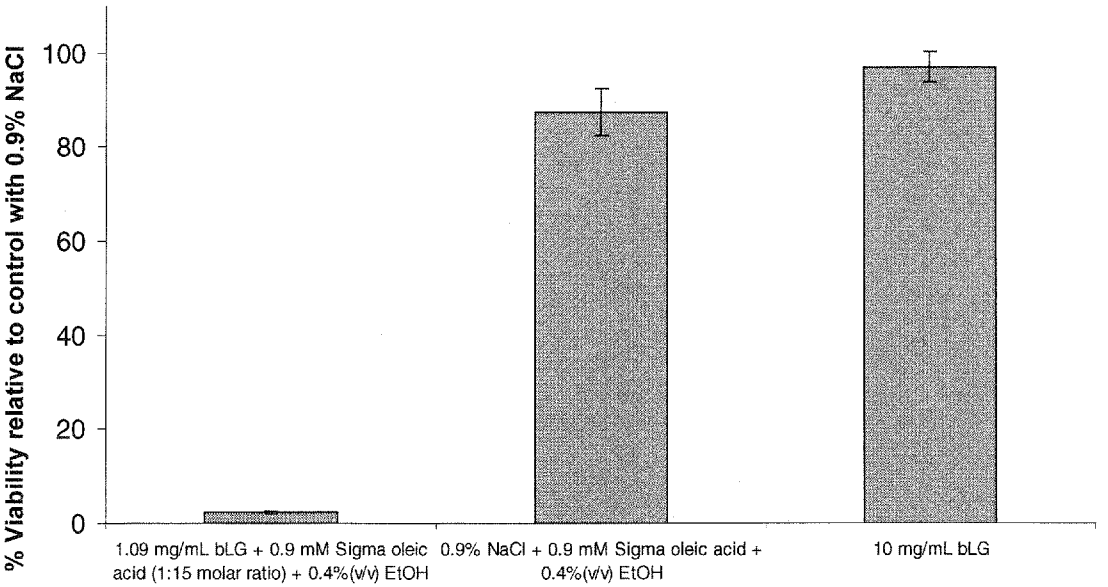
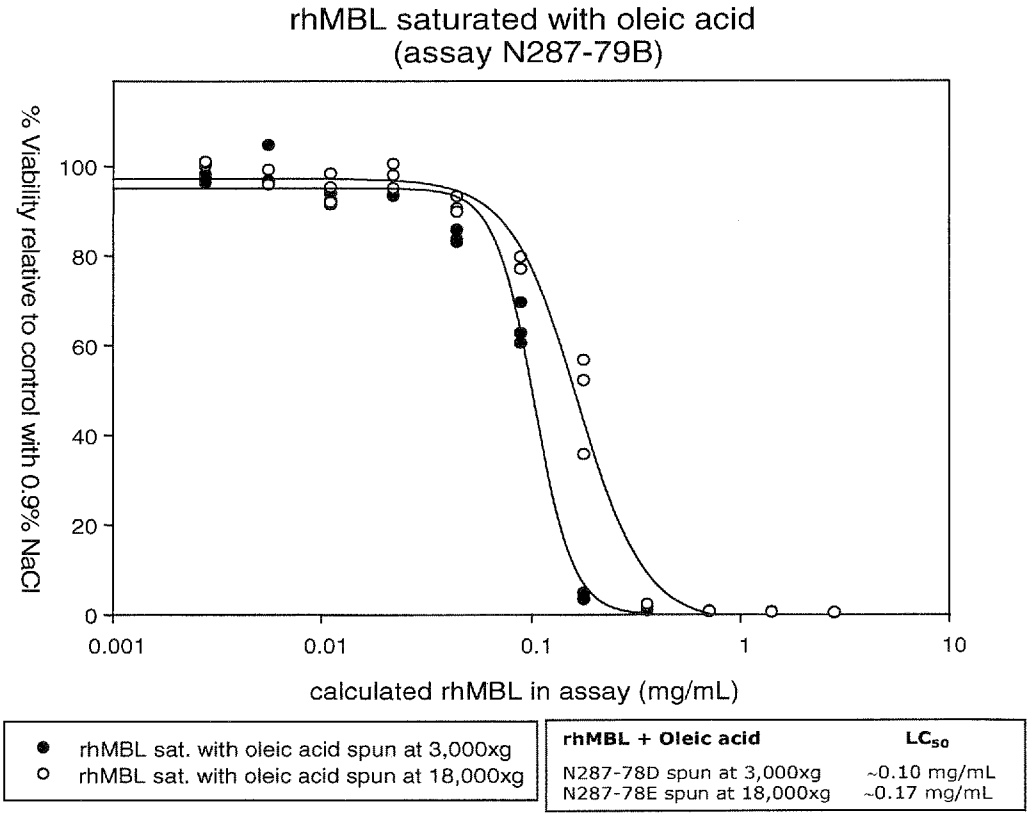
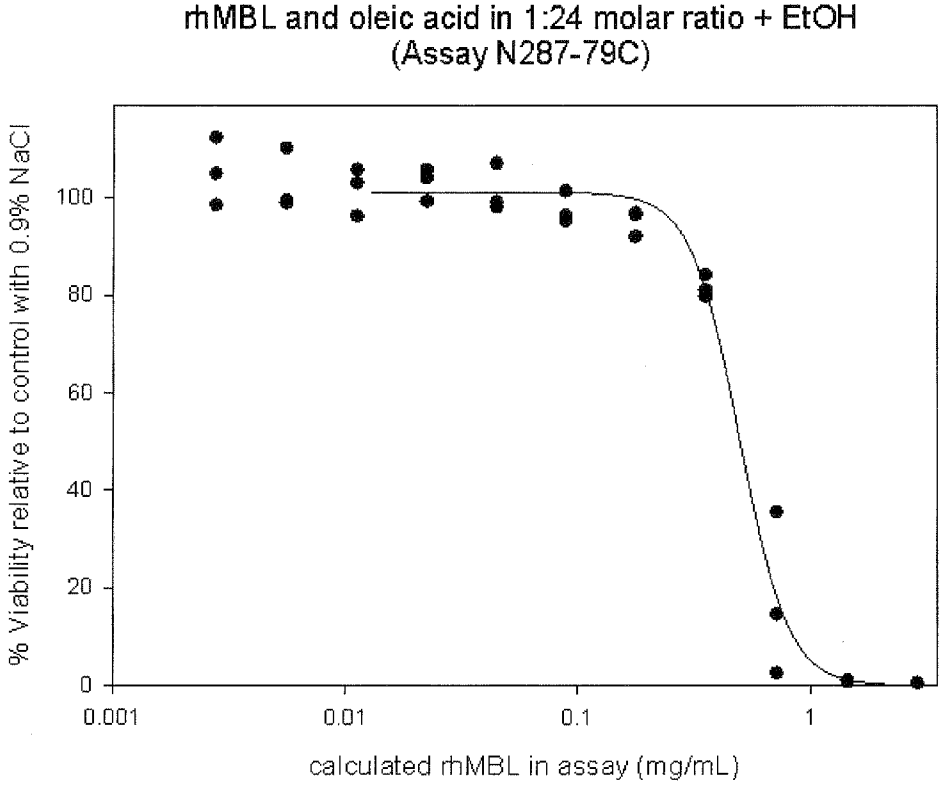

