(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 June 2008 (12.06.2008)

PCT

(10) International Publication Number
WO 2008/068762 A2

(51) International Patent Classification:
A61K 31/722 (2006.01)

(21) International Application Number:
PCT/IL2007/001512

(22) International Filing Date:
6 December 2007 (06.12.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/873,152 7 December 2006 (07.12.2006) US

(71) Applicant and
(72) Inventor: **BEN-SHALOM, Noah** [IL/IL]; Revadim 12, 69278 Tel-aviv (IL).

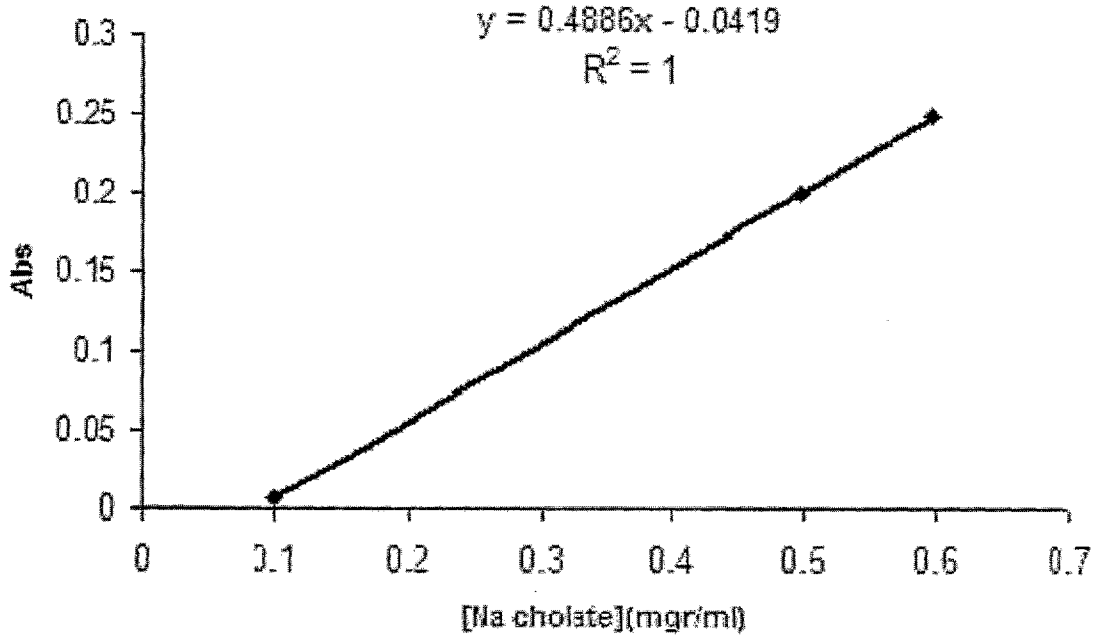
(74) Agent: **DR. D. GRAESER LTD.**; 13 HaSadna St, PO Box 2496, 43650 Raanana (IL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

(54) **Title:** CHITOSAN FORMULATION FOR CHOLESTEROL REDUCTION IN HUMANS



(57) **Abstract:** A formulation for reducing cholesterol levels in humans, comprising chitosan and an anionic or non-ionic surfactant, methods of preparation and uses thereof.

WO 2008/068762 A2

CHITOSAN FORMULATION FOR CHOLESTEROL REDUCTION IN HUMANS

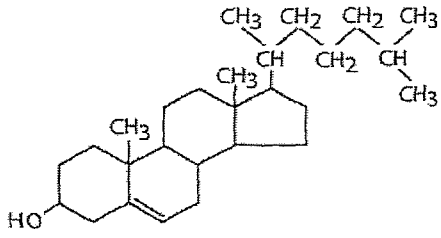
FIELD OF THE INVENTION

The present invention relates to the field of cholesterol-reducing formulations. More particularly, the present invention relates to a cholesterol-reducing formulation comprising hydrophobic microparticles of a positively charged polymer such as chitosan and an anionic or non-ionic surfactant, such as lecithin, methods of preparation and uses thereof.

BACKGROUND OF THE INVENTION

Cholesterol is a sterol lipid found in the bloodstream and in the cell membranes of all body tissues.

The structure of cholesterol is as follows:



Cholesterol is composed of three regions: a hydrocarbon tail; a ring structure region with 4 hydrocarbon rings; and a hydroxyl group. The hydroxyl group is polar, which makes it soluble in water. The ring region and tail region are non-polar, so are soluble in organic solvents, but insoluble in water.

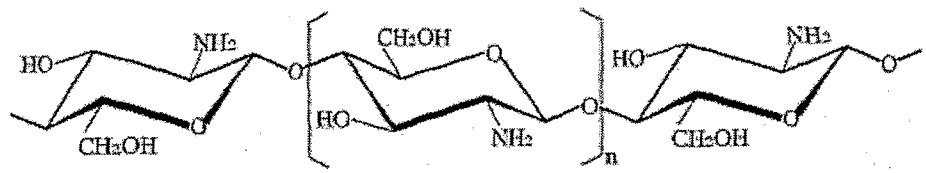
Most of the cholesterol found in humans is produced by the body, while some is of dietary origin. Genetic factors and dietary cholesterol intake both play a role in determining the blood cholesterol level of an individual.

Cholesterol is unable to dissolve in the blood, and must therefore be transported to and from cells by lipoprotein carriers. Low-density lipoprotein (LDL), is known as "bad" cholesterol. High-density lipoprotein (HDL), is known as "good" cholesterol. These two types of lipids, along with triglycerides and Lp(a) cholesterol, make up the total blood cholesterol count.

An elevated LDL blood cholesterol level (hypercholesterolemia) may lead to slow build up of cholesterol deposits in the walls of the arteries feeding the heart and

brain, forming a plaque which can clog these arteries, a condition known as artherosclerosis. A clot (thrombus) that forms near this plaque can block the blood flow to part of the heart muscle and cause a heart attack. If such a clot blocks the blood flow to part of the brain, a stroke results. A high level of LDL cholesterol (160 mg/dL and above) reflects an increased risk of heart disease.

Chitosan is the only naturally occurring, positively charged polysaccharide, and is generally produced by deacetylation of chitin, a naturally occurring biopolymer, found in the cytoskeleton and hard shells of marine organisms such as crustacea, shrimps, crabs, fungi, etc.



Chitosan

Chitosan is biocompatible, non-toxic, and non-immunogenic, allowing its use in the medical, pharmaceutical, and cosmetic fields.

The soluble form of chitosan contains positively charged amino groups that are able to form ionic bonds with anionic compounds, including proteins and fatty acids. Additionally, chitosan may form hydrophobic bonds.

In order to use chitosan in aqueous solution, dissolution of the crystalline structure must take place. In hydrated crystalline chitosan, water molecules form columns between chitosan sheets and contribute to stabilizing the structure by making water-bridges between polymer chains. The hydrogen bonds are broken during the dissolution process of the chitosan using weak organic acids like acetic acid.

The mechanism of dissolution of polyelectrolyte powders is believed to involve the formation of a spherical grain structure. In pure water, this includes the rapid formation of a gel layer around the particle, followed by the slow release of polymer chains into the solvent. The slow process of polymeric chains leaving an aggregate was explained as being due to the effect of an attractive potential forming between the charged individual polymer and the electroneutral aggregate.

It is known that equilibration times for the dissolution of polyelectrolytes are often in the order of hours and even many days (Michel, RC et al. *Biopolymers* 53:19-39, 2000; Reed WF et al. *Ber Bunzen Phys Chem* 100: 685-695, 1996). The duration time of stirring of the chitosan solution is reported to be 12-24 h at room temperature Fredheim GE et al *Biomacromolecules* 4:232-239, 2003.

It has been found that chitosan is able to absorb blood cholesterol in small animals (particularly mice and rats), as well as bile lipids, thereby lowering the blood levels of these molecules [*J. Nutr. 2000; 130: 2753-2759*] A number of studies have shown that chitosan has the unique ability to lower levels of "bad" LDL cholesterol, while boosting "good" HDL cholesterol levels.

Cholesterol is a precursor of bile acids, which are steroid acids found mainly in the bile of mammals, having both a hydrophilic and a hydrophobic face. It has been suggested that chitosan reduces blood cholesterol by absorption of bile acids, causing increased use of cholesterol in further synthesis of bile acids, thereby removing cholesterol from the blood.

A food fiber supplement comprising chitosan and glucomannan has been shown to lower blood cholesterol in rats [*J. Nutr. 2000; 130: 2753-2759*] and in humans [*J. Am. College Nutrition 2002; 21(5): 428-433].* However, large amounts of the fiber supplement were required in order to produce the cholesterol-lowering effect in humans, requiring the ingestion of fifteen capsules per day, providing 1.2 g/day each of chitosan and glucomannan. The total serum cholesterol in the human study was lowered by only about 7%, and the LDL cholesterol by 10%.

U.S Patent Nos. 7,067,146 and 6,814,975; U.S. Patent Application No. 20050079204; and European Patent No. 1233682 to Eritocap teach use of chitosan together with eritadenine in the preparation of a foodstuff for reduction of cholesterol.

U.S. Patent No. 6,323,189 and European Patent No. 1100344 teach a stable chitosan-containing liquid suspension for weight treatment.

U.S. Patent Application No. 20050175763 teaches a phospholipd-containing stable matrix consisting of a supporting material in the form of a carbohydrate, such as chitosan.U.S. Patent Application No. 20050100619 teaches a cholesterol-lowering supplement which may include chitosan and a phospholipid, together with a further composition capable of inhibiting cholesterol biosynthesis and a composition capable of increasing cholesterol metabolism.

Chitosan is unable to reduce cholesterol in the stomach, since it cannot absorb non-emulsified fats in the absence of bile salts, which are secreted only in the small intestine. Furthermore, chitosan dissolves and becomes positively charged in the acid conditions of the stomach, due to its functional amino groups. The highly charged polymer can react strongly with negatively-charged materials, such as phospholipids, which are present in the stomach, and become partially saturated. As a result, the amount of ingested chitosan having positively-charged groups available for interaction with the negatively charged bile acids after passing through the stomach is decreased.

There is thus a widely recognized need for, and it would be highly advantageous to have an improved formulation comprising chitosan which provides delivery of an increased proportion of unsaturated chitosan to the small intestine, for reducing blood cholesterol in humans.

SUMMARY OF THE INVENTION

The present invention provides formulations for reduction of cholesterol in humans, the formulation comprising chitosan and an anionic or non-ionic surfactant, methods of preparation and uses thereof.

