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(54) Titre : AMELIORATIONS APPORTEES A DES LIPOSOMES AMPHOTERES
(54) Title: IMPROVEMENTS IN OR RELATING TO AMPHOTERIC LIPOSOMES

(57) Abrégé/Abstract:
A serum-stable mixture of lipids capable of encapsulating an active agent to form a liposome, said mixture comprising phosphatidylcholine and phosphatidylethanolamine in a ratio in the range of about 0.5 to about 8. The mixture may also include pH sensitive anionic and cationic amphiphiles, such that the mixture is amphoteric, being negatively charged or neutral at pH 7.4 and positively charged at pH 4. Amphoteric liposomes comprising such a mixture may be used for encapsulating nucleic acid therapeutics, such as oligonucleotides and DNA plasmids. The drug/lipid ratio may be adjusted to target the liposomes to particular organs or other sites in the body.

ABSTRACT

A serum-stable mixture of lipids capable of encapsulating an active agent to form a liposome, said mixture comprising phosphatidylcholine and phosphatidylethanolamine in a ratio in the range of about 0.5 to about 8. The mixture may also include pH sensitive anionic and cationic amphiphiles, such that the mixture is amphoteric, being negatively charged or neutral at pH 7.4 and positively charged at pH 4. Amphoteric liposomes comprising such a mixture may be used for encapsulating nucleic acid therapeutics, such as oligonucleotides and DNA plasmids. The drug/lipid ratio may be adjusted to target the liposomes to particular organs or other sites in the body.

Improvements in or Relating to Amphoteric Liposomes

Field of the invention

The present invention relates to amphoteric liposomes and has particular reference to such liposomes having improved stability in human or animal serum. The present invention also comprehends mixtures of lipids capable of encapsulating active agents or ingredients such, for example, as drugs to form liposomes and pharmaceutical compositions comprising such liposomes.

Background of the invention

Oligonucleotides represent a novel class of drugs that can very specifically down-regulate or interfere with protein expression. Such oligonucleotides include antisense, locked nucleic acids (LNA), peptide nucleic acids (PNA), morpholino nucleic acids (Morpholinos), small interfering RNAs (siRNA) and transcription factors decoys of various chemistries. A detailed description of the different mechanisms of action of such oligonucleotide therapeutics can be found in the literature (e.g., Crooke in BBA (1999), 1489(1), 31-44; Tijsterman, *et al.* in Cell (2004), 117(1), 1-3; and Mann, *et al.* in J Clin Invest, (2000), 106(9), 1071-5).

The use of oligonucleotides for gene repair applications (see, e.g., Richardson, *et al.* in Stem Cells (2002), 20, 105-118) and micro RNAs are other examples from this rapidly growing field.

It is known in the art that nucleic acid therapeutics, irrespective of their actual chemical origin, may lack therapeutic efficacy owing to their instability in body fluids or because of inefficient uptake into cells, or both. Chemical modifications of such oligonucleotide, including the above-mentioned variants, as well as the formation of conjugates with ligands or polymers, represent one strategy to overcome such practical limitations.

A second set of strategies involves the use of carrier systems, in particular liposomes, for protecting, targeting and affording enhanced uptake into cells. Liposomes are artificial single, oligo or multilamellar vesicles having an aqueous core and being formed from amphiphilic molecules having both hydrophobic and hydrophilic components (amphiphiles). The cargo may be trapped in the core of the liposome, disposed in the membrane layer or at the membrane surface. Such carrier systems should meet an optimum score of the following criteria: high encapsulation efficiency and economical manufacture, colloidal stability, enhanced uptake into cells and of course low toxicity and immunogenicity.

Anionic or neutral liposomes are often excellent in terms of colloidal stability, as no aggregation occurs between the carrier and the environment. Consequently their biodistribution is excellent and the potential for irritation and cytotoxicity is low. However, such carriers lack encapsulation efficiency and do not provide an endosomolytic signal that facilitates further uptake into cells (Journal of Pharmacology and experimental Therapeutics (2000), 292, 480-488 by Klimuk, *et al.*).

A great many of publications deal with cationic liposomal systems; see, e.g., Molecular Membrane Biology (1999), 16, 129-140 by Maurer, *et al.*; BBA (2000) 1464, 251-261 by Meidan, *et al.*; Reviews in Biology and Biotechnology (2001), 1(2), 27-33 by Fiset & Gounni. Although cationic systems provide high loading efficiencies, they lack colloidal stability, in particular after contact with body fluids. Ionic interactions with proteins and/or other biopolymers lead to *in situ* aggregate formation with the extracellular matrix or with cell surfaces. Cationic lipids have often been found to be toxic as shown by Filion, *et al.* in BBA (1997), 1329(2), 345-356; Dass in J. Pharm. Pharmacol. (2002), 54(5), 593-601; Hirko, *et al.* in Curr. Med. Chem., 10(14), 1185-1193.

These limitations were overcome by the addition of components that provide a steric stabilisation to the carriers. Polyethylenglycols of various chain length, for example, are known to eliminate aggregation problems associated with the use of cationic components in body fluids, and PEGylated cationic liposomes show enhanced circulation times *in vivo* (BBA (2001) 1510, 152-166 by Semple, *et al.*). However, the use of PEG does not solve the intrinsic toxicity problems associated with cationic lipids. It is also known that PEG substantially inhibits the productive entry of such liposomes into the cells or their intracellular delivery (Song, *et al.* in BBA (2002), 1558(1), 1-13). Quite recently, Morrissey, *et al.* (Nature Biotechnology (2005), 23 (8), 1002 - 1007) described a diffusible PEG-lipid for a cationic vector that is able to transfer siRNA into liver cells *in vivo*. However, the huge demand for such solutions and the given attrition rate of clinical development more than motivates the development of conceptually independent solutions.

Amphoteric liposomes represent a recently described class of liposomes having an anionic or neutral charge at pH 7.5 and a cationic charge at pH 4. WO 02/066490, WO 02/066012 and WO 03/070735, all to Panzner, *et al.*

give a detailed description of amphoteric liposomes and suitable lipids therefor. Further disclosures are made in WO 03/070220 and WO 03/070735, also to Panzner, *et al.* which describe further pH sensitive lipids for the manufacture of such amphoteric liposomes.

Amphoteric liposomes have an excellent biodistribution and are very well tolerated in animals. They can encapsulate nucleic acid molecules with high efficiency.

The use of amphoteric liposomes as carriers for drugs for the prevention or treatment of different conditions or diseases in mammals requires stability of the liposomes after their injection into the bloodstream. For systemic applications especially, the drug must be stably encapsulated in the liposomes until eventual uptake in the target tissue or cells. The FDA's guidelines prescribe specific preclinical tests for drugs comprising liposomal formulations. For example, the ratio of encapsulated drug to free drug must be determined during the circulation time in the bloodstream.

After the injection of liposomes into the bloodstream, serum components interact with the liposomes and may lead to permeabilisation of the liposomal membrane. However, the release of a drug that is encapsulated by the liposome also depends upon the molecular dimensions of the drug. This means that a plasmid drug with a size of thousands of base pairs, for example, may be released much more slowly than smaller oligonucleotides or other small molecules. For liposomal delivery of drugs it is essential that the release of the drug during the circulation of the liposomes is as low as possible.

Objects of the invention

An object of the present invention therefore is to provide liposomes and mixtures of lipids capable of forming such liposomes having improved stability upon contact with human or animal serum.

In particular, an object of the present invention is to provide amphoteric liposomes having such improved serum stability.

Another object of the invention is to provide pharmaceutical compositions comprising such liposomes as a carrier for the targeted delivery of active agents or ingredients, including drugs such as nucleic acid drugs, e.g., oligonucleotides and plasmids.

A particular object of the present invention is to provide such a pharmaceutical composition for the treatment or prophylaxis of inflammatory, immune or autoimmune disorders of humans or non-human animals.

Yet another object of the present invention is provide methods for the treatment of human or non-human animals in which a pharmaceutical composition comprising an active agent is targeted to a specific organ or organs, tumours or sites of infection or inflammation.

Summary of the invention

According to one aspect of the present invention therefore there is provided a mixture of lipids capable of encapsulating an active agent to form a liposome, said mixture comprising phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in a ratio of phosphatidylethanolamine to phosphatidylcholine in the range of about 0.5 to about 8.

Suitably, said ratio range from about 0.75 to about 5, preferably from about 1 to about 4.

In some embodiments, said phosphatidylcholine may be selected from DMPC, DPPC, DSPC, POPC or DOPC, or from phosphatidylcholines from natural sources such, for example, as soy bean PC and egg PC.

Said phosphatidylethanolamines may be selected from DOPE, DMPE and DPPE.

Preferred neutral lipids include DOPE, POPC, soy bean PC and egg PC.

It is known that cholesterol may stabilise phosphatidylcholine bilayers against serum attack. However, neither POPC nor DOPE form serum stable structures by themselves. It has now been found surprisingly that mixtures of DOPE and POPC may form serum stable liposomes.

Accordingly, in a particular aspect of the present invention, said mixture of lipids may be neutral. In some embodiments said mixture may consist or consist essentially of phosphatidylcholine and phosphatidylethanolamine in a ratio in the aforementioned range.

In another aspect of the present invention there are provided neutral liposomes comprising a mixture of lipids in accordance with the invention. Such liposomes may be used as a serum-stable excipient or carrier for active agents such as drugs.

In a different aspect of the present invention however, said mixture may further comprise one or more charged amphiphiles.

Preferably said one or more charged amphiphiles are amphoteric, being negatively charged or neutral at pH 7.4 and positively charged at pH 4.