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
1.09 mg/mL bLG + 0.9 mM Sigma oleic acid (1:15 molar ratio) + 0.4%(v/v) EtOH
0.9% NaCl + 0.9 mM Sigma oleic acid + 0.4%(v/v) EtOH
10 mg/mL bLG

Fig. 9



rhMBL + Oleic acid	LC_{50}
N287-78D spun at 3,000xg	~0.10 mg/mL
N287-78E spun at 18,000xg	~0.17 mg/mL

Fig. 10



LC_{50}: ~0.50 mg/mL

Fig. 11

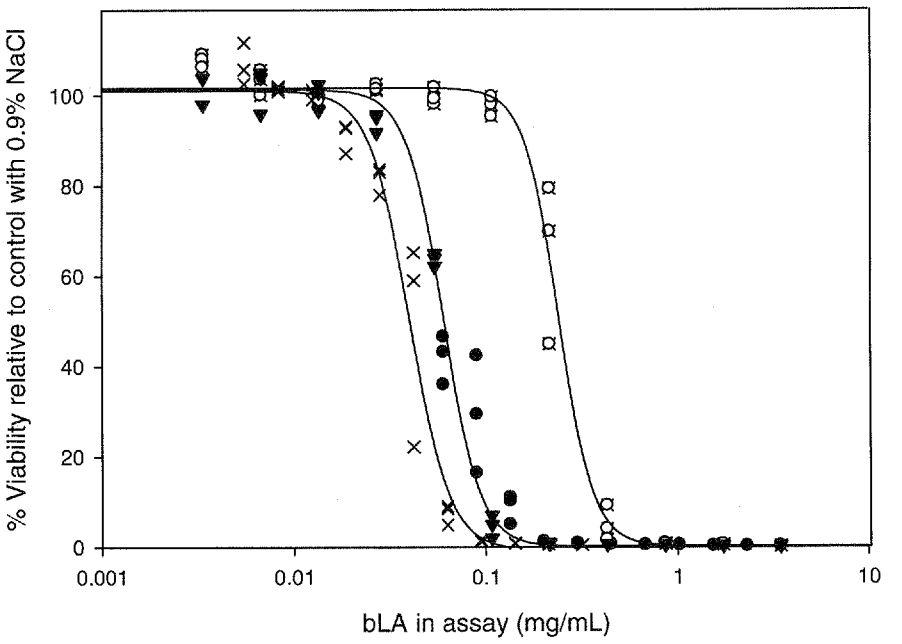

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
bLA in assay (mg/mL)