According to one aspect of the present invention there is provided a formulation for the reduction of cholesterol in humans, the formulation comprising composite, hydrophobic particles of chitosan and an anionic or non-ionic surfactant.

According to another aspect of the present invention there is provided a method of producing a formulation for the reduction of cholesterol in humans, the method comprising preparing a first solution comprising chitosan by mixing with an acid for at least 24 hours; preparing a second solution comprising an anionic or non-ionic surfactant; mixing the first and second solutions in acid conditions to form composite particles; and preparing a powder of the composite particles.

Optionally and preferably, the first solution is prepared in an acid selected from the group consisting of hydrochloric acid or an organic acid. Further optionally, the organic acid is selected from the group consisting of lactic acid and glutamic acid.

Optionally and preferably, the second solution is prepared in water.

The chitosan concentration in the first solution may comprise, for example, about 0.7%. The pH of the solution is preferably adjusted to a value of about 3.5.

The method may optionally further comprise the step of adjusting the pH of the suspension medium, after removal of said particles, to a value of about 7.

The suspension medium may optionally be allowed to stand for about 30 minutes, then filtered to remove the particles. Further optionally, the removed particles may be resuspended in acid medium.

The powder may be prepared, for example, by spray drying or lyophlization.

Optionally, mixing of the first and second solutions is carried out at a temperature of 25° for a time of no greater than 15 minutes.

According to another aspect of the present invention there is provided a method for increasing cholesterol reduction in humans by chitosan, the method comprising increasing the hydophobicity of said chitosan. Optionally, the hydrophobicity is increased by reacting chitosan with an anionic or non-ionic surfactant.

According to yet another aspect of the present invention, there is provided a method for the reduction of cholesterol in a human subject, the method comprising administering to the subject a formulation comprising composite, hydrophobic particles of chitosan and an anionic or non-ionic surfactant.

According to further features in preferred embodiments of the present invention, the composite, hydrophobic particles may comprise microparticles.

According to yet another aspect of the present invention, there is provided a formulation for the reduction of cholesterol in humans, the formulation comprising composite, hydrophobic microparticles comprising a cationic polymer and an anionic or non-ionic surfactant. Optionally and preferably, the cationic polymer comprises chitosan.

According to further features in preferred embodiments of the invention described below the anionic surfactant is preferably selected from the group consisting of phospholipids; bile salts; sodium lauryl ether sulfate; citric acid esters of monoglycerides; sodium, calcium or acid stearoyl lactylate; stearyl citrate; fatty acids or salts thereof; diacetyl tartaric acid esters of monoglycerides; or combinations thereof. More preferably, the phospholipids comprises lecithin.

According to still further features in the described preferred embodiments, the non-ionic surfactant is a fatty alcohol.

According to further features in the preferred embodiments, the chitosan has a degree of acetylation of from about 70% to about 95%.

According to further features in the preferred embodiments, the chitosan comprises high molecular weight chitosan having a molecular weight in the range of from about $5x10^5$ to about $3x10^6$ daltons,.

A ratio of surfactant:chitosan is optionally and preferably in the range of from about 0.2:1 to about 5:1. More preferably, the ratio is in the range of from about 3:1 to about 4:1.

A concentration of chitosan is preferably about 0.7% w/w. More preferably, the concentration is in the range of from about 0.7% w/w to about 3.5% w/w.

According to further features in the preferred embodiments, the chitosan and the surfactant in the composite particles are connected by ionic and hydrophobic interactions,

The formulation may optionally further comprise a cholesterol-reducing agent selected from the group consisting of a statin, a fibrate, niacin, a bile acid sequestrant, ezetimibe, or a phytosterol, or combinations thereof.

The formulation may optionally further comprise an enteric coating layer.

The formulation or method of the present invention may be used in the treatment or prevention of a condition selected from the group consisting of hypercholesterolemia, artherosclerosis, myocardial infarction, and cerebrovascular accident.

The formulation or method of the present invention may be used for reduction of body fat, such as, for example, in the treatment of obesity.

According to further features in the preferred embodiment, upon introducing the composite particles at a concentration of about 0.25% for about 30 minutes into an aqueous solution comprising a bile acid or its analog at a concentration of about 0.5%, at about pH 6.8 and about 25 degrees Celsius, th particles are capable of hydrophobically binding and removing bile acids or their analogs from solution by at least about 47% at a surfactant:chitosan ratio of from about 2:1 to about 3:1, and by at least about 80% at a surfactant:chitosan ratio of from about 4:1 to about 5:1.Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those

described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the figures:

FIG. 1 presents a flowchart of a method of reducing cholesterol in a human subject, in accordance with the principles of the present invention;

FIG. 2 shows a photographic recording of lecithin-chitosan particles at different lecithin:chitosan ratios after 3 hours in phosphate buffer with SK10 chitosan;

FIGS. 3a-3b show photographic recordings of lecithin-chitosan particles at different lecithin:chitosan ratios after 5 minutes, 30 minutes, and 3 hours, respectively, in phosphate buffer, with SK100 chitosan;

FIG. 4 shows the relationship between particle size and lecithin concentration in the lecithin-chitosan particles of the present invention; and

FIG. 5 shows a graph of the relationship between sodium cholate concentration in solution and absorbance by colorimeteric analysis.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a formulation for reduction of cholesterol in humans, the formulation comprising composite, hydrophobic, microparticles of a

positively charged polymer such as chitosan, and an anionic or non-ionic surfactant, such as lecithin.

The chitosan and the surfactant in the composite particles are chemically bound to form composite particles, due to ionic interactions between the cationic polymer, and the anionic surfactant, as well as hydrophobic interactions between the hydrodphobic regions of the polymer and the lipid fraction of the lecithin during drying of the particles. The interaction between the cationic polymer and non-ionic surfactants involves non-ionic interactions.

Chitosan is less effective in reducing cholesterol in humans than in rats. It was considered by the present inventor that absorption of bile acids by chitosan in small animals occurs as a result of non-specific ionic interactions between the positively-charged chitosan polymer (which competes with many other positively-charged blood proteins) and the negatively-charged bile acids, while binding of chitosan to the hydrophobic face of bile acids would be much more specific. The difference in intestinal pH between humans and rats may be significant in this respect. In humans, the pH is around 7.0-7.3, while in rats the pH is around 6.2-6.5. This range is significant because chitosan is insoluble at pH values above 6.5, such that a semi-gel precipitate is formed. Insoluble chitosan has very little effect on binding bile acids.

It was hypothesized by the present inventor that non-ionic surfactants may absorb chitosan molecules via interaction with the hydrophobic tail region, resulting in the formation of an uncharged chitosan molecule that is water-insoluble at the pH of the intestine. It was thus considered that increasing the hydrophobicity of chitosan would increase its binding to bile acids in humans.

It is therefore an object of the present invention to provide a chitosan formulation having increased hydrophobicity.

The formulation therefore comprises particles having a strong hydrophobic region, which are soluble in the acid conditions of the stomach, entering the small intestine in solution, wherein the microparticles bind bile salts and then precipitate.

It is a further object of the present invention to provide a chitosan formulation which is soluble in the stomach and delivered to the human small intestine in an effective form for binding bile acids. Furthermore, the formulation should be stable and unreactive in the environment of the human stomach, such that it is delivered to the intestine in soluble, unsaturated form.

It is a further object of the present invention to provide a method for the reduction of cholesterol in a human subject, the method comprising administering to the subject a formulation comprising composite particles of chitosan and an anionic or non-ionic surfactant.

It has been shown by Magdassi et al. of the Hebrew University, Jerusalem, Israel (see, for example, *G. Nizri and S. Magdassi: Solubilization of hydrophobic molecules in nanoparticles formed by polymer–surfactant interactions, J. Colloid Interface Sci. 291,169-174 (2005)*) that when a polycationic polymer is mixed with an anionic surfactant, electrostatic interactions lead to precipitation of nanoparticles. Such particles are capable of solubilizing hydrophobic molecules due to the formation of hydrophobic pockets in the polycationic polymer. Magdassi did not teach or suggest the use of hydrophobic microparticles comprising chitosan for reducing cholesterol in humans. Furthermore, Magdassi only studied nanoparticles and not microparticles.

Magdassi teaches, in U.S. Patent No. 5,753,264, preparation of a positively charged chitosan-containing aqueous emulsion of an oil, wherein an aqueous chitosan solution is added to an oil-in-water emulsion comprising an anionic emulsifier, to produce an insoluble surfactant-chitosan complex. The emulsifier may comprise lecithin. The purpose of the patent to Magdassi is stabilization of the oil. No particles of chitosan and lecithin are formed, but rather a stable emulsion of lecithin is first formed, to which chitosan is then added. The ratio of chitosan to lecithin used by Magdassi is 0.5:033. The process was carried out at pH 6. If the pH of the emulsion described by Magdassi were to be increased to above 7, at the chitosan:lecithin ratio used, precipitation of chitosan would immediately result, before interaction with bile acids could occur.

It has surprisingly been found by the present inventor that a formulation comprising a positively charged polymer such as chitosan and an anionic or non-ionic surfactant, such as lecithin, provides composite hydrophobic microparticles, wherein chitosan is prevented from undergoing ionic interactions with positively charged molecules in the stomach, such that the chitosan is delivered to the intestine in state which is readily available for binding bile acids.

The formulation of the present invention preferably comprises particles that are able to interact with bile salts to form a complex with passes through the small intestine in the form of a stable, insoluble suspension, in order to prevent digestion

and disintegration by free bile salts which are able to break down the particles and interact with the lecithin.

A formulation comprising such composite microparticles would therefore be highly useful in binding bile acids and thereby reducing LDL cholesterol levels in humans.

The formulation of the present invention is soluble in the acidic conditions of the stomach and forms suspended particles in the higher pH environment of the human small intestine. Such particles are able to interact with bile salts through hydrophobic interactions with the hydrophobic part of the bile salts, resulting in precipitation to form an insoluble agglomerate, within which the bile salts are trapped. The particles of the present invention must therefore be sufficiently hydrophobic to provide strong hydrophobic interactions with the hydrophobic region of bile salts.

The formulation is designed such that upon entry into the human intestine, the particles remain for a short time as a stable particle suspension in intestinal medium, while interaction with bile salts takes place. The particles then precipitate as an agglomerate with the bile salts. Bile salts are thus trapped inside the polymer through hydrophobic interactions, and prevented from undergoing hydrolysis during passage through the intestine.