By "amphoteric" herein is meant a substance, a mixture of substances or a supra-molecular complex (e.g., a liposome) comprising charged groups of both anionic and cationic character wherein:

(i) at least one of the charged groups has a pK between 4 and 8,

(ii) the cationic charge prevails at pH 4, and

(iii) the anionic charge prevails at pH 8,

resulting in an isoelectric point of neutral net charge between pH 4 and pH 8. Amphoteric character is by this definition different from zwitterionic character, as zwitterions do not have a pK in the range mentioned above. In consequence, zwitterions are essentially neutrally charged over a range of pH values; phosphatidylcholines and phosphatidylethanolamines are neutral lipids with zwitterionic character.

Suitably therefore, said mixture may comprise a plurality of charged amphiphiles which in combination with one another have amphoteric character. Preferably said one or more charged amphiphiles comprise a pH sensitive anionic lipid and a pH sensitive cationic lipid. Herein, such a combination of a chargeable cation and chargeable anion is referred to as an "amphoteric II" lipid pair. Said chargeable cation may have a pK value of between about 4 and about 8, preferably between about 5.0 or 5.5 and about 7.0 or 7.5.Said chargeable anion may have a pK value of between about 3.5 and about 7, preferably between abour 4 or 4.5 and about 6.0 or 6.5. Examples include MoChol/CHEMS, DPIM/CHEMS and DPIM/DGSucc.

An "amphoteric I" lipid pair comprises a stable cation (e.g., DDAB/CHEMS, DOTAP/CHEMS and DOTAP/DOPS) and a chargeable anion, while an "amphoteric III" lipid pair comprises a stable anion and a chargeable cation (e.g., MoChol/DOPG and MoChol/Chol-SO_4).

It is of course possible within the scope of the present invention to use amphiphiles with multiple charges such, for example, as amphipathic dicarboxylic acids, phosphatidic acid, amphipathic piperazine derivatives and the like. Such multi-charged amphiphiles may be pH sensitive amphiphiles or stable anions or cations, or they may have "mixed" character.

Suitably, said anionic lipid may be selected from DOGSucc, POGSucc, DMGSucc, DPGSucc and CHEMS.

Said cationic lipid may be selected from MoChol, HisChol and CHIM.

In yet another aspect of the present invention there are provided amphoteric liposomes comprising phosphatidylcholine and phosphatidylethanolamine in a ratio in the aforementioned range, a pH sensitive anionic lipid and a pH sensitive cationic lipid.

Said amphoteric liposomes may be negatively or neutrally charged at pH 7.4 and cationic at pH 4.

In another particular aspect of the present invention, said liposomes encapsulate at least one active agent. Said active agent may comprise a drug. In some embodiments said active agent may comprises a nucleic acid such, for example, as an oligonucleotide or DNA plasmid that is capable of being transcribed in a vertebrate cell into one or more RNAs, said RNAs being mRNAs, shRNAs, miRNAs or ribozymes, said mRNAs coding for one or more proteins or polypeptides.

Said oligonucleotide or other nucleic acid based drug may be encapsulated in said amphoteric liposomes. A substantial portion or all of said oligonucleotides may be physically entrapped in the amphoteric liposomes. The serum stable amphoteric liposomal formulations can be used for the intracellular delivery of drugs or for the prevention or treatment of a condition and/or disease in mammals or part of mammals, especially humans or their organs.

In some embodiments, said oligonucleotide may be adapted to target a nucleic acid encoding CD40, thereby to modulate expression of CD40 in mammalian cells. Suitably, said oligonucleotide may be directed against the mRNA of CD40.

In yet another aspect of the present invention there is provided a pharmaceutical composition comprising active agent-loaded amphoteric liposomes in accordance with the present invention and a pharmaceutically acceptable vehicle therefor.

Said composition may be formulated for high or low lipid doses, and suitably therefore the drug / lipid ratio may be adjusted to a desired lipid concentration. In some embodiments, said composition may further comprise empty liposomes to decrease said drug / lipid ratio, said empty liposomes having the same or similar size and composition to said active agent-loaded liposomes. Said empty liposomes may comprise a mixture of lipids according to the present invention.

In yet another aspect, the present invention comprehends the use of a pharmaceutical composition according to the present invention for the prevention or treatment of an inflammatory, immune or autoimmune disorder of a human or non-human animal, wherein said composition comprises an oligonucleotide adapted to target a nucleic acid encoding CD40 for modulating the expression of CD40 in mammalian cells.

Said composition may be formulated for systemic or local administration. When used systemically, the present invention comprises the use of said composition *inter alia* for the prevention or treatment of graft rejection, graft-versus-host disease, diabetes type I, multiple sclerosis, systemic lupus erythematosous, rheumatoid arthritis, asthma, inflammatory bowel disease, psoriasis or thyroiditis.

When formulated for local application, the invention comprises the use of said composition *inter alia* for the prevention or treatment of graft rejection, graft-versus-host disease, inflammatory bowel disease, asthma, Crohn's disease or ulcerative colitis.

Detailed description of the invention

As mentioned above, the amphoteric liposomes of the present invention may comprise anionic and cationic components, wherein both components are pH-sensitive, as disclosed in WO 02/066012.

Cationic lipids that are sensitive to pH are disclosed in WO 02/066489 and WO 03/070220, and in Budker, *et al.* 1996, Nat Biotechnol. 14(6):760-4.

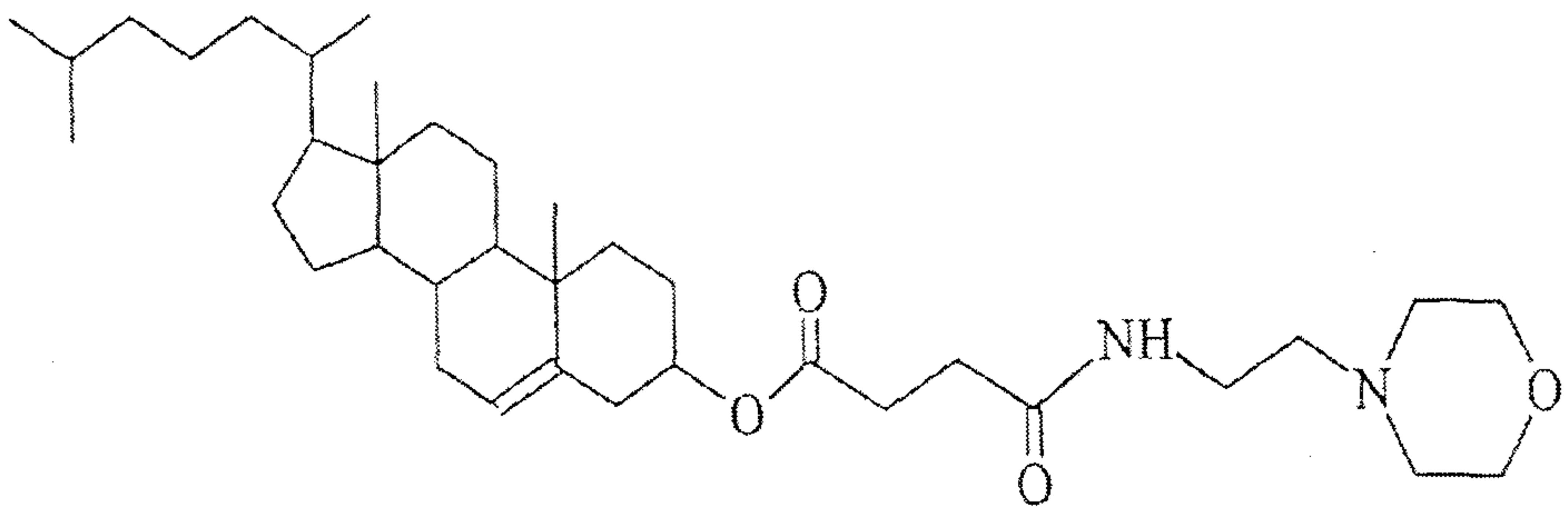
Preferred cationic components are MoChol, HisChol and CHIM, especially MoChol.

Preferred anionic lipids are selected from the group comprising: DOGSucc, POGSucc, DMGSucc, DPGSucc and CHEMS, especially DOGSucc, DMGSucc and CHEMS.

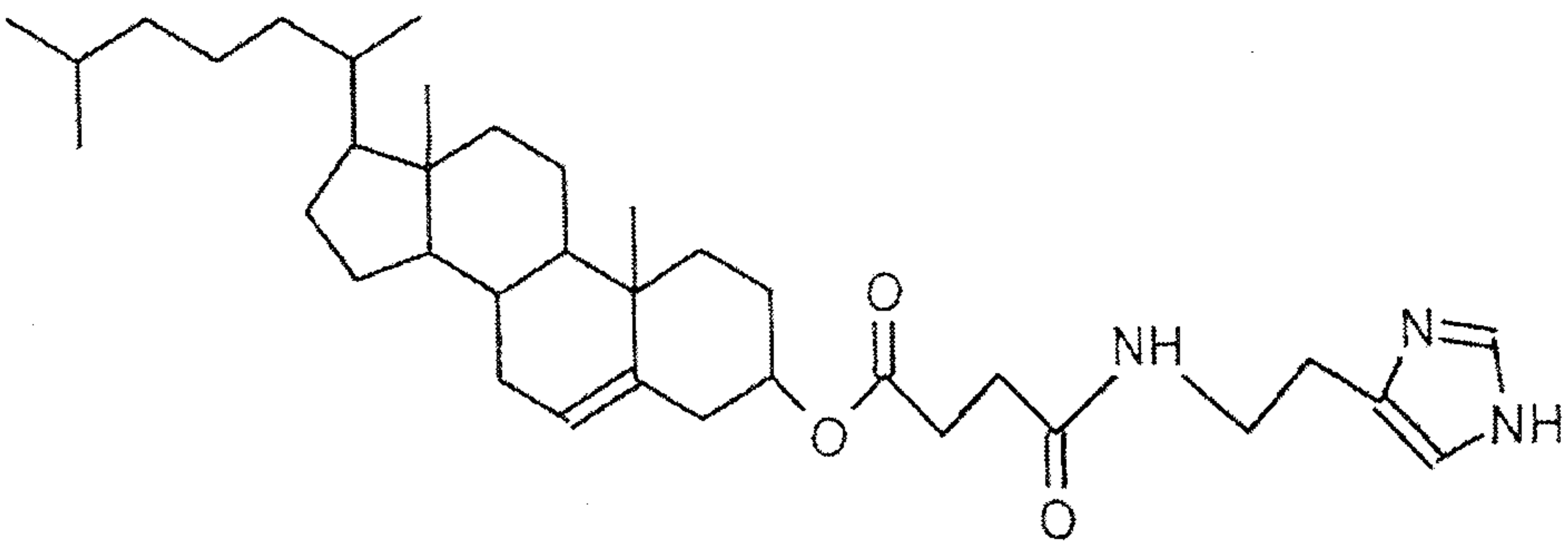
The following abbreviations for lipids are used herein, the majority of which abbreviations are in standard use in the literature:

PC	Phosphatidylcholine, unspecified membrane anchor
PE	Phosphatidylethanolamine, unspecified membrane anchor
DMPC	Dimyristoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylphosphatidylcholine
POPC	Palmitoyl-oleoylphosphatidylcholine
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DMPE	Dimyristoylphosphatidylethanolamine
DPPE	Dipalmitoylphosphatidylethanolamine
CHEMS	Cholesterolhemisuccinate
CHIM	Cholesterol-(3-imidazol-1-yl propyl)carbamate
DDAB	Dimethyldioctadecylammonium bromide
DOTAP	(1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium salt
DOPS	Dioleoylphosphatidylserine
DOPG	Dioleoylphosphatidylglycerol
Chol-SO_4	cholesterol sulfate

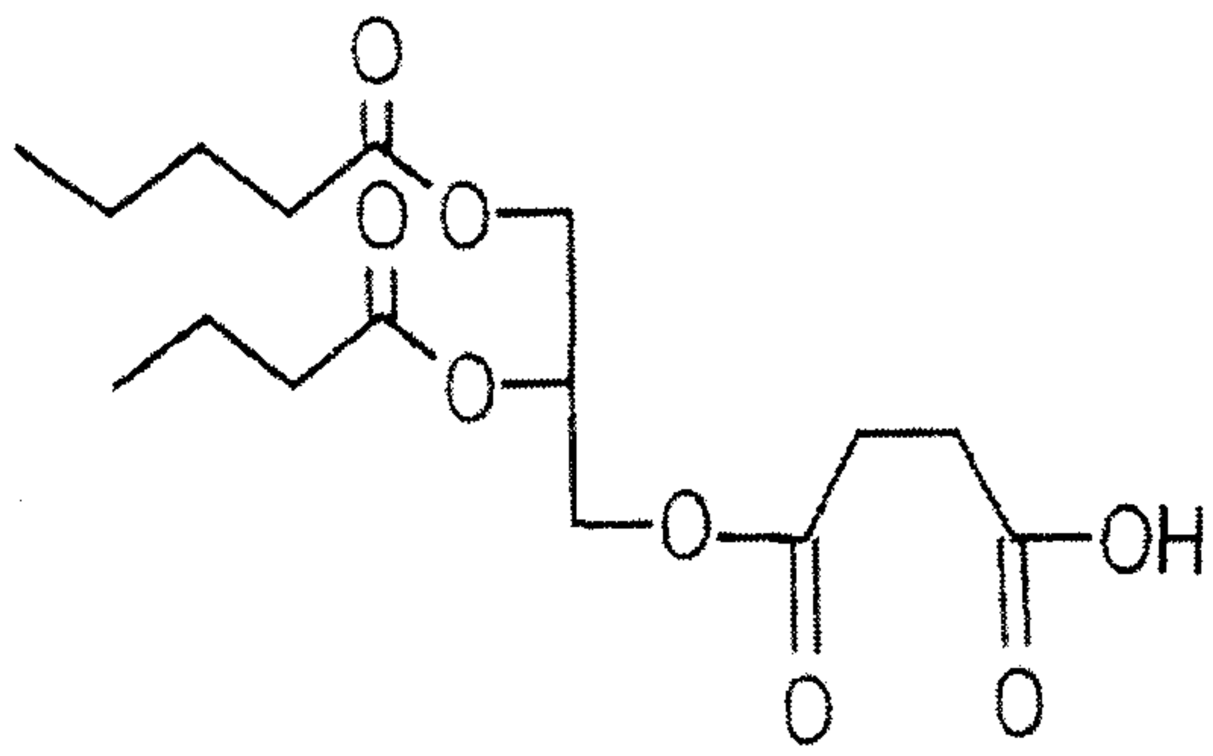
MoChol 4-(2-Aminoethyl)-Morpholino-Cholesterolhemisuccinate:



HisChol Histaminyl-Cholesterolhemisuccinate:



DGSucc 1,2-Dipalmitoyglycerol-3-hemisuccinate (& Distearoyl-, dimyristoyl- Dioleoyl or palmitoyl-oleoylderivatives) (in the structure below the acyl chain is shown schematically)



It has been found that the ratio between the cationic and anionic lipids (the charge ratio) not only determines the isoelectric point, but may also affect the serum stability of the composition. Accordingly, said charge ratio may vary from 4:1 to 1:4, preferably between 3:1 and 1:3 (cation : anion).

In some embodiments of the invention, the cation may be present in excess over the anion. Preferably said charge ratio is between 3:1 and 2:1. The total amount of charged lipids may vary from 5 to 95 mol.% of the lipid mixture, preferably from 30 to 80 mol.%, and more preferably from 45 or 50 mol.% to 75 mol.%, with the remaining lipids being formed from the neutral phospholipids PC and PE.

Alternatively, the cation and anion may be present in substantially equal amounts. The total amount of charged lipids may vary from 5 to 75 mol.% of the lipid mixture, preferably from 20 to 65 mol.%, with the remaining lipids being formed from the neutral phospholipids PC and PE.

In another alternative, the anion may be present in excess over the cation. Said charge ratio may be between 1:3 and 1:2, preferably about 1:2 (cation : anion). The total amount of charged lipids may vary from 40 mol.% to 75 or 80 mol.% of the lipid mixture, preferably from 45 or 50 mol.% to 70 or 75 mol.%, and more preferably from 55 to 65 mol.%, with the remaining lipids being formed from the neutral phospholipids PC and PE.

A number of different combinations of cations and anions may be selected from the lists of suitable components given above. Advantageously, the invention may be practised using MoChol or CHIM as a chargeable cation and CHEMS, DMGSucc or DOGSucc as a chargeable anion.

Presently preferred liposomes are made from a mixture of lipids comprising POPC and DOPE in a ratio between 1:1 and 1:4 and an amphoteric lipid pair selected from MoChol and CHEMS, MoChol and DMGSucc, MoChol and DOGSucc, CHIM and CHEMS, and CHIM and DMGSucc, in a ratio between 3:1 and 1:1, wherein the amount of charged lipids is between 30 and 80 mol.% of the lipid mixture.

Specific examples of such liposomes in accordance with the present invention include, but are not limited to:

POPC/DOPE/MoChol/CHEMS	6 : 24: 53: 17
POPC/DOPE/MoChol/CHEMS	6 : 24 : 47 : 23
POPC/DOPE/MoChol/CHEMS	15 : 45 : 20 : 20
POPC/DOPE/MoChol/CHEMS	10 : 30 : 30 : 30
POPC/DOPE/MoChol/CHEMS	24.5 : 35.5 : 20 : 20
POPC/DOPE/MoChol/CHEMS	16 : 24 : 30 : 30
POPC/DOPE/MoChol/DMGSucc	6 : 24 : 53 : 17
POPC/DOPE/MoChol/DMGSucc	6 : 24 : 47 : 23
POPC/DOPE/MoChol/DMGSucc	15 : 45 : 20 : 20
POPC/DOPE/MoChol/DMGSucc	10 : 30 : 30 : 30
POPC/DOPE/MoChol/DMGSucc	24.5 : 35.5 : 20 : 20
POPC/DOPE/MoChol/DMGSucc	16 : 24 : 30 : 30
POPC/DOPE/MoChol/DOGSucc	12.5 : 37.5 : 33 : 17
POPC/DOPE/MoChol/DOGSucc	7.5 : 22.5 : 47 : 23
POPC/DOPE/CHIM/CHEMS	12.5 : 37.5 : 33 : 17
POPC/DOPE/CHIM/CHEMS	7.5 :22.5 :47 : 23
POPC/DOPE/CHIM/DMGSucc	12.5 : 37.5 : 33 : 17
POPC/DOPE/CHIM/DMGSucc	7.5 : 22.5 : 47 : 23

Further presently preferred liposomes comprise a mixture of lipids comprising POPC and DOPE in a ratio between 1:1 and 1:4, DMGSucc or DOGSucc, and MoChol, wherein the molar amount of DMGSucc or DOGSucc exceeds the molar amount of MoChol and the amount of charged lipids is between 30 and 80 mol.%.

Preferably, the charge ratio is between 1:2 and 1:3 and charged components constitute between 45 or 50 mol.% and 70 or 75 mol.% of the lipid mixture.