Fig. 12

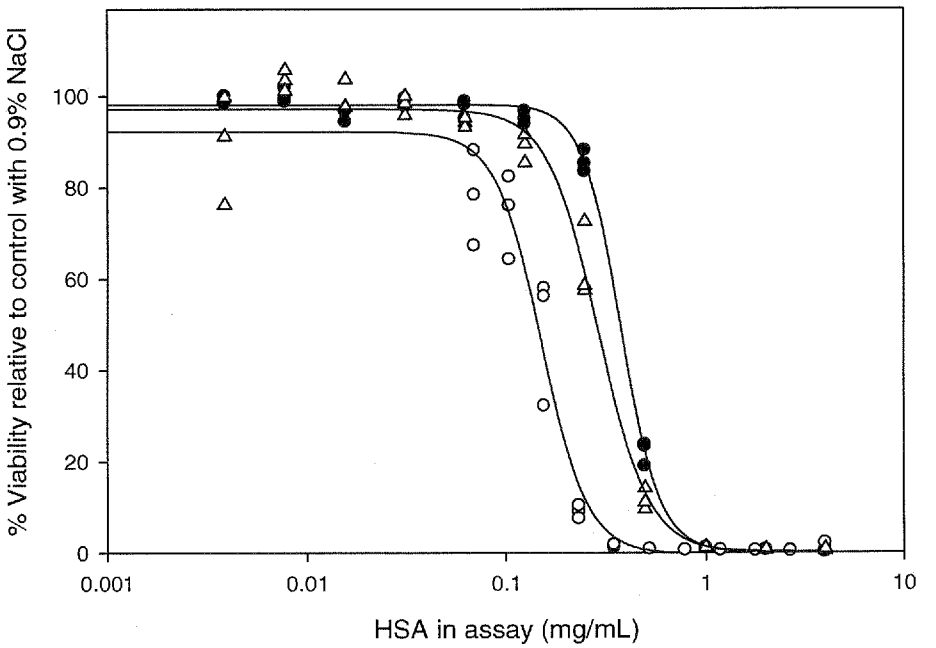

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
HSA in assay (mg/mL)

Fig. 13

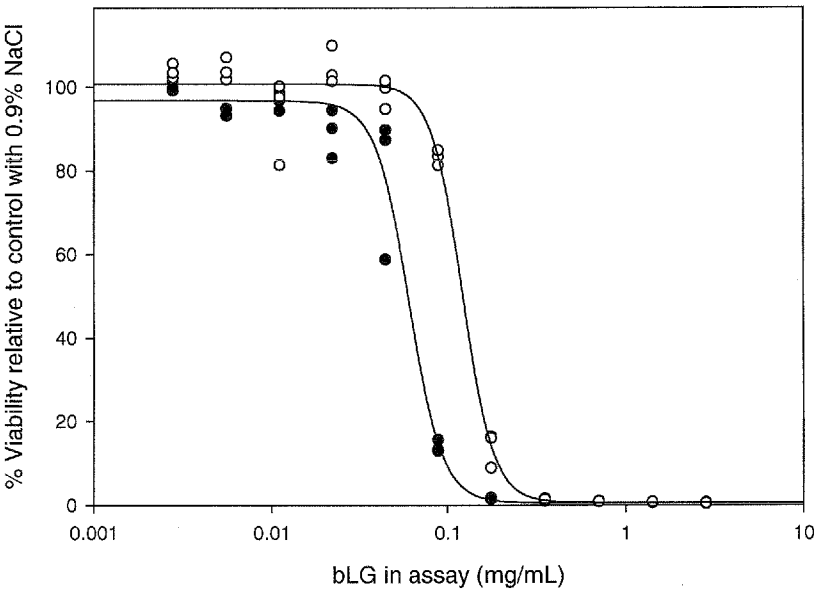

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
bLG in assay (mg/mL)