The time for which the particles remain in suspension must be sufficient to enable interaction with bile salts to occur. Particles which are insufficiently unstable in solution will undergo rapid flocculation in intestinal fluid, such that binding and entrapment of bile salts is not able to occur.

The particles of the present invention must therefore be capable of providing a suspension in intestinal medium, be sufficiently hydrophobic to react strongly with the hydrophobic region of bile salts, and form a precipitate with bile salts. It was found that absorption of bile salts by the lecithin-chitosan particles of the present invention is affected by hydrophobic degree and particle size. The hydrophobic degree is primarily controlled by the lecithin concentration in the particles.

The formulation thus provides for effective absorption of bile acids by chitosan under the high pH conditions of the human small intestine. The formulation also limits emulsification of dietary fats, which are thus inaccessible to lipase, and are therefore excreted in greater amounts in the feces. Microparticles are particularly suitable for the formulation of the present invention. Microparticles are solid particles

having a size range of from about 1 μm to about 1 mm, as opposed to nanoparticles, which range in size from about 10 nm to about 1 μm. Nanoparticles are stable in suspension, whereas microparticles are only partially stable. The greater stability of nanoparticles in suspension would not permit such particles to precipitate in the intestine.

Preferably, the particles of the present invention are in the range of from about 0.3 to about 1.5 μm.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Figure 1 presents a flow-chart of a method of reducing cholesterol in a human subject, in accordance with the principles of the present invention. As seen in the Figure, the method comprises first dissolving chitosan in acid (step **10**) and dissolving lecithin in water (step **12**). As discussed in detail in the Background section above, chitosan must be mixed for at least 24 hours in acid in order for solubilization to occur. Chitosan which has been dissolved under such conditions and dried may be easily re-dissolved in the acid conditions of the stomach.

The two solutions are then mixed at a lecithin:chitosan ratio of from about 1:1 to about 5:1 at pH 3.5 (step **14**), such that the lecithin and chitosan chemically react to form composite particles of chitosan and lecithin.

A powder formulation is then prepared by drying (step **16**). The formulation is administered to the subject (step **18**). The particles of the formulation dissolve in the stomach of the subject (step **20**) and enter the intestine of the subject in soluble form, forming a temporarily stable suspension (step **22**). The suspended particles form a complex with bile acids through hydrophobic interactions (step **24**), which precipitates and leaves the body via the feces (step **26**). This removal of bile acids results in a reduction of bile salts in the intestine (step **28**), causing increased hydrolysis of LDL in the blood in order to form new bile salts (step **30**), resulting in decreased blood LDL levels (step **32**).

The present invention further provides a method of producing a formulation for the reduction of cholesterol in humans. The method comprises preparing a first solution of chitosan in acid, by mixing for at least 24 hours, preparing a second solution of an anionic or non-ionic surfactant, mixing the solution of chitosan with the solution of anionic or non-ionic surfactant to form composite, soluble hydrophobic particles of chitosan and surfactant and drying the particles to form a powder.

Preferably, the chitosan solution is prepared in hydrochloric acid and the surfactant solution is prepared in water. More preferably, the pH of each of the solutions is adjusted to a value of about 3.5 prior to mixing the two solutions together.

It was further noted by the present inventors that the size of particles produced by mixing of chitosan and lecithin increases with mixing time and with reaction temperature. Particles which are too large are less stable at pH 7, so will therefore precipitate at intestinal pH, before binding of bile salts can occur. Mixing time is therefore preferably limited to no greater than 15 minutes, at a temperature of 25°C, or to 5 minutes at 45°C. Hydrophobic microparticles produced by this method are preferably then converted into powder form, for example by spray drying or lyophilization. The powder may optionally and preferably be compressed into a tablet, using any tabletting device known in the art, such as, for example, using a single punch tabletting machine (WICK)..

The method generally produces a powder comprising soluble solids at a concentration of from about 4% to about 8% w/w.

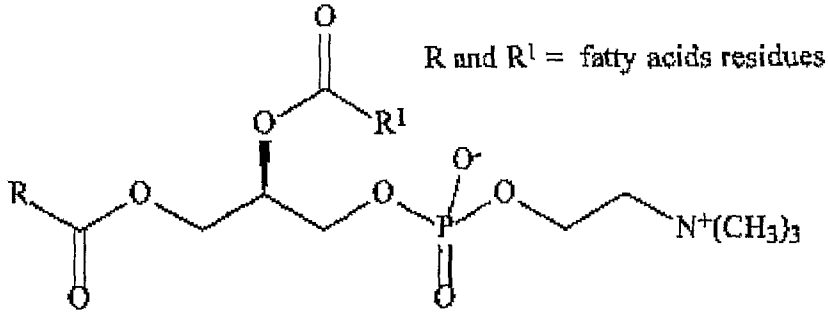
The pH of the solution of particles obtained under acid conditions may be increased to a pH value of about 7.0, and precipitation allowed to occur for about 30 minutes, after which the supernatant is filtered to obtain a pellet. The pellet may then be resuspended in a minimal amount of acid medium, and added to the particles obtained in the first precipitation step, prior to spray drying.

The hydrophobic microparticles of any of the embodiments of the present invention preferably have diameters in the range of from about 0.3 μm to about 1.5 μm.

According to any of the embodiments of the present invention, any anionic or non-ionic surfactant may be used. Examples of suitable anionic surfactants include phospholipids; bile salts; sodium lauryl ether sulfate; citric acid esters of monoglycerides; sodium, calcium or acid stearoyl lactylate; stearyl citrate; fatty acids

or salts thereof; diacetyl tartaric acid esters of monoglycerides; or combinations thereof. Examples of non-ionic surfactants include cetyl alcohol or oleyl alcohol.

Preferably, the anionic surfactant comprises the phospholipids, lecithin (phosphatidylcholine), also known as 1, 2-diacyl-sn-glycero-3-phosphocholine, or PtdCho, which is represented by the following chemical structure:



The term lecithin itself has different meanings when used in chemistry and biochemistry than when used commercially. Chemically, lecithin is phosphatidylcholine. Commercially, it refers to a natural mixture of neutral and polar lipids. Phosphatidylcholine, which is a polar lipid, is present in commercial lecithin in concentrations of 20 to 90%. Most of the commercial lecithin products contain about 20% phosphatidylcholine.

Lecithins containing phosphatidylcholine are produced from vegetable, animal and microbial sources, but mainly from vegetable sources. Soybean, sunflower and rapeseed are the major plant sources of commercial lecithin. Soybean is the most common source. Plant lecithins are considered to be GRAS (generally regarded as safe). Egg yolk lecithin is not a major source of lecithin in nutritional supplements. Eggs themselves naturally contain from 68 to 72% phosphatidylcholine, while soya contains from 20 to 22% phosphatidylcholine.

The fatty acid makeups of phosphatidylcholine from plant and animal sources differ. Saturated fatty acids, such as palmitic and stearic, make up 19 to 24% of soya lecithin; the monounsaturated oleic acid contributes 9 to 11%; linoleic acid provides 56 to 60%; and alpha-linolenic acid makes up 6 to 9%. In egg yolk lecithin, the saturated fatty acids, palmitic and stearic, make up 41 to 46% of egg lecithin, oleic acid 35 to 38%, linoleic acid 15 to 18% and alpha-linolenic 0 to 1%. Soya lecithin is

clearly richer in polyunsaturated fatty acids than egg lecithin. Unsaturated fatty acids are mainly bound to the second or middle carbon of glycerol.

Lecithin has a number of advantages over other surfactants. For example, lecithin is an intrinsic part of the bile salt complex, it is cheap, and it is well recognized by bile acids.

As an alternative to chitosan in any of the embodiments of the present invention, any suitable positively charged polymer may be used. Examples of suitable positively charged polymers include polyamines such as polylysine and polyamidoanine. According to any of the embodiments of the present invention, high molecular weight chitosan is preferably used, since low molecular weight chitosan has low hydrophobicity, and is therefore less effective in the formulation of the present invention. The preferred range for high molecular weight chitosan is about $5x10^5$-$3x10^6$ daltons.

As shown in the Examples section and Figures 2 and 3a-3c below, precipitation of microparticles increased as the lecithin:chitosan ratio increased, due to increasing hydrophobicity of the particles. A ratio of from about 2:1 to about 3:1 was found to provide partially stable particles, which underwent gradual flocculation.

However, at such ratios, less bile salt was found to be bound, since particles formed at lower ratios were not sufficiently hydrophobic to bind the hydrophobic portion of the bile salt. Furthermore, it is considered a ratio of from about 4:1 may result in excessively rapid precipitation of particles, such that insufficient time for the particles to interact with bile acids would be allowed. Hence it is concluded that a ratio of from about 3:1 to about 4:1 is preferable. Increasing the hydrophobic part of the microparticles improves the affinity of the particles for the hydrophobic region of the bile acids and not for the hydrophilic region, leading to increased interaction with, and precipitation of, bile acids. As further shown in Table 3 below, an increased lecithin:chitosan ratio, and the subsequent increase in hydrophobic character of the microparticles, resulted in an improved affinity for bile acids.

It was intended to quantify the parameters that control the amount of bile salts which will be trapped by the formulation of the present invention and consequently excreted from the body, based on colorimetric analysis of absorbed bile salts, as described in the Examples section below.

It was found, based on measurements of particle size and particle stability at pH 6.8, that absorption of bile salts by lecithin-chitosan particles is affected by a combination of hydrophobic degree and particle size. The ratio of lecithin to chitosan in the formulation of the present invention is optionally and preferably in the range of from about 1:0.2 to about 5:1. Hence, the range may comprise, for example, 0.2:1, 0.25:1, 0.4:1, 0.6:1, 1:1, 1.2:1, 2:1, 2.8:1, 3:1, 4:1 or 5:1. More preferably, the range is from about 3:1 to about 4:1, such as, for example, 3:1, 3.3:1, 3.5:1, 3.7:1 or 4:1.

The concentration of chitosan in the formulation is optionally and preferably no greater than about 2% (w/w), such as, for example, about 0.1%, about 0.2%, about 0.25%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 1.1%, about 1.2%, about 1.25%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9% or about 2% (w/w).

The concentration of lecithin in the formulation is optionally and preferably in the range of from about 0.5% to about 5% (w/w).

The formulation of the present invention may optionally further comprise a pharmaceutically acceptable carrier, and may optionally further comprise one or more excipients, such as, for example, a binder, a filler, or a disintegrant.