Specific examples of such further liposomes include, but are not limited to:

POPC/DOPE/MoChol/DMGSucc	6 : 24 : 23 : 47
POPC/DOPE/MoChol/DMGSucc	8 : 32 : 20 : 40
POPC/DOPE/MoChol/DMGSucc	10 : 40 : 17 : 33
POPC/DOPE/MoChol/DMGSucc	10 : 20 : 23 : 47
POPC/DOPE/MoChol/DMGSucc	13 : 27 : 20 : 40
POPC/DOPE/MoChol/DMGSucc	10 : 30 : 20 : 40
POPC/DOPE/MoChol/DMGSucc	17 : 33 : 17 : 33
POPC/DOPE/MoChol/DOGSucc	12.5 : 37.5 : 17 : 33

Without being limited to such use, the materials described in the present invention are well suited for use as carriers for nucleic acid-based drugs such for example as oligonucleotides and DNA plasmids. These drugs are classified into nucleic acids that encode one or more specific sequences for proteins, polypeptides or RNAs and into oligonucleotides that can specifically regulate protein expression levels or affect the protein structure through *inter alia* interference with splicing and artificial truncation.

In some embodiments of the present invention, therefore, the nucleic acid-based therapeutic may comprise a nucleic acid that is capable of being transcribed in a vertebrate cell into one or more RNAs, which RNAs may be mRNAs, shRNAs, miRNAs or ribozymes, wherein such mRNAs code for one or more proteins or polypeptides. Such nucleic acid therapeutics may be circular DNA plasmids, linear DNA constructs, like MIDGE vectors (Minimalistic Immunogenically Defined Gene

Expression) as disclosed in WO 98/21322 or DE 19753182, or mRNAs ready for translation (e.g., EP 1392341).

In another embodiment of the invention, oligonucleotides may be used that can target existing intracellular nucleic acids or proteins. Said nucleic acids may code for a specific gene, such that said oligonucleotide is adapted to attenuate or modulate transcription, modify the processing of the transcript or otherwise interfere with the expression of the protein. The term "target nucleic acid" encompasses DNA encoding a specific gene, as well as all RNAs derived from such DNA, being pre-mRNA or mRNA. A specific hybridisation between the target nucleic acid and one or more oligonucleotides directed against such sequences may result in an inhibition or modulation of protein expression. To achieve such specific targeting, the oligonucleotide should suitably comprise a continuous stretch of nucleotides that is substantially complementary to the sequence of the target nucleic acid.

Oligonucleotides fulfilling the abovementioned criteria may be built with a number of different chemistries and topologies. Oligonucleotides may be single stranded or double stranded.

The mechanisms of action of oligonucleotides may vary and might comprise effects on *inter alia* splicing, transcription, nuclear-cytoplasmic transport and translation.

In a preferred embodiment of the invention single stranded oligonucleotides may be used, including, but not limited to, DNA-based oligonucleotides, locked nucleic acids, 2'-modified oligonucleotides and others, commonly known as antisense oligonucleotides. Backbone or base or sugar modifications may include, but are not limited to, Phosphothioate DNA (PTO), 2'O-methyl RNA (2'Ome), 2' O- methoxyethyl-RNA (2'MOE), peptide nucleic acids (PNA), N3'-P5' phosphoamidates (NP), 2'fluoroarabino nucleic acids (FANA), locked nucleic acids (LNA), Morpholine phosphoamidate (Morpholino), Cyclohexene nucleic acid (CeNA), tricyclo-DNA (tcDNA) and others. Moreover, mixed chemistries are known in the art, being constructed from more than a single nucleotide species as copolymers, block-copolymers or gapmers or in other arrangements. In addition to the aforementioned oligonucleotides, protein expression can also be inhibited using double stranded RNA molecules containing the complementary sequence motifs. Such RNA molecules are known as siRNA molecules in the art (e.g., WO 99/32619 or WO 02/055693). Again, various chemistries were adapted to this class of oligonucleotides. Also, DNA / RNA hybrid systems are known in the art.

In another embodiment of the present invention, decoy oligonucleotides can be used. These double stranded DNA molecules and chemical modifications thereof do not target nucleic acids but transcription factors. This means that decoy oligonucleotides bind sequence-specific DNA-binding proteins and interfere with the transcription (e.g. Cho-Chung, *et al.* in Curr. Opin. Mol. Ther., 1999).

In a further embodiment of the invention, oligonucleotides that may influence transcription by hybridizing under physiological conditions to the promoter region of a gene may be used. Again various chemistries may adapt to this class of oligonucleotides.

In a still further alternative of the invention, DNAzymes may be used. DNAzymes are single-stranded oligonucleotides and chemical modifications therof with enzymatic activity. Typical DNAzymes, known as the "10-23" model, are capable of cleaving single-stranded RNA at specific sites under physiological conditions. The 10-23 model of DNAzymes has a catalytic domain of 15 highly conserved deoxyribonucleotides, flanked by 2 substrate-recognition domains complementary to a target sequence on the RNA._Cleavage of the target mRNAs may result in their destruction and the DNAzymes recycle and cleave multiple substrates.

In yet another embodiment of the invention, ribozymes can be used. Ribozymes are single-stranded oligoribonucleotides and chemical modifications thereof with enzymatic activity. They can be operationally divided into two components, a conserved stem-loop structure forming the catalytic core and flanking sequences which are reverse complementary to sequences surrounding the target site in a given RNA transcript. Flanking sequences may confer specificity and may generally constitute 14-16 nt in total, extending on both sides of the target site selected.

In a still further embodiment of the invention, aptamers may be used to target proteins. Aptamers are macromolecules composed of nucleic acids, such as RNA or DNA, and chemical modifications thereof that bind tightly to a specific molecular target and are typically 15-60 nt long. The chain of nucleotides may form intramolecular interactions that fold the molecule into a complex three-dimensional shape. The shape of the aptamer allows it to bind tightly against the surface of its target molecule including but not limited to acidic proteins, basic proteins, membrane proteins, transcription factors and enzymes. Binding of aptamer molecules may influence the function of a target molecule.

All of the above-mentioned oligonucleotides may vary in length between as little as 10, preferably 15 and even more preferably 18, and 50, preferably 30 and more preferably 25, nucleotides. The fit between the oligonucleotide and the target sequence is preferably perfect with each base of the oligonucleotide forming a base pair with its complementary base on the target nucleic acid over a continuous stretch of the abovementioned number of oligonucleotides. The pair of sequences may contain one or more mismatches within the said continuous stretch of base pairs, although this is less preferred. In general, the type and chemical composition of such nucleic acids is of little impact for the performance of the inventive liposomes as vehicles be it *in vivo* or *in vitro*, and the skilled artisan may find other types of oligonucleotides or nucleic acids suitable for combination with the inventive liposomes.

In a preferred embodiment of the invention however, oligonucleotides may used that are adapted to target a nucleic acid encoding the CD40 gene, its sense or antisense strand, any exons or introns or untranslated regions thereof, thereby to modulate expression of CD40 in mammalian cells.

In another preferred embodiment of the invention, said oligonucleotides may directed against any mRNA of CD40, wherein such mRNAs include pre-mRNA and their subsequently matured forms.

Protein expression can be specifically down-regulated using oligonucleotides such, for example, as antisense, locked nucleic acids (LNA), peptide nucleic acids (PNA), morpholino nucleic acids (Morpholinos) and small interfering RNAs (siRNA) of various chemistries.

CD40 was first described by Pauli, *et al.* 1984 (Cancer Immunol. Immunotherapy 17: 173-179). The protein is primarily expressed on dendritic cells, endothelia cells and B-cells and interacts with its ligand (CD40 ligand or CD154) on T-cells. The signalling between CD40 and CD154 is crucial for the development of a humoral immune response. Overstimulation of the pathway may lead to a variety of immune-associated disorders, including graft rejection, graft-versus-host disease, multiple sclerosis, systemic lupus erythematosous, rheumatoid arthritis, asthma, inflammatory bowel disease, psoriasis and thyroiditis. CD40 over-expression might also be involved in tumour growth (Gruss, *et al.* 1997, Leuk. Lymphoma. 24(5-6): 393-422) and enhanced levels of a soluble form of CD40 were reported to be associated with Alzheimers disease (Mocali et al.2004, Exp Gerontol. 39(10):1555-61. CD40 signals into the NF-κB pathway, consequently leading to activation of the transcription factor and the eventual release of cytokines such as IL-1, TNFα and IFNγ, which in turn activate other cells, thus promoting inflammation using a positive feedback mechanism.

Inhibition of the early events in the pathway described above has been proposed as an effective strategy to inhibit immune disorders or inflammation processes. Examples include the competitive binding of TNFα using antibodies, receptor blocking using antibodies against the TNFα-receptor and competitive inhibition of NF-κB binding. Since CD40 signals through its interaction with the trimeric ligand, CD154, inhibition of the signalling event with small molecule inhibitors is unlikely and therapeutic developments have therefore focused on the use of blocking antibodies. More specifically, the CD40/CD154 interaction may be blocked using antibodies targeted against one of the components, as described by Holstager, *et al.* 2000 (J. Biol. Chem. 275:15392-15398) or Baccam & Bishop 1999 (Eur. J. Immunol. 29: 3855-3866). However, the CD40 antibodies under development give rise to side reactions, and there is therefore an need for alternative means to cut the inflammatory feedback loop at this point.

A number of oligonucleotide sequences targeted against CD40 mRNA have been validated *in vitro* so far. US 2004/0186071 and US 6197584, both to Bennett, *et al.*, for example, give a detailed description of such oligonucleotides based on antisense mechanisms. Pluvinet, *et al.* in Blood, 2004 first described the down-regulation of CD40 using siRNA against the human target. Further, WO 2004/090108 to Manoharan describes the applicability of novel oligonucleotides to inhibit the expression of CD40 protein. Indirect means to down-regulate the CD40 expression are described in DE 10049549 to Hecker and Wagner, using the inhibition of transcription factor IFR-1. Suitable specific nucleic acids for modulating the expression of CD40 are set forth in Example 11 below.