Fig. 14

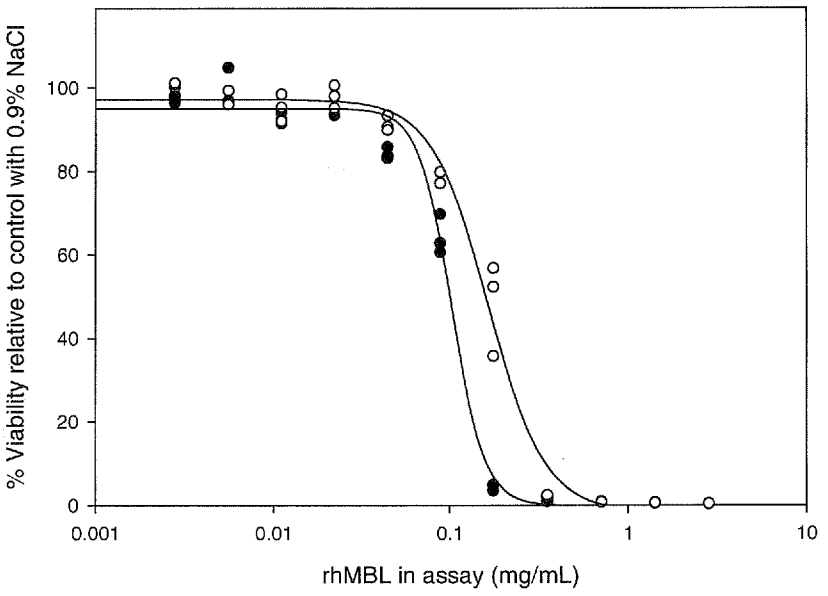

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
rhMBL in assay (mg/mL)

Fig. 15

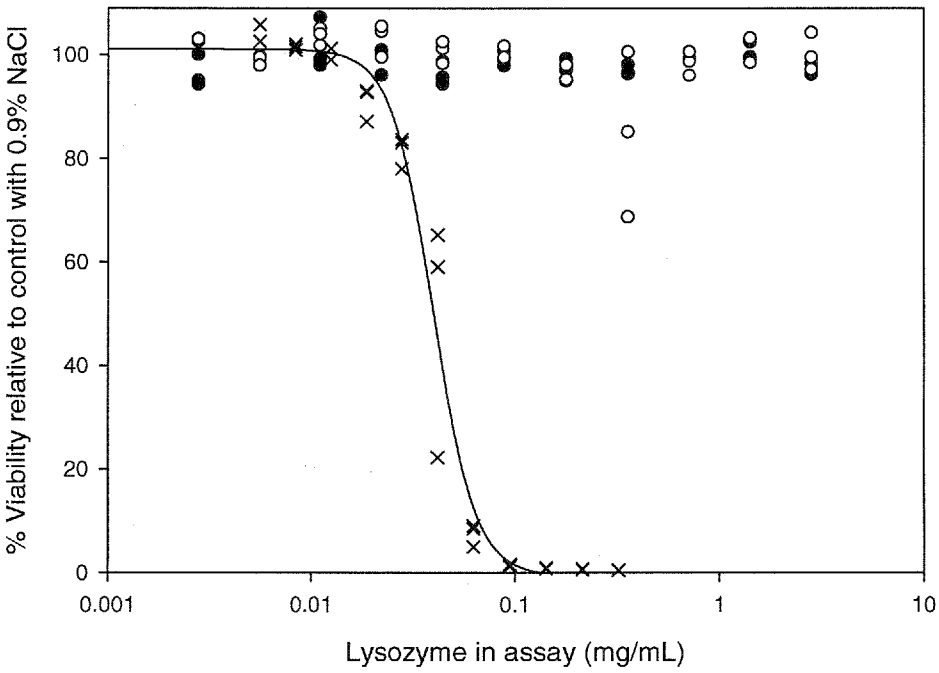

% Viability relative to control with 0.9% NaCl
100
80
60
40
20
0
0.001
0.01
0.1
1
10
Lysozyme in assay (mg/mL)