Suitable binders may include, for example, Povidone (PVP: polyvinyl pyrrolidone), low molecular weight HPC (hydroxypropyl cellulose), low molecular weight HPMC (hydroxypropyl methylcellulose), carboxy methyl cellulose, hydroxylethyl cellulose, ethylcellulose, gelatin polyethylene oxide, acacia, dextrin, magnesium aluminum silicate, starch, and polymethacrylates.

Suitable fillers may include, for example, at least one of sugars such as lactose, glucose, fructose, or sucrose; dicalcium phosphate; sugar alcohols such as sorbitol, manitol, mantitol, lactitol, xylitol, isomalt, erythritol, and hydrogenated starch hydrolysates; malto dextrines; corn starch, potato starch, sodium carboxymethycellulose, ethylcellulose and cellulose acetate, or a mixture thereof.

The disintegrant is optionally and preferably at least one of low-substituted carboxymethyl cellulose sodium, cross-linked polyvinyl pyrrolidone, sodium starch glycolate, cross-linked sodium carboxymethyl cellulose, pregelatinized starch, microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, and low substituted hydroxypropyl cellulose magnesium aluminum silicate.

The formulation of the present invention may be used in the treatment or prevention of any condition involving hypercholesterolemia, such as, for example, artherosclerosis, myocardial infarction, and cerebrovascular accident.

The formulation of the present invention may also be used as in the treatment of obesity by reducing body fat. Reduction of bile salts in the intestine reduces emulsification of fat, such that fat absorption by the body is decreased. According to any of the embodiments of the present invention, the formulation or method may further comprise an additional agent for cholesterol reduction, such as, for example, a statin, a fibrate, niacin, a bile acid sequestrant, ezetimibe, or a phytosterol, or combinations thereof.

The additional agent may optionally be provided in a combined dosage form, together with the chitosan-lecithin microparticles. Alternatively, the additional agent may be provided in a separate dosage form, for co-administration or sequential administration, either before or after administration of the chitosan-lecithin microparticles.

Examples of suitable statins include atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin, or combinations thereof.

Examples of suitable fibrates include, for example, bezafibrate, cirpofibrate, clofibrate, gemfibrozil, and fenobirate, or combinations thereof.

Examples of suitable bile acid sequestrants, include, for example, cholestyramine, colesevlam, and colestipol, or combinations thereof.

Examples of suitable phytosterols include, for example, β-sitosterol, campesterol, stigmasterol, brassicasterol, ergosterol, or combinations thereof.

Preferably, the additional cholesterol-reducing agent is a statin; more preferably simvastatin. Since the statins affect cholesterol levels by an entirely different mechanism from that described herein for cholesterol reduction by chitosan, the effects of chitosan and statin would be expected to be additive, or even synergistic.

The formulation of the present invention may optionally further comprise an enteric coating layer. Enteric coating layers are formed by use of enteric polymers, such as cellulose, vinyl, and acrylic derivatives. These polymers exhibit resistance to gastric fluids, yet are readily soluble or permeable in intestinal fluid. Enteric polymeric materials are primarily weak acids containing acidic functional groups,

which are capable of ionization at elevated pH. In the low pH of the stomach, the enteric polymers are unionized, and therefore, insoluble. As the pH increases in the intestinal tract, these functional groups ionize, and the polymer becomes soluble in the intestinal fluids. Thus, an enteric polymeric film coating allows the coated solid to pass intact through the stomach to the small intestine, where the drug is then released for absorption through the intestinal mucosa into the human body where it can exert its pharmacologic effects.

Examples of enteric polymers include cellulose acetate phthalate, cellulose acetate succinate, methylcellulose phthalate, ethylhydroxycellulose phthalate, polyvinylacetatephthalate, polyvinylbutyrate acetate, vinyl acetate-maleic anhydride copolymer, styrene-maleic mono-ester copolymer, methyl acrylate-methacrylic acid copolymer, methacrylate-methacrylic acid-octyl acrylate copolymer.

An animal study involving guinea pigs will be carried out, as described in the Examples section below. Guinea pigs are useful models for investigation of the hypocholesterolemic effects of drugs. As in humans, most of the plasma cholesterol in guinea pigs is in the LDL form, making them a unique animal model with which to study hepatic cholesterol and lipoprotein metabolism. Guinea pigs show other striking similarities to humans in terms of hepatic cholesterol and lipoprotein metabolism. Guinea pig responses to dietary factors, drug treatment, ascorbic acid deficiency, oxidative stress, exercise, gender and hormonal status, undoubtedly mimic the human situation. In addition, many of the mechanisms by which guinea pigs regulate cholesterol and lipoprotein metabolism as a response to diet or drug treatment are analogous to those reported in clinical experiments. These studies clearly document the suitability and appropriateness of the guinea pig model and reinforce the importance of the use of alternatives to the more-established and more widely used animal models.

Diets rich in lauric and myristic acids are known to cause endogenous hypercholesterolemia in guinea pigs. This particular model is required for the study of the hypocholesterolemic effects of new drugs (*Maria Luz Fernandez et al, 2001* JTT-130, a microsomal triglyceride transfer protein (MTP) inhibitor lowers plasma triglycerides and LDL cholesterol concentrations without increasing hepatic triglycerides in guinea pigs, BMC Cardiovasc Disord. 2005; 5: 30).

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

EXAMPLES

MATERIALS AND METHODS

Materials

Low molecular weight chitosan 100,000 g/mol (SK-10), having a degree of acetylation of 20% and high molecular weight chitosan, 2.8×10^6 g/mol (SK-100), having a degree of acetylation of 9% were obtained from Koyo, Japan.

Partially hydrolyzed lecithin, resulting in a rich phospholipid (HL 50 IP) was obtained from Cargill, Germany.

Method of preparation

a. SK-10 chitosan

Solutions of low molecular weight SK-10 chitosan (1%) in 0.5 % HCl and lecithin (10%) in water were prepared by stirring for 24 hours at 25°C. The pH of each solution was adjusted to 3.2.

The two solutions were mixed for 15 minutes at 25°C at the following lecithin: chitosan ratios: 1:1, 2:1; 3:1; 4:1; and 5:1 to form a suspension.

Samples were withdrawn in order to study the size distribution and zeta potential of the particles thus formed. Results are shown in Table 1.

The suspension was stirred for one hour and than transferred to a spray dryer (BUCHI mini spray dryer B-290). The temperature at the entrance of the spray dryer was 120°C, the degree of the aspirator was 70% and the pumping degree was 25%.

The powder that formed was first resuspended in hydrochloric acid (pH 3.0) to form a suspension of particles comprising chitosan at a concentration of 0.25% w/w, with varying concentrations of lecithin, to provide different ratios of lecithin:chitosan, as follows:

Ratio of lecithin/SK-10 chitosan in the powder	Concentration of the lecithin in the dispersion (% weight)
0.6	0.15
1.2	0.3
2.8	0.7
4	1
5	1.25

0.25 g was removed from each of the powders obtained and resuspended in 20 ml HCl (pH 3.0) for 30 mins, to simulate conditions in the human stomach. The solutions were then neutralized with NaOH and 30 ml phosphate buffer containing (in mM): 108 NaCl, 4.7KCl, 1.8 NaH_2PO_4, 15 $NaHCO_3$, 1.2$MgSO_4$, 1.25 $CaCl_2$, to bring the pH of the solution to 6.8, which stimulates human intestinal conditions.

The dispersion and the stability of the particles were recorded by digital photography after 3 hours in phosphate buffer. The results are shown in Figure 2.

b. SK-100 chitosan

Solutions of high molecular weight SK-100 chitosan (1%) in 0.5 % HCl and lecithin (10%) in water were prepared as described above for SK-10.

The powder that formed was first resuspended in hydrochloric acid (pH 3.0) to form a suspension of particles comprising chitosan at a concentration of 0.25% w/w, with varying concentrations of lecithin, to provide different ratios of lecithin:chitosan, as follows:

Ratio of lecithin/SK-100 chitosan in the powder	Concentration of the lecithin in the dispersion (% weight)
1.25	0.3
2	0.5
2.8	0.7
4	1
5	1.25

Samples were withdrawn in order to study the size distribution of the particles thus formed. Results are shown in Table 2 and in Figures 4a and 4b.

0.25 g was removed from each of the powders obtained and resuspended in HCl, then in phosphate buffer at 6.8, as described above with regard to SK-10 chitosan.

The dispersion and the stability of the particles were recorded by digital photography after 5 minutes (Fig. 2a), 30 minutes (Fig. 2b) and 3 hours (Fig. 2c) in phosphate buffer.

Exemplary formulation

An exemplary formulation comprises the following components in 100 g tablets:

Chitosan	0.075 g
Lecithin	0.225 g
Microcrystalline cellulose	99.7 g

In vitro model of interaction between chitosan-lecithin particles and bile acids.

In order to study the possible interaction between bile acids and the chitosan-lecithin particles of the present invention, an in vitro model was used, based on the assumption that the soluble free bile acid derivative, sodium cholate, will be bound by

the particles, such that the amount of sodium cholate in the supernate would be reduced. The method measures the free bile acids that remain in the supernatant after mixing particles of lecithin and chitosan in different ratios with sodium cholate, after centrifugation and filtration to remove bound cholate from soluble, unbound cholate remaining in solution.

Colorimetric analysis of cholate in phosphate buffer was performed as described by *Paul et al.(Journal of Biological Chemistry, pp. 73-82, 1948)* Briefly, the method is based on the color produced when acetic acid solutions comprising cholate are treated with furfural and sulfuric acid . The relationship between absorbance and sodium cholate concentration was first studied in order to establish that colorimetric analysis is a suitable method for measuring cholate concentration. The results are presented in Figure 5.

High molecular weight (SK-100) chitosan at 0.25% w/w, and pure sodium cholate at 0.5% w/w were used, with different amounts of lecithin, to give the following lecithin:chitosan ratios:

Ratio of lecithin/ chitosan in the powder	Concentration of the lecithin in the dispersion (% weight)
1.25	0.3
2	0.5
2.8	0.7
4	1
5	1.25

The particles were prepared as described above, and suspended in HCl for 30 minutes. The particles were then transferred to phosphate buffer at pH 6.8 and test solutions prepared by mixing with sodium cholate for 30 minutes. Particles were then centrifuged and filtered in order to separate between the particles and the free cholate in the supernatant, before being subjected to colorimetric analysis. Controls consisted of chitosan-lecithin particles in phosphate buffer, without sodium cholate, which have some absorbance in intestinal media. Colorimetric results are presented in Table 2 below.