In a particular aspect of the present invention therefore there is provided a pharmaceutical composition comprising an oligonucleotide directed against CD40 as an active agent and an amphoteric liposome of the present invention as an excipient. Such formulations have been found to be therapeutically active in the treatment of inflammations and autoimmune disorders, and accordingly the invention further comprehends the use of the composition of the invention for the prevention or treatment of inflammations, immune or autoimmune disorders, including graft rejection, graft-versus-host disease, multiple sclerosis, systemic lupus erythematosous, rheumatoid arthritis, asthma, asthma bronchiale, inflammatory bowel disease, psoriasis, thyroiditis, Morbus Crohn, Colitis ulcerosa, COPD and atopic dermatitis.

The pharmaceutical composition of the present invention may also be used for topical treatments, for example the treatment of inflamed mucosa. In particular, the composition of the invention may be used for the treatment or prophylaxis of inflammatory bowel disease or graft rejection. The composition of the present invention may also be adapted for topical application to the skin or lungs.

Liposomes have been widely used to alter the pharmacokinetic and biodistribution profile of encapsulated drugs *in vivo*. The liposomes of the present invention, together with their cargo, may be cleared rapidly and to a great extent by the liver. However, the pharmacokinetic parameters as well as the biodistribution pattern may be controlled by adjusting the size of the liposomes and/or the lipid dose as illustrated in the examples below.

In some embodiments, the liposomes of the present invention may have a size greater than about 150 nm. Such liposomes may be administered at a low lipid dose. Said liposomes may be unilamellar, oligolamellar or multilamellar. Such a dosing scheme allows for effective and rapid targeting to the liver and avoids the accumulation of liposomes and drug in other organs, such as the spleen.

Alternatively, such liposomes having a size greater than about 150 nm may be administered at a high lipid dose, leading to saturation of the liver and an alteration of the biodistribution pattern to an accumulation of the liposomes in the spleen and more distal sites in the circulation, such as sites of infection or inflammation or tumours. These areas of the body have fenestrated or incomplete capillaries through which liposomes may be filtered out. Furthermore, it is known that the spleen and such other areas of infection or inflammation and many tumors often have high contents of macrophages which can remove the liposomes from the circulation.

Said pharmaceutical composition according to the present invention may be provided with a high lipid dose by different methods. In some embodiments, the drug / lipid ratio of the composition can be lowered to achieve the desired lipid concentration. Alternatively, the lipid concentration of the pharmaceutical composition may be controlled by adding empty liposomes of comparable composition and size to the drug loaded liposomes.

In some embodiments, the liposomes according to the present invention may have a size of less than about 150 nm. Said liposomes may be unilamellar, oligolamellar or multilamellar. The spleen acts as a filter which removes unwanted red blood cells and particles from the blood. Large liposomes are also retained by the reticular filter in the same way. However, small liposomes may escape and thus do not accumulate in spleen. Accordingly, liposomes according to the present invention, having a size of less than 150 nm may circumvent the spleen as an organ.

Such liposomes having a size of less than 150 nm may be administered at a low lipid dose in order to target liver cells. Such liposomes are particularly well adapted to penetrate fully the entire liver and to reach a substantial portion of the parenchymal cells of the liver such as hepatocytes.

Alternatively, said liposomes having a size of less than 150 nm may be administered at a high lipid dose to target more distal sites in the circulation, such as areas of infection or inflammation or solid tumours, and simultaneously to circumvent the spleen.

In general, the pharmacokinetic profile and the biodistribution of the liposomes of the present invention may depend upon many factors. Next to the lipid composition of the liposomes, the size and lipid dose determine the *in vivo* fate of the liposomes. The liposomes of the invention may be unilamellar, oligolamellar or multilamellar, irrespective of their size.

In some embodiments, the liposomes of the present invention may be used to target an inflamed lung by systemic administration to a human or non-human animal patient.

Starting from the data presented herein, those skilled in the art will be able to establish appropriate dosage regimens for other species, in particular for other mammals or humans. Specifically, whether a lipid dose in another species (e.g. human) is "low" or "high" can be determined by pharmacokinetic data. The pharmacokinetic of liposomes follows a two compartment model. As mentioned above, high lipid doses lead to a saturation of the liver and an alteration of the biodistribution pattern. This leads to enhanced Cmax values in the terminal part of the pharmacokinetic curve.

The pharmaceutical composition of the present invention may be formulated for use as a colloid in a suitable pharmacologically acceptable vehicle. Vehicles such as water, saline, phosphate buffered saline and the like are well known to those skilled in the art for this purpose.

In some embodiments, the composition of the present invention may be administered at a physiological pH of between about 7 and about 8. To this end, the composition comprising the active agent, excipient and vehicle may be formulated to have a pH in this range.

Methods for manufacturing liposomes are known to those skilled in the art. They include, but are not limited to, extrusion through membranes of defined pore size, injection of lipid solutions in ethanol into the water phase containing cargo or high pressure homogenisation.

Also, it is known in the art that nucleic acid therapeutics can be contacted with the lipids at neutral pH, resulting in volume inclusion of a certain percentage of the solution containing the nucleic acid. High concentrations of lipids ranging from 50 mM to 150 mM are preferred to achieve substantial encapsulation of the drug.

In contrast to such standard procedures, amphoteric liposomes offer the distinct advantage of binding nucleic acids at or below their isoelectric point, thereby concentrating the drug at the liposome surface. Such a process is described in WO 02/066012 in more detail. Upon elevating the pH of the liposomes to physiological pH (about pH 7.4) the negatively charged nucleic acids dissociate from the liposomal membrane. Irrespective of the actual production process, the non-encapsulated active drug can be removed from the liposomes after the initial production step, wherein liposomes are formed as tight containers. Again, the technical literature and the references included here describe such methodology in detail and suitable process steps may include, but are not limited to, size exclusion chromatography, sedimentation, dialysis, ultrafiltration, diafiltration and the like.

In some embodiments of the invention, more than 80 wt.% of the drug may be disposed inside said liposomes.

However, such removal of non-encapsulated material is not mandatory and in some embodiments the composition may comprises entrapped as well as free drug.

The particle size of the liposomes may be between 50 and 500 nm, preferably between 50 and 300 nm.

Following is a description by way of example only with reference to the accompanying drawings of embodiments of the present invention.

In the drawings:

Figure 1 is a graph of carboxyfluorescein (CF) release from the MoChol/CHEMS formulations of Table 1 below after incubation in full human serum for 4 hours. CF release is expressed as % of the unquenched CF signal. The x-axis shows the total amount of charged lipid at a 1:1 ratio between MoChol and CHEMS.

Figure 2 is a graph of CF release from the MoChol/DMGSucc formulations of Table 4 below after incubation in full human serum for 4 hours. CF release is expressed as % of the unquenched CF signal. The x-axis shows total amount of charged lipid at a 1:1 ratio between MoChol and DMGSucc.

Figure 3 is graph of CF release from liposomes containing MoChol/CHEMS or MoChol/DMGSucc after incubation in full human serum at 37°C. CF release is expressed as % of the unquenched CF signal. Excess cation stabilises the liposomes against serum attack. DMGSucc is notably more stable then the CHEMS counterpart.

Figure 4 is a graph of CF release from the MoChol/CHEMS and MoChol/DMGSucc formulations of Tables 3 and 6 below after incubation in full human serum at 37°C. The formulations have DOPE/POPC ratios of 2 and 4 and the ratio cationic to anionic lipids is less than 1. Release is expressed as % of the unquenched CF signal.

Figure 5 is a bar chart showing the biodistribution of the formulation POPC/DOPE/MoChol/CHEMS 15:45:20:20 having a size > 150 nm when administered at low and high lipid doses in rat liver and spleen (see Example 7 below)

Figure 6 is a bar chart showing the biodistribution of the formulation POPC/DOPE/MoChol/CHEMS 15:45:20:20 having a size < 150 nm when administered at low and high lipid doses in rat liver and spleen (see Example 7 below)

Figure 7 is a set of photographs of the limbs of sacrificed collagen-induced arthritic mice obtained by NIR-imaging and showing the biodistribution of amphoteric liposomes encapsulating Cy5.5 labelled CD40 antisense (see Example 8 below)

Figure 8 is a graph showing the effect of treatment with amphoteric liposomes containing CD40 antisense on the paw swelling of inflamed mice.

Figure 9 is a graph of the assessed clinical score of mice treated with amphoteric liposomes containing CD40 antisense.

Figure 10 is a porcine CD40 cDNA sequence (SEQ ID NO:4) for targeting in accordance with the present invention

Example 1: Preparation of carboxyfluorescein (CF) loaded liposomes with the amphoteric II lipids MoChol and CHEMS

Stock solutions of lipids in chloroform were mixed and finally evaporated in a round bottom flask to dryness under vacuum. Lipid films were hydrated with 100 mM CF in PBS pH 7.5. The resulting lipid concentration was 20 mM. The suspensions were hydrated for 45 minutes in a water bath at room temperature, sonicated for 5 minutes following by three freeze/thaw cycles at -70°C. After thawing the liposomal suspensions were extruded 15 times through polycarbonate membranes with a pore size of 100 nm. Non-encapsulated CF was removed by gel filtration, whereas the liposomes were diluted by a factor three. Lipid recovery and concentration was analysed by organic phosphate assay. Particle size was measured by dynamic light scattering on a Malvern Zetasizer 3000 HSA.