Fig. 16

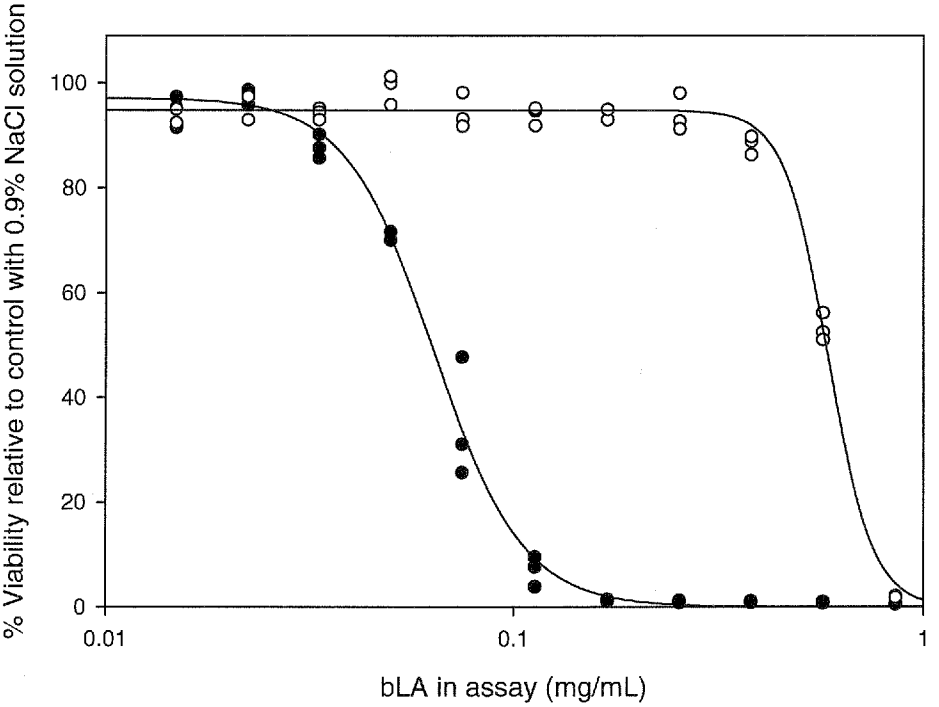

% Viability relative to control with 0.9% NaCl solution
100
80
60
40
20
0
0.01
0.1
1
bLA in assay (mg/mL)

Fig. 17

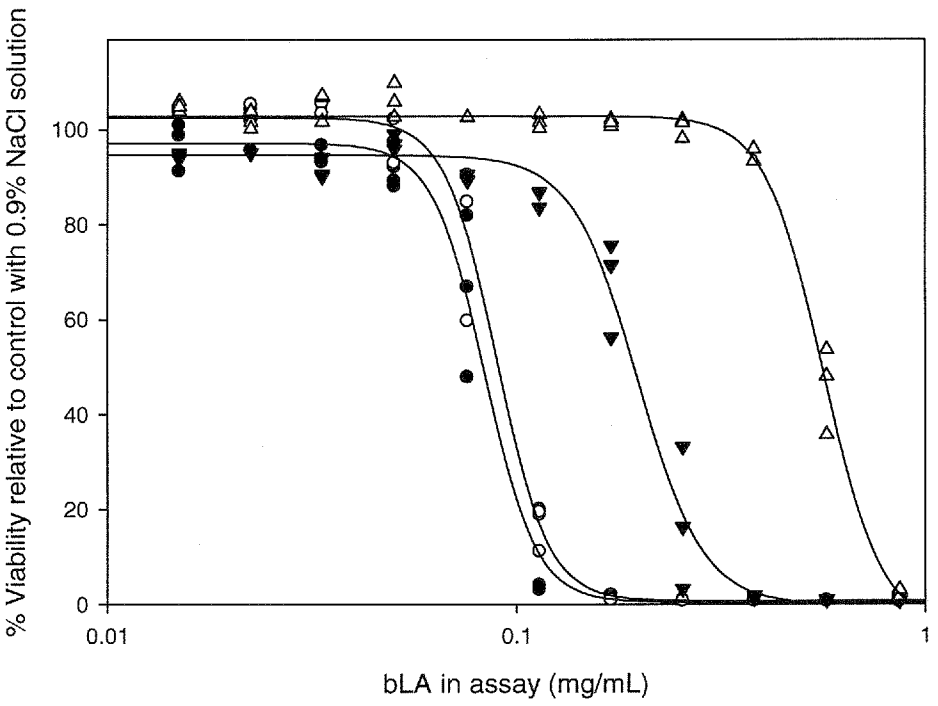

% Viability relative to control with 0.9% NaCl solution
100
80
60
40
20
0
0.01
0.1
1
bLA in assay (mg/mL)