Hypocholesterolemic effects of chitosan formalations in the guinea pig hypercholesterolemia-induced model

The cholesterol blood levels of guinea pigs, fed *ad libitum* a high level fat diet rich in lauric and myristic acids alone, or in combination with various orally-administered chitosan formulations is examined.

Animals: Twenty male guinea pigs are used, each of body weight about 300-400 g, such that the body weight of the group does not exceed ±20% of the group mean body weight.

Commercial guinea pig diet: Harlan Teklad Guinea Pig Diet, cat. No. 2040S

Controlled diet: Guinea Pig Diet with 15% Fat Mix L With 0.25% Cholesterol, D22316, (Research Diets inc.):

Product #	**D22316**	
	gm	**Kcal**
Protein	22.2	22.6
Carbohydrate	40.5	42.2
Fat	15.1	35.3
Total		100
Kcal/gm	3.85	
Ingredient		
Soy protein	80	320
Casein, lactic	120	480
L-Methionine	5	0.0
Corn starch	125	500
Maltodextrin 10	25	100
Sucrose	214	856
Cellulose	125	0
Olive oil	33.36	300.2
Palm kernel oil	68.11	613
Safflower oil	37.53	337.8
Mineral mix S20001	75	0
Vitamin mix V20001	10	40
Cholin bitartrate	2	0
Cholesterol, USP	2.3	0
FD&C blue dye #1	0.1	0

Total	**922.4**	**3547.0**

Preparation of lecithin-chitosan particles for animal feeding

In order to produce sufficient quantities of particles for use in the animal study, a spray drier of capacity 16 liters per hour is used. By using such a spray drier, from about 0.5 to about 1 kg can be produced per day. A solution of chitosan (1%) is dissolved in 1% lactic acid for 24 hours with stirring at 25°C. Lecithin (10%) in water is prepared by stirring for 24 hours at 25°C. The pH of each solution is adjusted to 3.3.

The two solutions are mixed for 5 minutes at 45°C at lecithin: chitosan ratios of from about 1:1 to about 4:1 and then transferred to a spray drier. Samples of lecithin:chitosan ratios 1:1, 2:1 and 3:1 are dried with 50% maltodextrin in order to obtain dry samples. Samples of ratio 3.5:1 and 4:1 are dried with 80% maltodextrin.

Animal care: Animals are handled according to the National Institute of Health and the Association for Assessment and Accreditation of Laboratory Animal Care standards. Housing consists of polyethylene cages (1 animal per cage), measuring 35 x 30 x 15 cm, with stainless steel top grill having facilities for drinking water in a glass bottle. Animals are housed under standard laboratory conditions, air conditioned and filtered (HEPA F6/6) with adequate fresh air supply (31 air changes/hour). Animals are kept in a climate controlled environment. Temperature range is between 20 – 24^{0}C and RH is between 30 – 70% with 12 hours light and 12 hours dark cycle.

Pelleted food is placed in hoppers on the bottom of the cage. Bedding is steam sterilized clean paddy husk (Harlan, Sani-chip, cat. No. 2018SC+F), and is changed along with the cage at least twice weekly.

Study methods: The animals are given a 5 day acclimation period. During acclimation, animals are fed *ad libitum* commercial guinea pig diet. Animals have free access to drinking water obtained from the municipality supply.

Prior to commencement of the study, animals are randomized using a computer generated randomization program "Research Randomizer" and divided into 5 groups of 4 animals each.

Initially, all animals are fed *ad libitum* with the Controlled diet, D22316, detailed above, for 3 weeks. After this period, a control group is fed freely with D22316 alone. Test groups are fed with D22316 and lecithin-chitosan particles at lecithin:chitosan ratios of 3:1, 3.5:1, or 4:1 at concentrations of 0.3% lecithin, or statin at a concentration of 0.05% for a period of 4 weeks, as follows:

Group	D22316	Lecithin: chitosan ratio	Lecithin concentration	Statin concentration
Control	√	-	-	-
I	√	3:1	0.3%	-
II	√	3.5:1	0.3%	-
III	√	4:1	0.3%	-
IV	√	-	-	0.05%

Determination of individual body weights of animals will be made once weekly during acclimation and test periods. Measurements of food consumption will be made once per 24-hour period during the acclimation period, and at least once per 24-hour period during the study period. Determination of food consumption (g/animal) is based on diet provided in hoppers and unused diet including noticable scatter.

After the 4 week period, blood samples (approximately 3 ml/animal) are withdrawn for blood lipid profile determination, following overnight food deprivation. Blood collection is performed by abdominal aorta puncture under isoflurane anesthesia. Animals are then sacrificed.

Blood samples are maintained at room temperature for at least 30 minutes, then centrifuged (5000 RCF, 10 minutes) for serum separation.

Following serum separation, samples are transferred to clean vials, labeled with study code, animal number and date, and kept at -2-8°C until transferred for blood lipid profile analysis. Samples are packed in a closed Styrofoam receptacle containing ice pack and transferred within 24 hours of blood collection.

Hypocholesterolemic effects of chitosan formalations in combination with statins in the guinea pig hypercholesterolemia-induced model

The study is conducted as for the previous Example, except that test groups are provided with lecithin:chitosan particles in combination with statins. As a first control, animals are provided with D22316 alone. A second control group is provided with D23316 containing statin. Test groups are fed different concentrations of statins in combination with different combinations of lecithin-chitosan particles.

A synergistic effect is expected due to the reduction of cholesterol by two separate mechanisms.

RESULTS

Particle size and zeta potential at different lecithin:chitosan ratios (SK-10 chitosan)

The size of the particles formed and their zeta potential at different ratios of lecithin to chitosan is shown in Table 1.

Sample no.	lecithin concentration (%)	Ratio (w/w) lecithin/chitosan	Size (microns)	Zeta potential (mV)
1	0.05	0.2	1.2	+43
2	0.07	0.25	1.8	+42
3	0.1	0.4	1.95	+42
4	0.3	1.2	1.25	+39
5	0.7	2.8	0.95	+33

Table 1

The zeta potential represents the charge of the particle. As shown in the Table, zeta potential decreases with increased lecithin:chitosan ratio.

Particle size at different lecithin:chitosan ratios (SK-100 chitosan)

Table 2 shows particle size for different concentrations of lecithin. The concentration of chitosan in the experiment is 0.25%. The lecithin:chitosan ratios are therefore 1.2:1; 2:1; 2.8:1; and 4:1.

As shown in the Table, particle sizes generally increase with time and with increased lecithin:chitosan ratio.

Lecithin concentration (%)	Particle size (nm) after 15 minutes	Particle size (nm) after 1 hour

0.3	518	381
0.3	27	379
0.5	526	1030
0.5	771	1010
0.7	574	861
0.7	25.2	773
1	938	1270
1	905	1460
1.25	1170	1180
1.25	1240	785

Dispersion and stability of particles at different lecithin:chitosan ratios

Figure 2 shows dispersion of the SK-10 particles after 3 hours in phosphate buffer at lecithin:chitosan ratios of 1:1, 2:1, 3:1, 4:1 and 5:1.

Figures 3a-3c show dispersion of SK-100 particles, after 5 minutes, 30 minutes and 3 hours in phosphate buffer. As shown in Figure 3a-3c, particles were stable at a ratio of 1:1 lecithin:chitosan, and did not precipitate with time over at least 3 hours. The average size of these particles was found to be around 300-400 nano microns. As the lecithin:chitosan ratio was increased, it was found that particle size ranging from several hundred nanometers to one or two microns were obtained, and the particles lost their stability in the solution and begin to precipitate.

Precipitation of particles increased in proportion to the increase in the lecithin:chitosan ratio, as the result of increasing the hydrophobic portion of the particles. However, it is considered that too rapid precipitation of particles would not enable sufficient time for the particles to interact with bile acids, hence it is concluded that a ratio of from about 3:1 to about 4:1 is preferable.

Interaction between chitosan-lecithin particles and bile acids.

In vitro model of interaction between chitosan-lecithin particles and bile acids.

In order to study the possible interaction between bile acids and the chitosan-lecithin particles of the present invention, an in vitro model was used, based on the assumption that the soluble free bile acid derivative, sodium cholate, will be bound by the particles, such that the amount of sodium cholate in the supernate would be reduced. The method measures the free bile acids that remain in the supernatant after mixing particles of lecithin and chitosan in different ratios with sodium cholate, after

centrifugation and filtration to remove bound cholate from soluble, unbound cholate remaining in solution.

Colorimetric analysis of cholate in phosphate buffer was performed as described by *Paul et al.(Journal of Biological Chemistry, pp. 73-82, 1948)* Briefly, the method is based on the color produced when acetic acid solutions comprising cholate are treated with furfural and sulfuric acid . The relationship between absorbance and sodium cholate concentration was first studied in order to establish that colorimetric analysis is a suitable method for measuring cholate concentration. The results are presented in Figure 5.

As shown in Figure 5, a linear relationship was established between sodium cholate concentration in solution (mg/ml) and absorbance by colorimetric analysis, showing that this method is suitable for measurement of cholate concentration.

The results of colorimetric analysis of test solutions are shown in Table 3 below.

Lecithin (%)	control	treatment	Treat- Con
0.3	0.1549	0.3182	0.1633
0.5	0.1956	0.6141	0.4185
0.7	0.2235	0.6398	0.4163
1	0.3547	0.4024	0.0477
1.25	0.2786	0.4316	0.153

Table 3

As shown in the Table, increased lecithin content led to increased binding of cholate by the chitosan-lecithin particles, such that less cholate remained in the supernatant. Increasing the lecithin content in the particles increased the hydrophobic character of the particles, thereby improving the affinity of the particles for the hydrophobic site of the sodium cholate.

From the results of Table 3, the amount of cholate in mg remaining in solution at lecithin:chitosan ratios of 2:1 and 3:1 was calculated, based on an OD of 0.4185 being equal to 0.74 mg sodium cholate. It was found that 40% of this amount remained at a ratio of 1:1, 0.12% of this amount at a ratio of 4:1, and 37% at a ratio of 5:1. Hence, 60%, 88% and 63% of cholate is absorbed at ratios of 1:1, 4:1 and 5:1, respectively.