Table 1: *Variation of the ratio DOPE/POPC and the total amount of charged components*

Lipids	**Composition**
DOPE/MoChol/CHEMS	60:20:20
DOPE/MoChol/CHEMS	50:20:30
DOPE/MoChol/CHEMS	40:30:30
DOPE/MoChol/CHEMS	20:40:40
POPC/MoChol/CHEMS	60:20:20
POPC/MoChol/CHEMS	40:30:30
POPC/MoChol/CHEMS	20:40:40
POPC	100
POPC/DOPE	20:80
POPC/DOPE/MoChol/CHEMS	10:50:20:20
POPC/DOPE/MoChol/CHEMS	7:35:30:30
POPC/DOPE/MoChol/CHEMS	3:17:40:40
POPC/DOPE	25:75
POPC/DOPE/MoChol/CHEMS	15:45:20:20
POPC/DOPE/MoChol/CHEMS	10:30:30:30
POPC/DOPE/MoChol/CHEMS	5:15:40:40
POPC/DOPE	40:60
POPC/DOPE/MoChol/CHEMS	24.5:35.5:20:20
POPC/DOPE/MoChol/CHEMS	16:24:30:30
POPC/DOPE/MoChol/CHEMS	8:12:40:40
POPC/DOPE	57:43
POPC/DOPE/MoChol/CHEMS	34:26:20:20
POPC/DOPE/MoChol/CHEMS	22.8:17.2:30:30
POPC/DOPE/MoChol/CHEMS	11.4:8.6:40:40

Table 2: Variation of the ratio MoChol/CHEMS

Lipids	Composition
POPC/DOPE/MoChol/CHEMS	6:24:53:17
POPC/DOPE/MoChol/CHEMS	6:24:47:23
POPC/DOPE/MoChol/CHEMS	6:24:35:35
POPC/DOPE/MoChol/CHEMS	6:24:23:47

Table 3: Variation of ratio DOPE/POPC and the total amount of charged components

Lipids	Composition
POPC/DOPE/MoChol/CHEMS	4:16:27:53
POPC/DOPE/MoChol/CHEMS	6:24:23:47
POPC/DOPE/MoChol/CHEMS	8:32:20:40
POPC/DOPE/MoChol/CHEMS	10:40:17:33
POPC/DOPE/MoChol/CHEMS	7:13:27:53
POPC/DOPE/MoChol/CHEMS	10:20:23:47
POPC/DOPE/MoChol/CHEMS	13:26:20:40
POPC/DOPE/MoChol/CHEMS	17:33:17:33

Example 2: Preparation of carboxyfluorescein (CF) loaded liposomes with the amphoteric II lipids MoChol and DMGSucc

Liposomes were prepared as described in Example 1.

Table 4: Variation of the ratio DOPE/POPC and the total amount of charged components

Lipids	Composition
POPC/DOPE/MoChol/DMGSucc	15:45:20:20
POPC/DOPE/MoChol/DMGSucc	10:30:30:30
POPC/DOPE/MoChol/DMGSucc	5:15:40:40
POPC/DOPE/MoChol/DMGSucc	24.5:35.5:20:20

Lipids	**Composition**
POPC/DOPE/MoChol/DMGSucc	16:24:30:30
POPC/DOPE/MoChol/DMGSucc	8:12:40:40
POPC/DOPE/MoChol/DMGSucc	34:26:20:20
POPC/DOPE/MoChol/DMGSucc	22.8:17.2:30:30
POPC/DOPE/MoChol/DMGSucc	11.4:8.6:40:40

Table 5: Variation of the ratio MoChol/DMGSucc

Lipids	**Composition**
POPC/DOPE/MoChol/DMGSucc	6:24:53:17
POPC/DOPE/MoChol/DMGSucc	6:24:47:23
POPC/DOPE/MoChol/DMGSucc	6:24:35:35
POPC/DOPE/MoChol/DMGSucc	6:24:23:47

Table 6: Variation of ratio DOPE/POPC and the total amount of charged components

Lipids	**Composition**
POPC/DOPE/MoChol/DMGSucc	4:16:27:53
POPC/DOPE/MoChol/DMGSucc	6:24:23:47
POPC/DOPE/MoChol/DMGSucc	8:32:20:40
POPC/DOPE/MoChol/DMGSucc	10:40:17:33
POPC/DOPE/MoChol/DMGSucc	7:13:27:53
POPC/DOPE/MoChol/DMGSucc	10:20:23:47
POPC/DOPE/MoChol/DMGSucc	13:26:20:40
POPC/DOPE/MoChol/DMGSucc	17:33:17:33

Example 3 : Preparation of carboxyfluorescein (CF) loaded liposomes with the amphoteric II lipids MoChol and DOGSucc

Liposomes were prepared as described in Example 1.

Table 7: Variation of the ratio MoChol/DOGSucc and the total amount of charged components

Lipids	Composition	Serum stability
POPC/DOPE/MoChol/DOGSucc	12.5:37.5:17:33	+
POPC/DOPE/MoChol/DOGSucc	12.5:37.5:33:17	+
POPC/DOPE/MoChol/DOGSucc	7.5:22.5:23:47	-
POPC/DOPE/MoChol/DOGSucc	7.5:22.5:47:23	+

Example 4 : Preparation of carboxyfluorescein (CF) loaded liposomes with the amphoteric II lipids CHIM and CHEMS

Liposomes were prepared as described in Example 1.

Table 8: Variation of the ratio CHIM/CHEMS and the total amount of charged components

Lipids	Composition	Serum stability
POPC/DOPE/CHIM/CHEMS	12.5:37.5:17:33	-
POPC/DOPE/CHIM/CHEMS	12.5:37.5:33:17	+
POPC/DOPE/CHIM/CHEMS	7.5:22.5:23:47	-
POPC/DOPE/CHIM/CHEMS	7.5:22.5:47:23	+

Example 5 : Preparation of carboxyfluorescein (CF) loaded liposomes with the amphoteric II lipids CHIM and DMGSucc

Liposomes were prepared as described in Example 1.

Table 8: Variation of the ratio CHIM/DMGSucc and the total amount of charged components

Lipids	Composition	Serum stability
POPC/DOPE/CHIM/DMGSucc	12.5:37.5:17:33	-
POPC/DOPE/CHIM/DMGSucc	12.5:37.5:33:17	+
POPC/DOPE/CHIM/DMGSucc	7.5:22.5:23:47	-
POPC/DOPE/CHIM/DMGSucc	7.5:22.5:47:23	+

Example 6: Serum stability test of CF-loaded amphoteric liposomes of Examples 1 and 2

Carboxyfluorescein (CF) was used as model drug to determine the serum stability of amphoteric liposomes. As well as oligonucleotides, CF is negatively charged.

25µl of the CF-loaded liposomes were mixed with 100µl pre-warmed full human serum or PBS, respectively and incubated at 37°C. At defined time points 5 µl sample was transferred into a 96-well microtiter plate to 20 µl PBS, pH 7.5 or 20 µl 20% Triton X-100. Finally 275 µl PBS were added to each well and fluorescence intensity was measured at 475 / 530 nm.

The serum stability was observed over a period of 4 hours by determining the release of CF from the liposomes via the fluorescence measurement. The released amount of CF (in %) is measured at defined time points as well as after a treatment of the liposomes with a detergent (Triton X-100) to get a 100 % release value.

Results:

Mixtures of POPC and DOPE are stable in serum. POPC itself does not form liposomes that withstand attack from serum. In addition, DOPE does not form liposomes at all. Quite surprisingly, mixtures from both components were found to be very stable and resistant against serum attack. In this example, DOPE/POPC ratios from 0.75 to 5 were found to form stable structures with a broad optimum between 1.5 and 5 (see also Figures 1 and 2).

Charged components and neutral lipids are independent variables. Serum sensitivity for a 1:1 ratio of both MoChol/CHEMS or MoChol/DMGSucc is low to very low and stable particles are formed over a wide range of mixtures. At least 60 or 70 mol.% of total charged components was required to affect significantly the bilayer stability.

The serum stability of lipid mixtures containing 70 % of charged components (see Tables 2 and 5) is shown in Figure 3. In general, an excess of MoChol has a stabilising effect.

The formulations of Tables 3 and 6 that were tested for serum stability have DOPE and POPC in a ratio of either 2:1 or 4:1. The total amount of the charged lipids was titrated from 80 % down to 50 %. The results are shown in Figure 4.

Example 7: Biodistribution of serum stable amphoteric liposomes

Stock solutions of lipids (+/- 1 % 14C-DPPC) in chloroform were mixed and finally evaporated in a round bottom flask to dryness under vacuum. Lipid films were hydrated with 1.5 ml 3H-Inulin in PBS pH 7.5 or 5 ml PBS alone. The resulting lipid concentration was 100 mM. The suspensions were hydrated for 45 minutes in a water bath at room temperature, sonicated for 30 minutes following by three freeze/thaw cycles at -70°C. After thawing the liposomal suspensions were extruded 15 times through polycarbonate membranes with an appropriate pore size. Liposomes were separated from non-encapsulated 3H-Inulin by ultracentrifugation (twice).

Lipid recovery and concentration was analysed by organic phosphate assay and in case of radiolabelled particles, the encapsulation efficiency was measured by liquid scintillation. Particle size was measured by dynamic light scattering on a Malvern Zetasizer 3000 HSA. The resulting unlabelled and radiolabelled preparations were mixed up and diluted with PBS to the final lipid concentrations.

Formulations:

Number	Formulation	Size [nm]	Lipid [mM]	3H [kBq/ml]	14C [kBq/ml]
LD-1	POPC/DOPE/MoChol/CHEMS 15:45:20:20	229	12,3	332	52
HD-2	POPC/DOPE/MoChol/CHEMS 15:45:20:20	231	54,8	453	70
LD-3	POPC/DOPE/MoChol/CHEMS 15:45:20:20	148	10	173	53
HD-4	POPC/DOPE/MoChol/CHEMS 15:45:20:20	140	50	182	58

Biodistribution study

39 male Wistar rats (Charles River) were divided into five groups and injected intravenously via the tail vein. At specific time points blood samples (for PK) and/or tissue samples (for BD) were collected and analysed by catalytic oxidation under high temperature. Percentage of carry over between samples was determined and included into the analysis of the data set.