Fig. 18

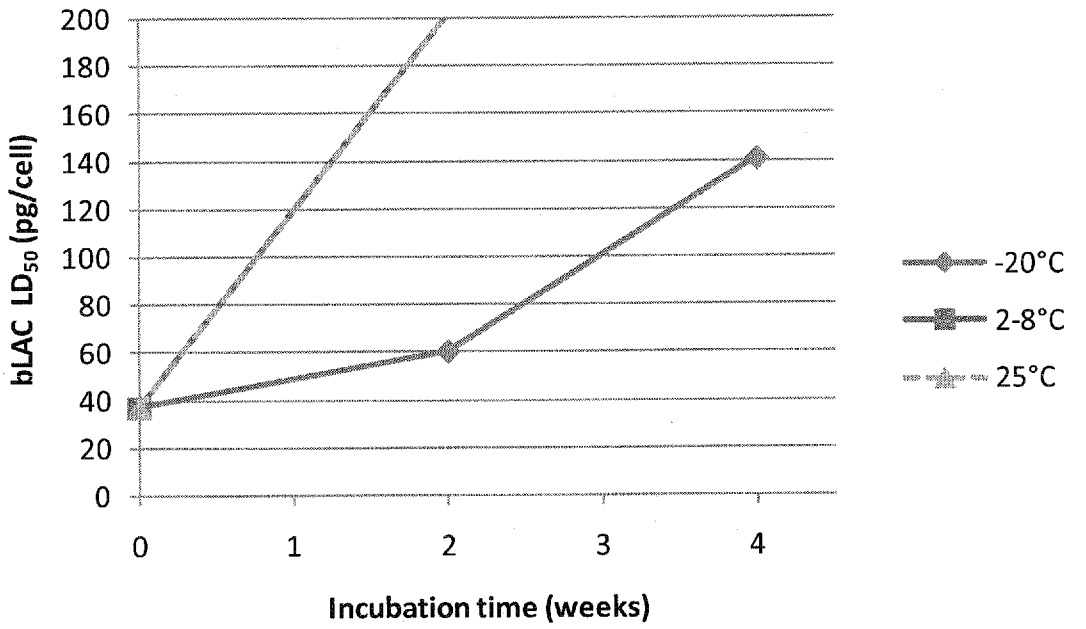

200
180
160
140
120
100
80
60
40
20
0
bLAC LD50 (pg/cell)
0
1
2
3
4
Incubation time (weeks)
-20°C
2-8°C
25°C

Fig. 19

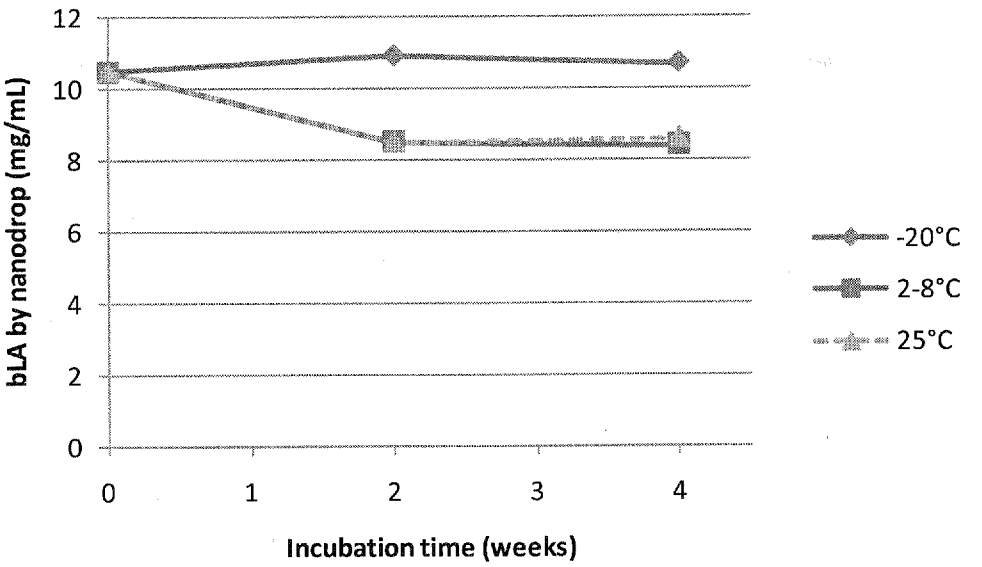

12
10
8
6
4
2
0
bLA by nanodrop (mg/mL)
0
1
2
3
4
Incubation time (weeks)
-20°C
2-8°C
25°C