It was further found that for particles having a lecithin:chitosan ratio of about 1:1, the effect of size is more significant than that of hydrophobic degree. Since nanoparticles (of diameter about 0.4 μm) have a high surface area these are more soluble, but less hydrophobic than the particles formed at higher ratios. These particles absorbed about 60% sodium cholate. However, bile salts which are absorbed by these nanoparticles will not be able to pass through the intestine without undergoing further hydrolysis, but will instead quickly separate from the particles and remain in the intestinal tract. Hence, it is desirable to use microparticles for the formulation of the present invention.

It is concluded that the in vitro test for measuring the degree of absorption of bile salts is not sufficient for quantification of the amount of bile salts which is trapped by the particles and subsequently expelled from the body via the feces. In vivo animal studies are required to accurately quantify the amount of bile salts expelled in the feces.

It is appreciated that certain features of the invention, which are, for clarity, described in the content of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application

shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A formulation for the reduction of cholesterol in humans, the formulation comprising composite, hydrophobic particles of chitosan and an anionic or non-ionic surfactant.

2. A method of producing a formulation for the reduction of cholesterol in humans, the method comprising:

preparing a first solution comprising chitosan by mixing with an acid for at least 24 hours;

preparing a second solution comprising an anionic or non-ionic surfactant;

mixing said first solution with said second solution in acid conditions to form composite particles; and

preparing a powder of said composite particles.

3. A method for the reduction of cholesterol in a human subject, the method comprising administering to the subject a formulation comprising composite, hydrophobic particles of chitosan and an anionic or non-ionic surfactant.

4. A formulation for the reduction of cholesterol in humans, the formulation comprising composite, hydrophobic microparticles comprising a cationic polymer and an anionic or non-ionic surfactant.

5. The formulation of claim 4, wherein said cationic polymer comprises chitosan.

6. The formulation of claims 4 or 5, wherein said cationic polymer comprises a polyamine.

7. The formulation of claim 6, wherein said polyamine comprises polylysine or polyacrylamide.

8. The formulation or method of any of claims 1 to 7, wherein said particles are microparticles.

9. The formulation or method of any of claims 1 to 8, wherein said anionic surfactant is selected from the group consisting of phospholipids; bile salts; sodium lauryl ether sulfate; citric acid esters of monoglycerides; sodium, calcium or acid stearoyl lactylate; stearyl citrate; fatty acids or salts thereof; diacetyl tartaric acid esters of monoglycerides; or combinations thereof.

10. The formulation or method of claim 9, wherein said phospholipid comprises lecithin.

11. The formulation or method of any of the claims 1 to 9, wherein said non-ionic surfactant is a fatty alcohol.

12. The formulation or method of any of claims 1 to 9, wherein said chitosan has a degree of acetylation of from about 50 % to about 95%.

13. The formulation or method of any of claims 1-12, wherein said chitosan comprises molecular weight chitosan having a molecular weight in the range of from about $0.5x10^5$ to about $3x10^6$ daltons,.

14. The formulation or method of any of claims 1 to 13, wherein said chitosan and said surfactant in said composite particles are connected by ionic and hydrophobic interactions.

15. The formulation or method of any of claims 1 to 14, wherein a ratio of surfactant:chitosan is in the range of from about 0.2:1 to about 5:1.

16. The formulation or method of claim 15, wherein said ratio is in the range of from about 3:1 to about 4:1.

17. The formulation or method of claim 16, wherein a concentration of said chitosan is about 0.7% w/w.

18. The formulation or method of claim 16, wherein a concentration of said chitosan is about 1.5% w/w.

19. The formulation or method of claim 10, wherein a concentration of said lecithin is in the range of from about 0.5% % w/w to about 7.5% w/w.

20. The formulation or method of claim 19, wherein a concentration of said lecithin is in the range of from about 0.7% % w/w to about 3.5% w/w.

21. The formulation or method of any of claims 1-20, wherein a concentration of said chitosan is in the range of from about 0.1% % w/w to about 2.0% w/w.

22. The formulation or method of any of the preceding claims, wherein said formulation further comprises a cholesterol-reducing agent selected from the group consisting of a statin, a fibrate, niacin, a bile acid sequestrant, ezetimibe, or a phytosterol, or combinations thereof.

23. The formulation or method of any of the preceding claims, wherein said formulation further comprises an enteric coating layer.

24. The formulation or method of any of the preceding claims, wherein said composite particles of said mixture are capable, upon mixing 2.5 grams of said composite particles with 1 liter of a sodium cholate solution having a sodium cholate concentration of 0.5%, of removing at least about 10% of free sodium cholate from said sodium cholate solution within 30 minutes.

25. The formulation or method of claim 24, wherein said mixture removes at least about 20% of free sodium cholate from said sodium cholate solution within 30 minutes.

26. The formulation or method of claim 25, wherein said mixture removes at least about 30% of free sodium cholate from said sodium cholate solution within 30 minutes.

27. The formulation or method of claim 26, wherein said mixture removes at least about 40% of free sodium cholate from said sodium cholate solution within 30 minutes.

28. The formulation or method of any of the preceding claims, wherein at least about 10%, by mass, of said composite particles of the mixture have a size between 0.3 microns and 2 microns.

29. The formulation or method of claim 28, wherein at least about 30%, by mass, of said composite particles of the mixture have a size between 0.3 microns and 2 microns.

30. The formulation or method of claim 29, wherein at least about 50%, by mass, of said composite particles have a size between 0.3 microns and 2 microns.

31. The formulation or method of claim 30, wherein at least about 70%, by mass, of said composite particles of the mixture have a size between 0.3 microns and 2 microns.

32. The formulation or method of claim 31, wherein at least about 90%, by mass, of said composite particles have a size between 0.3 microns and 2 microns.

33. The method of claim 2, wherein said first solution is prepared in an acid selected from the group consisting of hydrochloric acid.

34. The method of claim 33, wherein said organic acid is selected from the group consisting of lactic acid and glutamic acid

35. The method of claim 2, wherein said second solution is prepared in water.

36. The method of any of claims 33 to 35, wherein a pH of said solution is adjusted to a value of about 3.5.

37. The method of claim 2, further comprising adjusting a pH of said said suspension medium, after removal of said particles, to a value of about 7.

38. The method of claim 37, further comprising allowing said suspension medium to stand for about 30 minutes, then filtering to remove particles.

39. The method of claim 38, further comprising resuspending said removed particles in acid medium.

40. The method of claim 2, wherein said powder is prepared by a method selected from the group consisting of spray drying and lyophlization.

41. The method of claim 40, wherein said powder is prepared by spray drying.

42. A method for increasing cholesterol reduction in humans by chitosan, the method comprising increasing the hydophobicity of said chitosan.

43. The method of claim 42, wherein said hydrophobicity is increased by reacting chitosan with an anionic or non-ionic surfactant.

44. Use of the formulation or method of any of the preceding claims in the treatment or prevention of a condition selected from the group consisting of hypercholesterolemia, artherosclerosis, myocardial infarction, and cerebrovascular accident.

45. Use of the formulation or method of any of the preceding claims for reduction of body fat.

46. Use of the formulation or method of claim 45 in the treatment of obesity.

47. A formulation for the reduction of cholesterol in humans, the formulation comprising chitosan and an anionic or non-ionic surfactant.

48. A method of composite particle production, comprising:

Mixing low molecular weight chitosan in powder form into an acid for solubility;

preparing a second solution comprising an anionic or non-ionic surfactant;

mixing said first and second solutions under acidic conditions to form chitosan-surfactant composite particles including chitosan chemically bound to said surfactant; and

forming a powder from said chitosan-surfactant composite particles.

49. The method of claim 48, wherein said molecular weight is less than about 100 kilodaltons and said mixing is performed for at least about one hour.

50. The method of claim 48, wherein said molecular weight is from about 100 to about 400 kilodaltons and said mixing is performed for at least about two hours.

51. The method of claim 48, wherein said molecular weight is from about 400 to about 3000 kilodaltons and said mixing is performed for at least about three hours.

52. The method of any of claims 48 to 51 wherein said mixing is performed so that said first and second solutions are mixed together, before said powder-forming, for a time period that is at least 2 minutes and at most 30 minutes, and an average temperature during said time period is less than 40 degrees Celsius.

53. The method of any of claims 48 to 51 wherein said mixing is performed so that said first and second solutions are mixed together, before powder-forming, for a time period that is at least 2 minutes and at most 10 minutes, and an average temperature during said time period is less than 50 degrees Celsius.

54. The method of any of claims 48 to 51 wherein the method is performed at a temperature and mixing time duration so that at least 10% by mass of said formed composite particles have a size that is between 0.3 and 2 microns.