Study group	Formulation	Number	Animals
1	POPC/DOPE/MoChol/CHEMS 15:45:20:20	LD-1	9
2	POPC/DOPE/MoChol/CHEMS 15:45:20:20	HD-2	9
3	POPC/DOPE/MoChol/CHEMS 15:45:20:20	LD-3	9
4	POPC/DOPE/MoChol/CHEMS 15:45:20:20	HD-4	9
5	PBS	PBS	3

The results of the biodistribution study is shown in Figures 5-6 wherein biodistribution of the different liposomal formulations in liver and spleen is shown. The accumulation of the liposomes in other organs did not exceed 5 % and is therefore not shown. Figure 5 clearly demonstrates that amphoteric liposomes of the present invention having a size > 150 nm accumulate solely in the liver when administered in low lipid doses. In contrast, by administering the same liposomal formulation in a high lipid dose it could be shown that the biodistribution pattern is changed. Next to the liver the liposomes with a size > 150 nm accumulate in spleen as well.

Figure 6 shows the biodistribution of amphoteric liposomes of the present invention prepared in a size < 150 nm. Whereas the biodistribution of these liposomes administered at low lipid dose does not differ from the liposomes with a size > 150 nm, it can be demonstrated that an administration of the liposomes having a size < 150 nm in high lipid dose does not lead to an accumulation in spleen.

Example 8: Biodistribution of amphoteric liposomes encapsulating Cy5.5 labelled CD40 antisense in collagen induced arthritic mice

Stock solutions of lipids in chloroform were mixed and finally evaporated in a round bottom flask to dryness under vacuum. Lipid film was hydrated with Cy5.5 labelled CD40 antisense in 10 mM NaAc, 50 mM NaCl, pH 4.5. The resulting lipid concentration was 20 mM. The suspensions were hydrated for 45 minutes in a water bath at 50°C, sonicated for 5 minutes following by a freeze/thaw cycle at -70°C. After thawing the liposomal suspensions were extruded 19 times through 200 nm polycarbonate membranes. After the extrusion process the pH of the liposomal suspension was shifted to pH 7.5 by adding 1/10 Vol. 1M HEPES, pH 8. Non-encapsulated Cy5.5 labelled CD40 antisense was removed by high speed sedimentation (twice) and discarding the supernatant.

Lipid recovery and concentration was analysed by organic phosphate assay. Encapsulation efficiency was measured by fluorescence spectroscopy. Particle size was measured by dynamic light scattering on a Malvern Zetasizer 3000 HSA.

Empty liposomes were produced by injecting 10 Vol-% of an ethanolic lipid solution (a mixture of 15 mol.% POPC, 45 mol.% DOPE, 20 mol.% MoChol and 20 mol.% CHEMS) into 10 mM NaAc 50 mM NaCl pH 4.5. The resulting lipid concentration was 2 mM. The pH of this solution was immediately shifted with 1/10 volume 1M Hepes pH 8. To concentrate the diluted liposomes the suspension was diafiltered.

Formulation	Size [nm]	Lipid [mM]	Cargo	Encapsulation efficiency
POPC/DOPE/MoChol/CHEMS 15:45:20:20	192	19	Cy5.5 CD40-ODN	77 %
POPC/DOPE/MoChol/CHEMS 15:45:20:20	104	195	empty	---

For the biodistribution study in mice the filled and empty liposomes were mixed as follows:

200 µl Cy5.5 liposomes and 41 µl empty liposomes

DBA/1 mice were immunized by subcutaneous injections of type II collagen (200 µg/mouse) emulsified in complete Freund`s adjuvant. Mice were injected intravenously with the liposomal suspension (241 µl) at day 1 of arthritis induction (around day 21 after single immunization with collagen type II). Day one was defined as the day where the inflammation was obvious (clinical score after R.O. Williams of at least 2).

Mice were sacrificed ten hours after the injection of the liposomal suspension. Organs and paws were removed and immediately freezed in liquid nitrogen. The biodistribution of the Cy5.5 labelled CD40 antisense encapsulated in the liposomes was assessed by NIR-Imaging and compared with tissue samples of untreated mice. Specific enrichment was found for inflamed paws in mice with active disease. More specifically, accumulation of the amphoteric liposomes coincides with the highly active sites of the disease on individual paws or even toes or fingers (see Figure 7).

Example 9: Preparation of CD40-ODN-containing liposomes with the advanced loading procedure

Liposomes were produced by injecting 10 Vol-% of an ethanolic lipid solution (a mixture of 15 mol.% POPC, 45 mol.% DOPE, 20 mol.% MoChol and 20 mol.% CHEMS) into 10 mM NaAc 50 mM NaCl pH 4.5 containing 60 µg/ml of a 18 bp antisense against CD40.

The resulting lipid concentration was 2 mM. The pH of this solution was immediately shifted with 1/10 volume 1M Hepes pH 8. To concentrate the diluted liposomes the suspensions were sedimented for 2h and 5min at 65.000 rpm at 20°C in a T865 rotor (Sorvall Ultra Pro 80). Afterwards the formulation was sterile filtered through 0.45 µm.

Lipid	Mol.%	size	Polydisp. Index
POPC/DOPE/MoChol/CHEMS	15:45:20:20	178.5	0.317

Table 9: example for Smarticles formulation which encapsulate CD40 ODN

The amount of encapsulated ODN was measured by checking the optical density (OD) by 260 nm. The following amount of ODN was encapsulated in the Smarticles formulation.

Lipid	Mol.%	µg ODN/µmol lipid	Encapsulation efficacy
POPC/DOPE/MoChol/CHEMS	15:45:20:20	8,87	29,58%

Table 10: encapsulated amount of ODN in the Smarticles formulation

Example 10: therapeutic efficacy in arthritis

DBA/1 mice were immunized by subcutaneous injections of type II collagen (200 µg/mouse) emulsified in complete

Freund`s adjuvant. Treatment with Smarticles or controls was initiated at day 1 of arthritis induction (around day 21 after single immunization with collagen type II) and repeated at day 3 and 5. Day one was defined as the day where the inflammation was obvious (clinical score after R.O. Williams of at least 2).

For the treatment studies the liposomal CD40-ODN was injected intravenously into the tail vein of rats with established inflammation. Each dosage contains 4 mg CD40-ODN per kg bodyweight (encapsulated CD40-ODN).

During the experiment the swelling of paws were observed and the clinical arthritis score were determined.

As evidenced by Figures 8 and 9, there was a significant reduction of the swelling of the paws after a treatment with CD40-ODN encapsulated in the amphoteric liposomes. Also the clinical score was significant reduced after treatment with CD40-ODN encapsulated in such liposomes.

Example 11: Materials

This example provides non-limiting examples of CD40 nucleotide sequences that may be targeted by oligonucleotides that modulate the expression of CD40 and that are suitable for use in the compositions in accordance with the present invention.

Human CD40 mRNA (GenBank accession no. X60592)

Human CD40 mRNA sequence for targeting in accordance with the present invention is presented in SEQ ID NO: 1. Related sequence information is found in published patent application number US 2004/0186071 (i.e., SEQ ID NO: 85) to Bennett, et al. and in US patent no. 6197584 (i.e., SEQ ID NO: 85) to Bennett, *et al.* and in Pluvinet, *et al.*, Blood, 2004, 104(12), 3642-3646.

(SEQ ID NO: 1):

1 gcctcgctcg ggcgcccagt ggtcctgccg cctggtctca cctcgccatg gttcgtctgc
61 ctctgcagtg cgtcctctgg ggctgcttgc tgaccgctgt ccatccagaa ccacccactg
121 catgcagaga aaaacagtac ctaataaaca gtcagtgctg ttctttgtgc cagccaggac
181 agaaactggt gagtgactgc acagagttca ctgaaacgga atgccttcct tgcggtgaaa
241 gcgaattcct agacacctgg aacagagaga cacactgcca ccagcacaaa tactgcgacc
301 ccaacctagg gcttcgggtc cagcagaagg gcacctcaga aacagacacc atctgcacct
361 gtgaagaagg ctggcactgt acgagtgagg cctgtgagag ctgtgtcctg caccgctcat
421 gctcgcccgg ctttggggtc aagcagattg ctacaggggt ttctgatacc atctgcgagc
481 cctgcccagt cggcttcttc tccaatgtgt catctgcttt cgaaaaatgt caccсttgga
541 caagctgtga gaccaaagac ctggttgtgc aacaggcagg cacaaacaag actgatgttg
601 tctgtggtcc ccaggatcgg ctgagagccc tggtggtgat ccccatcatc ttcgggatcc
661 tgtttgccat cctcttggtg ctggtcttta tcaaaaaggt ggccaagaag ccaaccaata
721 aggcccccca ccccaagcag gaaccccagg agatcaattt tcccgacgat cttcctggct
781 ccaacactgc tgctccagtg caggagactt tacatggatg ccaaccggtc acccaggagg
841 atggcaaaga gagtcgcatc tcagtgcagg agagacagtg aggctgcacc cacccaggag
901 tgtggccacg tgggcaaaca ggcagttggc cagagagcct ggtgctgctg ctgcaggggt
961 gcaggcagaa gcggggagct atgcccagtc agtgccagcc cctc

Mus musculus CD40 mRNA

Murine CD40 mRNA sequence for targeting in accordance with the present invention is presented in SEQ ID NO: 2. Related sequence information is found in published patent application number US 2004/0186071 (i.e. SEQ ID NO: 132) to Bennett, et al.