Fig. 20

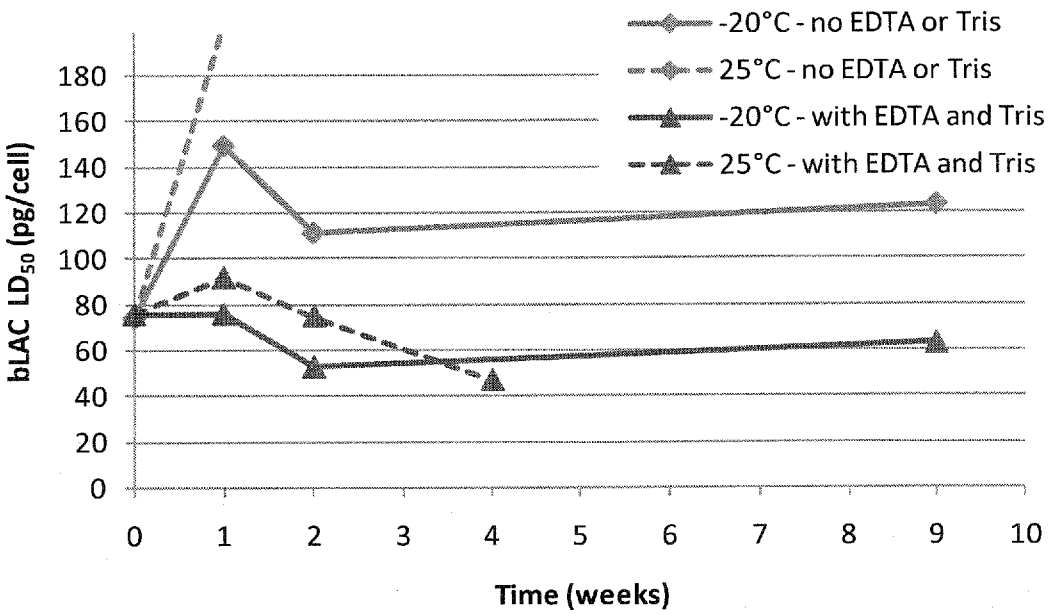

-20°C - no EDTA or Tris
25°C - no EDTA or Tris
-20°C - with EDTA and Tris
25°C - with EDTA and Tris
bLAC LD50 (pg/cell)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Time (weeks)

Fig. 21

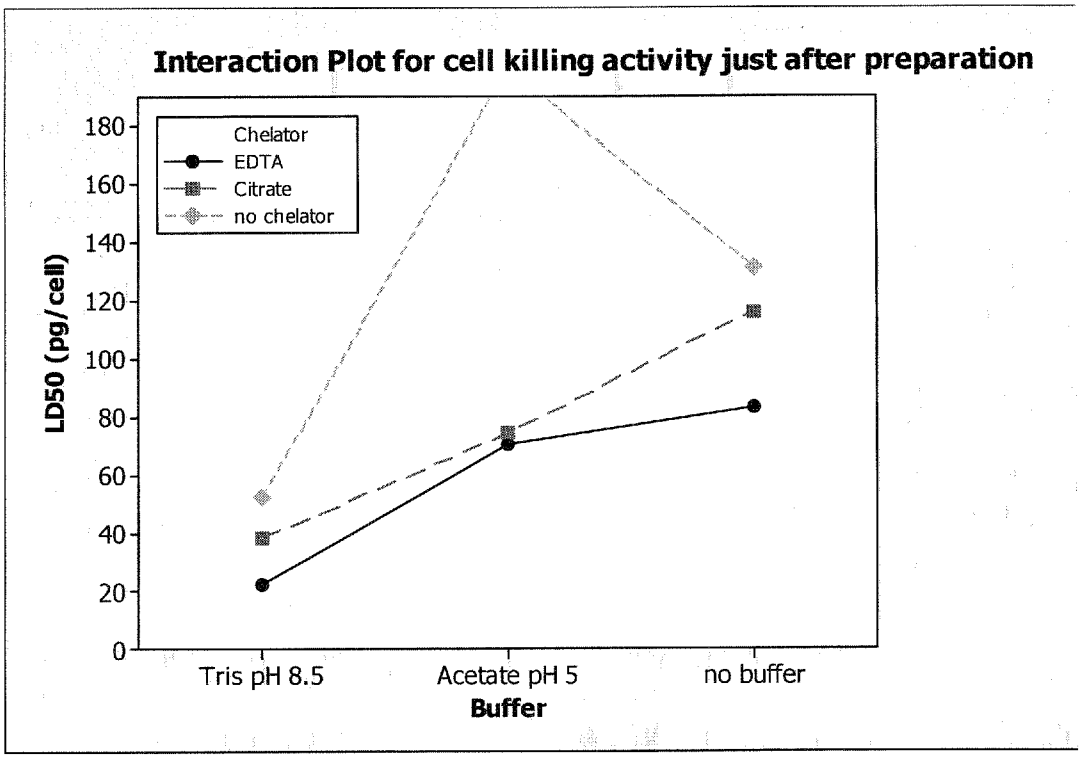

Interaction Plot for cell killing activity just after preparation
Chelator
EDTA
Citrate
no chelator
LD50 (pg/cell)
180
160
140
120
100
80
60
40
20
0
Tris pH 8.5
Acetate pH 5
no buffer
Buffer