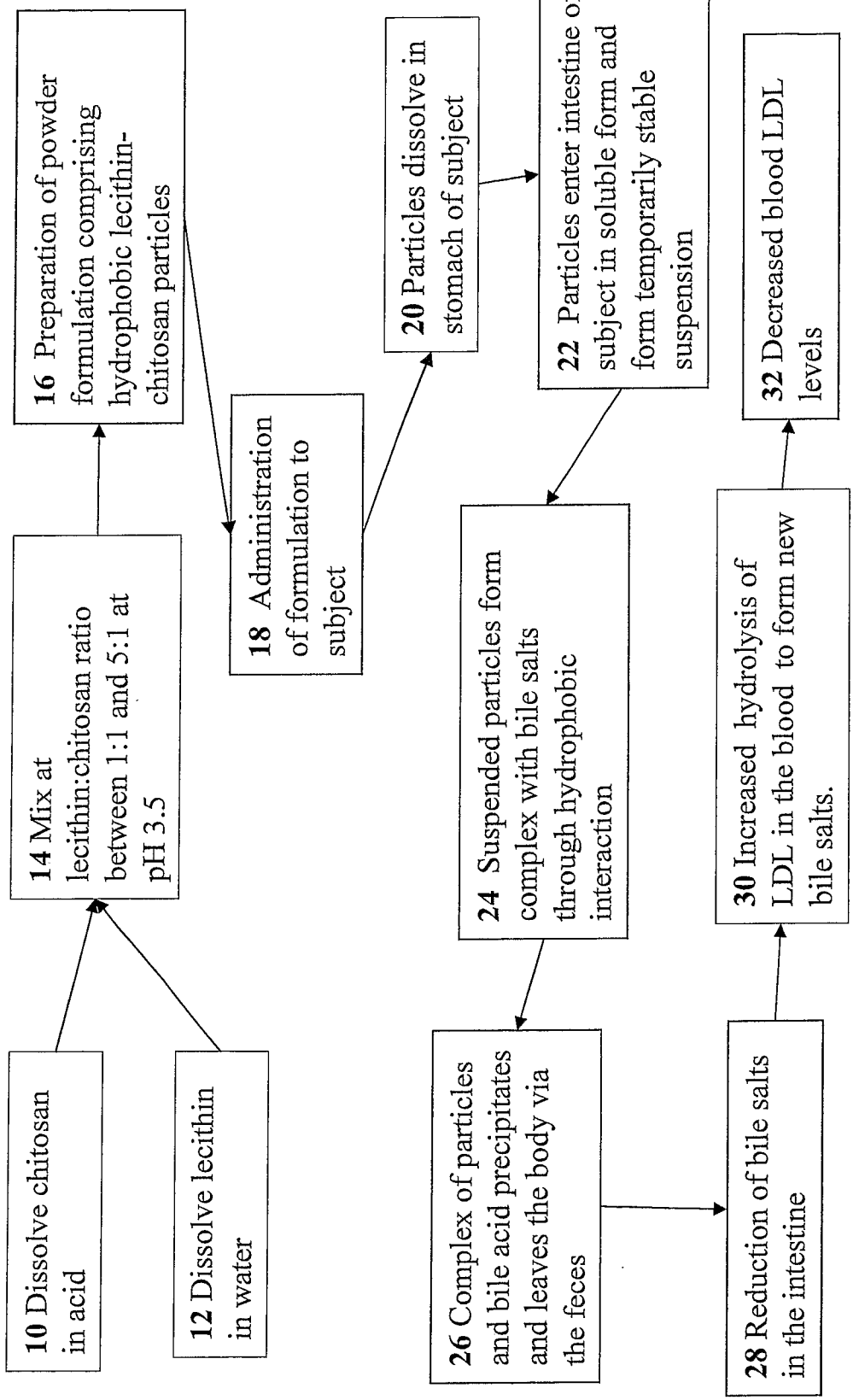

10 Dissolve chitosan in acid
12 Dissolve lecithin in water
14 Mix at lecithin:chitosan ratio between 1:1 and 5:1 at pH 3.5
16 Preparation of powder formulation comprising hydrophobic lecithin-chitosan particles
18 Administration of formulation to subject
20 Particles dissolve in stomach of subject
22 Particles enter intestine of subject in soluble form and form temporarily stable suspension
24 Suspended particles form complex with bile salts through hydrophobic interaction
26 Complex of particles and bile acid precipitates and leaves the body via the feces
28 Reduction of bile salts in the intestine
30 Increased hydrolysis of LDL in the blood to form new bile salts.
32 Decreased blood LDL levels

Figure 1

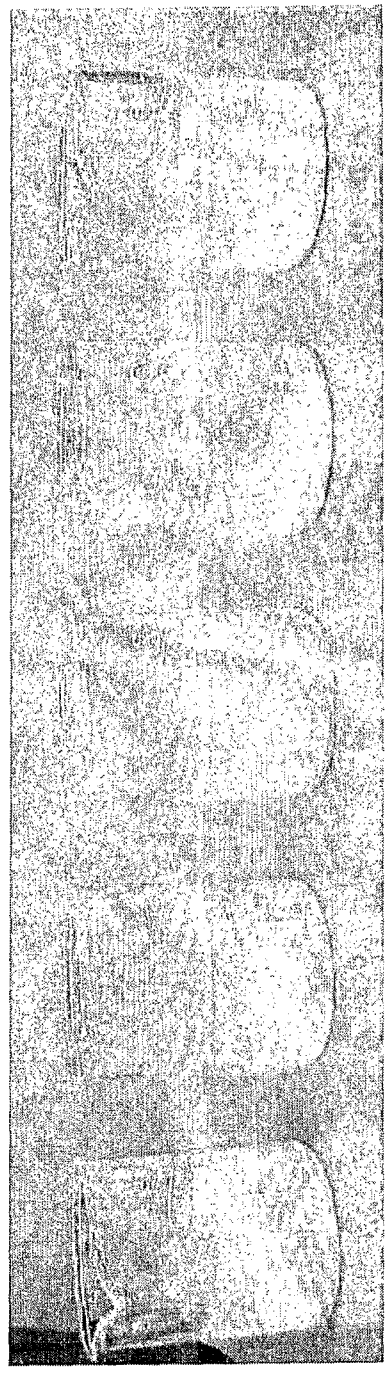


Figure 2

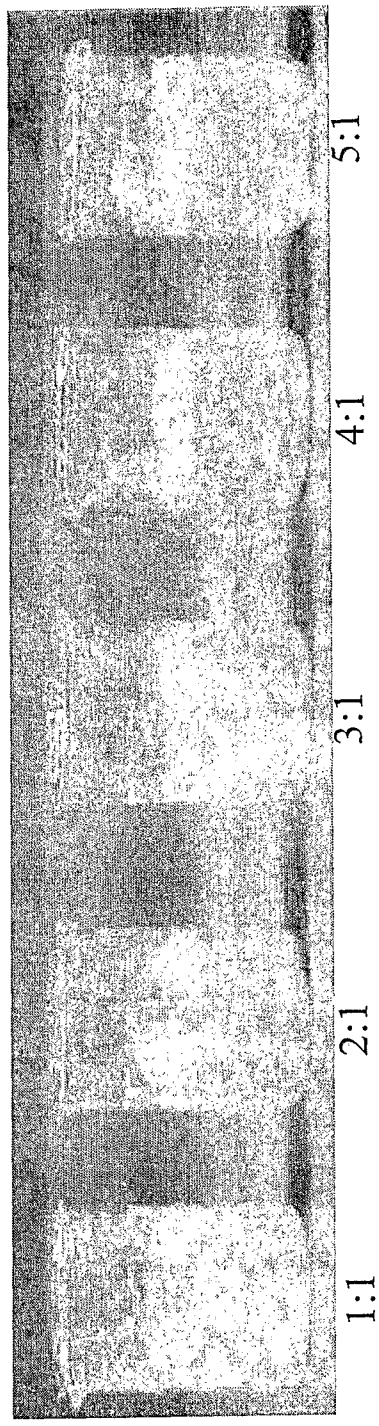


Figure 3A

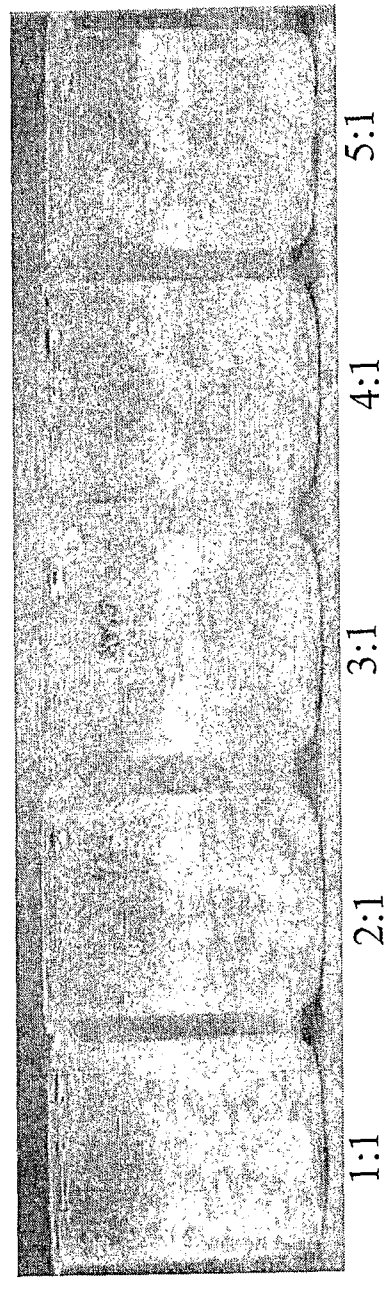


Figure 3B

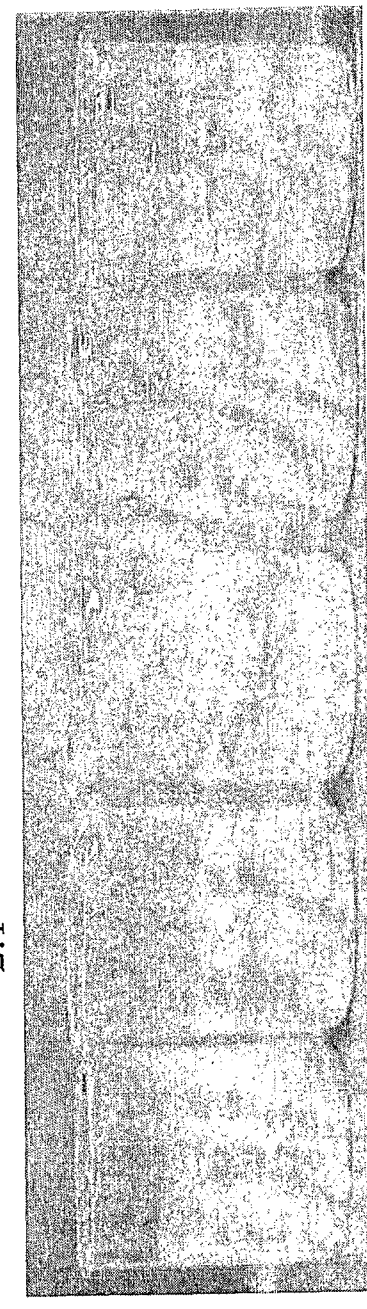


Figure 3C

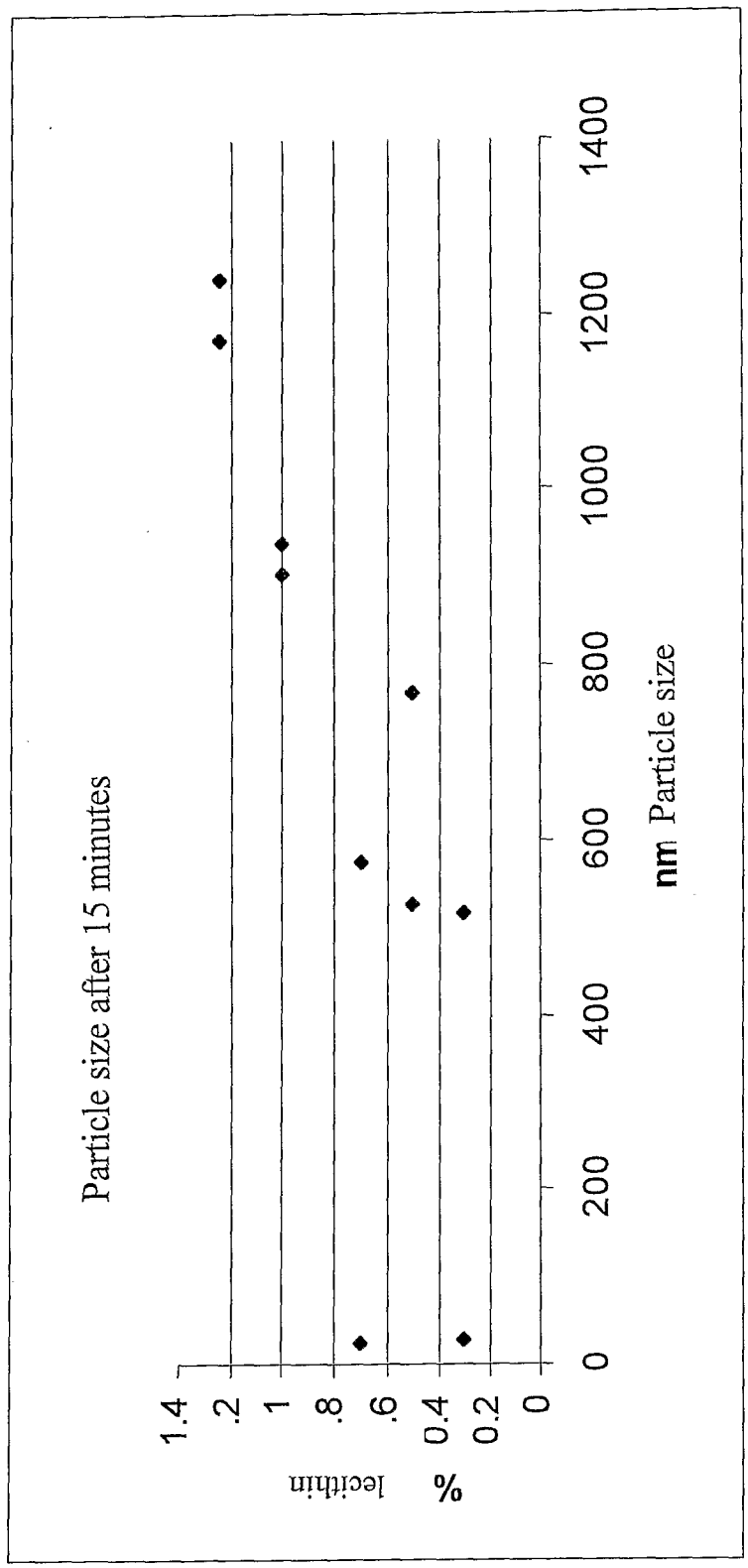

Particle size after 15 minutes
lecithin %
1.4
.2
1
.8
.6
0.4
0.2
0
0
200
400
600
800
1000
1200
1400
nm Particle size

Figure 4A

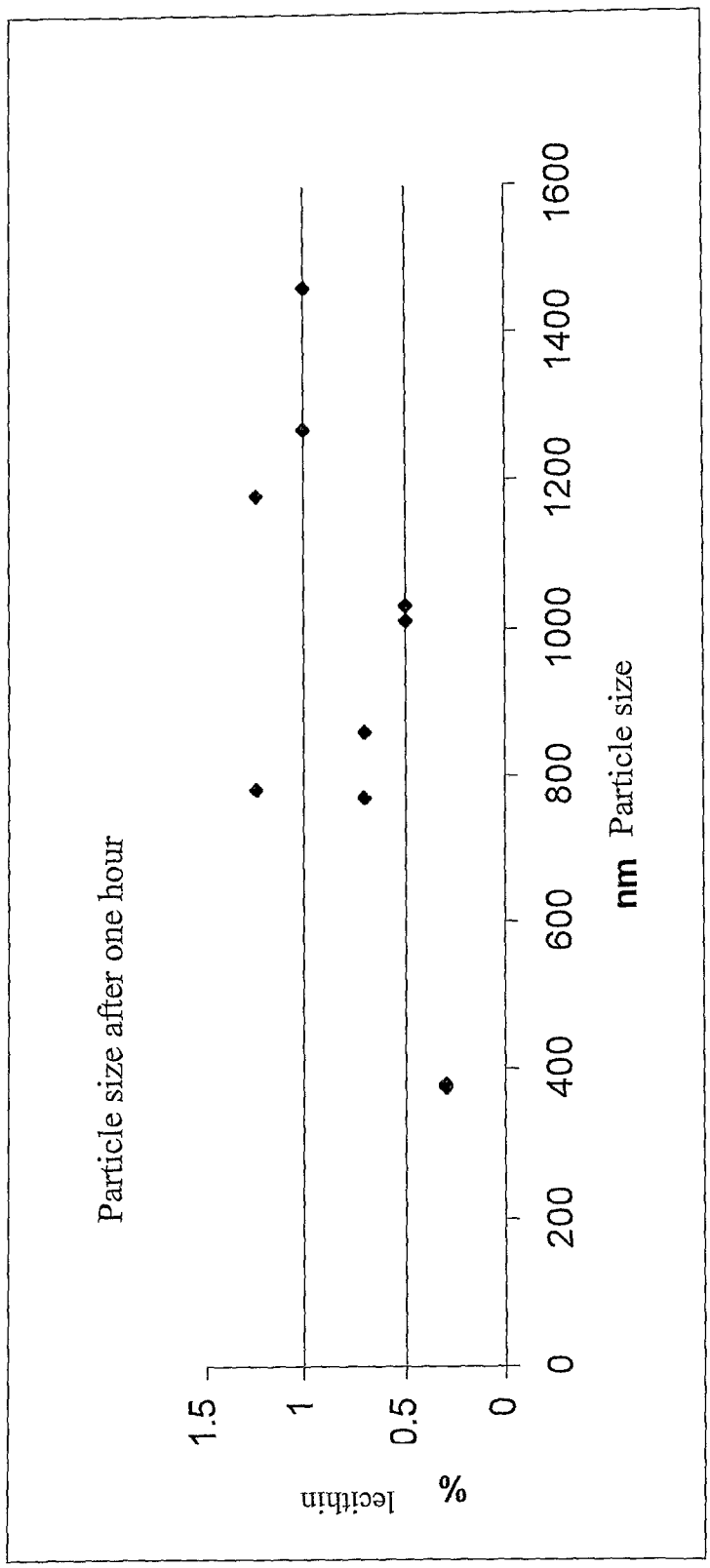

Particle size after one hour
lecithin %
1.5
1
0.5
0
0
200
400
600
800
1000
1200
1400
1600
nm Particle size

Figure 4B

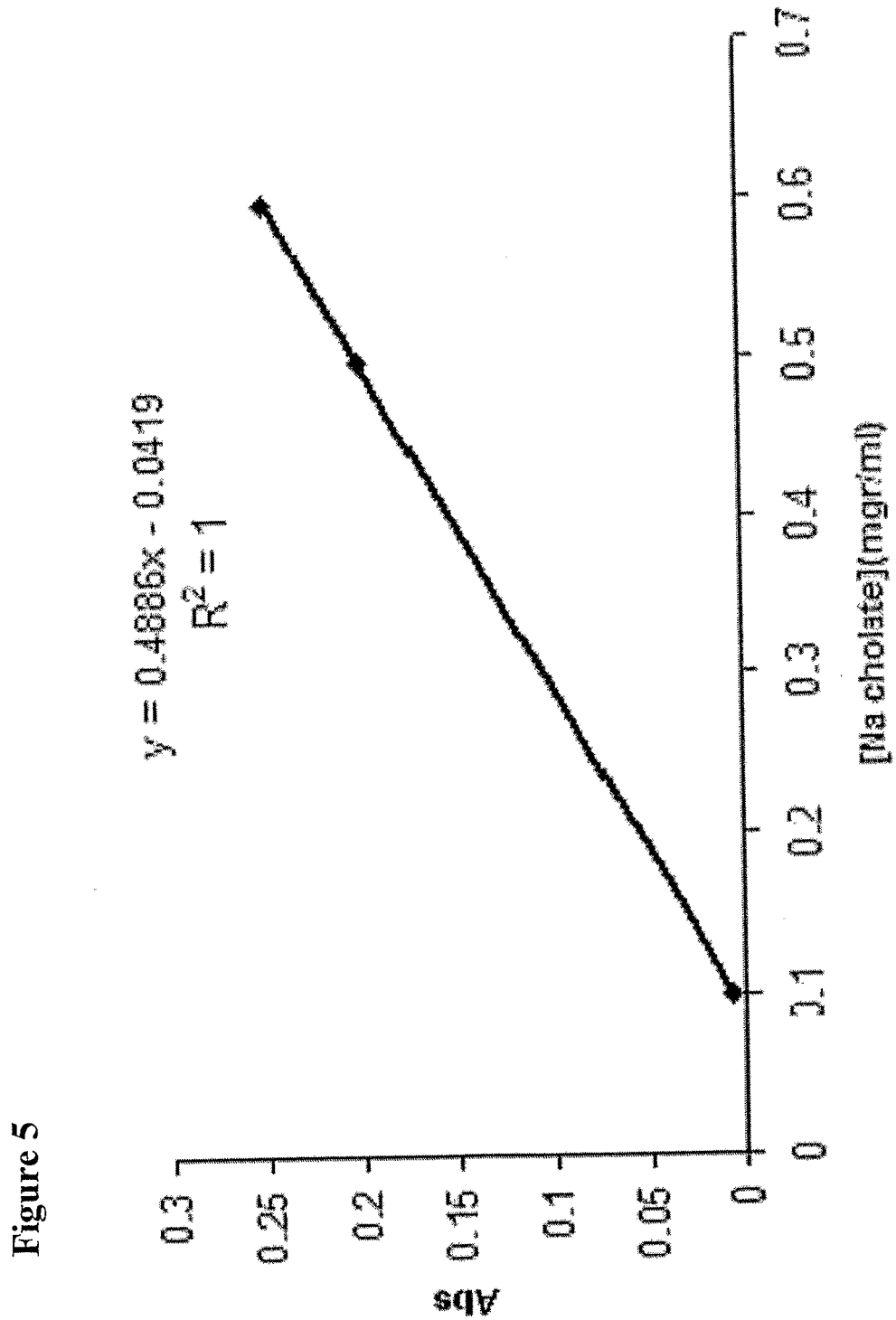

y = 0.4886x - 0.0419
R2 = 1
Abs
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
[Na cholate](mgr/ml)

Figure 5