Fig. 22

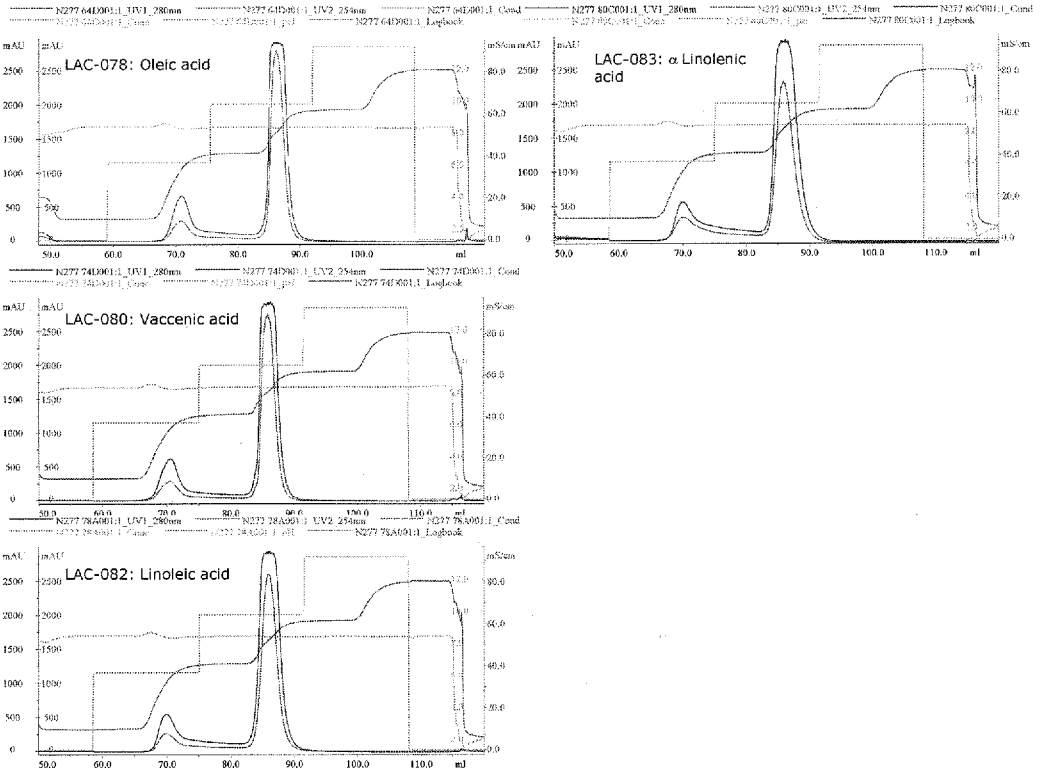
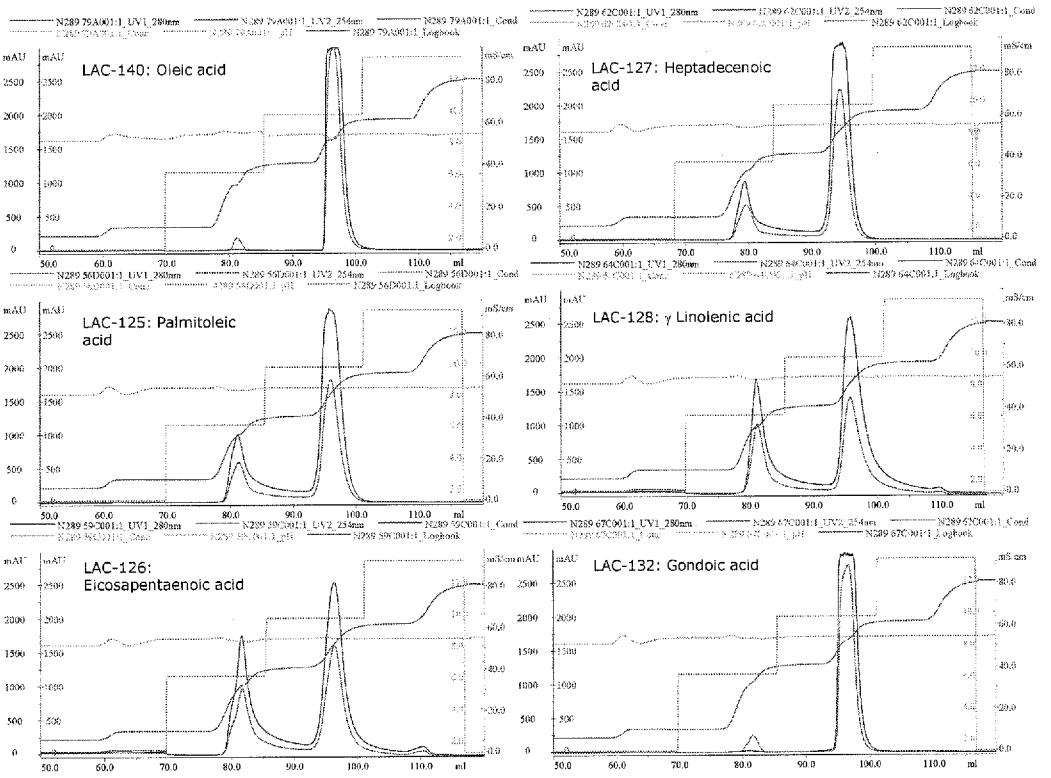

LAC-078: Oleic acid
LAC-083: α Linolenic acid
LAC-080: Vaccenic acid
LAC-082: Linoleic acid

Fig. 23


LAC-140: Oleic acid
LAC-127: Heptadecenoic acid
LAC-125: Palmitoleic acid
LAC-128: γ Linolenic acid
LAC-126: Eicosapentaenoic acid
LAC-132: Gondoic acid