(SEQ ID NO: 2):

```
gcctcctggc ccttcagctg tggtctttcc cgttttctga ctttgcggtg acactgggga    60
cttccttaga cctctctgga gacgctttcg gttctgcaga gattcccagg ggtattgtgg   120
gtggggtggg gtaacaatag tgtccctgtg gcgctcccag tccctatagt aatccttcac   180
ccctctgcta tcttgcaatc aggagagtcc ttagccctgc tataggtggc ttttgaggtc   240
ctggatgcga ggaggggac tgggggtgg gtcgggtaat gtaagaaaag ggctcctttt     300
gggaccctgg ctcctccagc caccttggtg cccatccctt aaactcttgg ggacaatcag   360
actcctggga aggtcctggg gaaatccctg ctcagtgact agccataggc ccaccgcgat   420
tggtgcccga agaccccgcc ctcttcctgg gcgggactcc tagcagggac tttggagtga   480
cttgtggctt cagcaggagc cctgtgattt ggctcttctg atctcgccct gcgatggtgt   540
ctttgcctcg gctgtgcgcg ctatggggct gcttgttgac agcggtgagt ggcttgtgtt   600
ctaacctcca agggagttag ggcttagaga gtgagagatg gaaagaggaa agaggagaca   660
agactttgga gatgagagat cttcctactg gaagcggcgg ttagtaggat gggcaagatc   720
tctcgcgtct tgacacacac acacacacac acaaatgagg tgggctgctc ctctttcctt   780
ccagaaggtc ggggttctgt tccacgaagc ccacagggaa ccttagggag ggcattcctc   840
cacagcggtg cctggacagc tttgtctgac ccaagccttg ctccggagct gactgcagag   900
actggaaagg gttagcagac aggaagcctg gctggggg                           938
```

Rat CD40 mRNA (GenBank accession no. AF 241231)

Rat CD40 mRNA sequence for targeting in accordance with the present invention is presented in SEQ ID NO: 3. (See, Gao, Ph.D. thesis, Goettingen 2003).

(SEQ ID NO: 3):

```
1   tgggacccct gtgatctggc tgctctgatc tcgctctgca atgctgcctt tgcctcagct
61  gtgcgcgctc tggggctgct tgttgacagc ggtccatcta ggacagtgtg ttacgtgcag
121 tgacaaacag tacctccaag gtggcgagtg ctgcgatttg tgccagccgg gaaaccgact
181 agttagccac tgcacagctc ttgagaagac ccaatgccaa ccgtgcgact caggcgaatt
241 ctcagctcac tggaacaggg agatccgctg ccaccagcac cgacactgcg aactcaatca
301 agggcttcag gttaagaagg agggcaccgc ggtntcagac actgtttgta cctgcaagga
361 agggcagcac tgcgccagca aggagtgcga gacgtgcgct cagcacaggc cctgtggccc
421 tggctttgga gtcgtgcaga tggccactga gactactgat accgtctgcc aaccctgccc
481 ggtcggattc ttctccaatg ggtcatcact tttgaaaag tgtcatccat ggacaagctg
541 tgaagat
```

Porcine CD40 cDNA

Porcine CD40 cDNA sequence for targeting in accordance with the present invention is presented in SEQ ID NO: 4. (FIG. 10). Related sequence information is found in Rushworth, et al., *Transplantation*, 2002, 73(4), 635-642.

In addition, the following provide non-limiting examples of anti-CD40 oligonucleotides, e.g., antisense CD40 nucleic acid sequences, that are suitable for use in the present invention:

Oligonucleotides against human CD40

Examples of human antisense CD40 oligonucleotides are presented below. Further sequence information is found in published patent application number US 2004/0186071 and US Patent No. 6197584 to Bennett, *et al.*, the contents of which are provided by reference herein. The SEQ ID NOS. referred to by Bennett, *et al.* are provided to the right.

SEQ ID NO:	5	ccaggcggca ggaccact	Seq ID No: 1	of Bennett et al.
SEQ ID NO:	6	gaccaggcgg caggacca	Seq ID No.:2	of Bennett et al.
SEQ ID NO:	7	aggtgagacc aggcggca	Seq ID No: 3	of Bennett et al.
SEQ ID NO:	8	gcagaggcag acgaacca	Seq ID No: 5	of Bennett et al.
SEQ ID NO:	9	gcaagcagcc ccagagga	Seq ID No: 6	of Bennett et al.
SEQ ID NO:	10	ggtcagcaag cagcccca	Seq ID No.:7	of Bennett et al.
SEQ ID NO:	11	gacagcggtc agcaagca	Seq ID No: 8	of Bennett et al.
SEQ ID NO:	12	gatggacagc ggtcagca	Seq ID No: 9	of Bennett et al.
SEQ ID NO:	13	tctggatgga cagcggtc	Seq ID No.:10	of Bennett et al.
SEQ ID NO:	14	ggtggttctg gatggaca	Seq ID No: 11	of Bennett et al.
SEQ ID NO:	15	gtgggtggtt ctggatgg	Seq ID No: 12	of Bennett et al.
SEQ ID NO:	16	gcagtgggtg gttctgga	Seq ID No: 13	of Bennett et al.
SEQ ID NO:	17	ctggcacaaa gaacagca	Seq ID No: 15	of Bennett et al.
SEQ ID NO:	18	gtgcagtcac tcaccagt	Seq ID No: 20	of Bennett et al.

SEQ ID NO:	19	attccgtttc agtgaact	Seq ID No: 23	of Bennett et al.
SEQ ID NO:	20	ttcaccgcaa ggaaggca	Seq ID No: 25	of Bennett et al.
SEQ ID NO:	21	ctctgttcca ggtgtcta	Seq ID No: 26	of Bennett et al.
SEQ ID NO:	22	ctggtggcag tgtgtctc	Seq ID No: 27	of Bennett et al.
SEQ ID NO:	23	ggtgcccttc tgctggac	Seq ID No: 31	of Bennett et al.
SEQ ID NO:	24	ctgaggtgcc cttctgct	Seq ID No: 32	of Bennett et al.
SEQ ID NO:	25	gtgtctgttt ctgaggtg	Seq ID No: 33	of Bennett et al.
SEQ ID NO:	26	acaggtgcag atggtgtc	Seq ID No: 35	of Bennett et al.
SEQ ID NO:	27	gtgccagcct tcttcaca	Seq ID No: 37	of Bennett et al.
SEQ ID NO:	28	tgcaggacac agctctca	Seq ID No: 40	of Bennett et al.
SEQ ID NO:	29	gagcggtgca ggacacag	Seq ID No: 41	of Bennett et al.
SEQ ID NO:	30	aatctgcttg accccaaa	Seq ID No: 43	of Bennett et al.
SEQ ID NO:	31	gctcgcagat ggtatcag	Seq ID No: 46	of Bennett et al.
SEQ ID NO:	32	gcagggctcg cagatggt	Seq ID No: 47	of Bennett et al.
SEQ ID NO:	33	gactgggcag ggctcgca	Seq ID No: 49	of Bennett et al.
SEQ ID NO:	34	gcagatgaca cattggag	Seq ID No: 52	of Bennett et al.
SEQ ID NO:	35	tcgaaagcag atgacaca	Seq ID No: 53	of Bennett et al.
SEQ ID NO:	36	gtccaagggt gacatttt	Seq ID No: 54	of Bennett et al.
SEQ ID NO:	37	caggtctttg gtctcaca	Seq ID No: 57	of Bennett et al.
SEQ ID NO:	38	ctgttgcaca accaggtc	Seq ID No: 58	of Bennett et al.
SEQ ID NO:	39	gtttgtgcct gcctgttg	Seq ID No: 59	of Bennett et al.
SEQ ID NO:	40	gtcttgtttg tgcctgcc	Seq ID No: 60	of Bennett et al.
SEQ ID NO:	41	caccaccagg gctctcag	Seq ID No: 64	of Bennett et al.
SEQ ID NO:	42	gggatcacca ccagggct	Seq ID No: 65	of Bennett et al.
SEQ ID NO:	43	gtcgggaaaa ttgatctc	Seq ID No: 71	of Bennett et al.
SEQ ID NO:	44	ggagccagga agatcgtc	Seq ID No: 73	of Bennett et al.
SEQ ID NO:	45	tggagccagg aagatcgt	Seq ID No: 74	of Bennett et al.
SEQ ID NO:	46	tggcatccat gtaaagtc	Seq ID No: 77	of Bennett et al.
SEQ ID NO:	47	ggtgcagcct cactgtct	Seq ID No: 81	of Bennett et al.
SEQ ID NO:	48	aactgcctgt ttgcccac	Seq ID No: 82	of Bennett et al.

The following siRNA sequences are suitable for use in the present invention. (See, e.g., Pluvinet, *et al.*, *Blood*, 2004, 104(12), 3642-3646).

(SEQ ID NO: 49):

5_-GCGAAUUCCUAGACACCUGUU-3_ (siRNA-2 of Pluvinet et al.)

3_-UUCGCUUAAGGAUCUGUGGAC-5_

(SEQ ID NO: 50):

5_-CUGGUGAGUGACUGCACAGUU-3_ (siRNA-6 of Pluvinet et al.)

3_-UUGACCACUCACUGACGUGUC-5_

(SEQ ID NO: 51):

5_-UACUGCGACCCCAACCUAGUU-3_ (siRNA-8 of Pluvinet et al.)

3_-UUAUGACGCUGGGGUUGGAUC-5_

All siRNA contain a 2 nucleotide overhang at 3'ends.

Oligonucleotides against murine CD40

Examples of murine antisense CD40 oligonucleotides are presented below. Further sequence information is found in published patent application number US 2004/0186071 to Bennett, et al.

The SEQ ID NOS. referred to by Bennett, *et al.* are provided to the right.

Murine

SEQ ID NO:	52	agacaccatc gcag	Seq. ID No. 116	of Bennett et al.
SEQ ID NO:	53	gcgagatcag aagag	Seq. ID No. 117	of Bennett et al.
SEQ ID NO:	54	cgctgtcaac aagca	Seq. ID No. 118	of Bennett et al.
SEQ ID NO:	55	ctgccctaga tggac	Seq. ID No. 119	of Bennett et al.
SEQ ID NO:	56	ctggctggca caaat	Seq. ID No. 120	of Bennett et al.
SEQ ID NO:	57	cttgtccagg gataa	Seq. ID No. 123	of Bennett et al.
SEQ ID NO:	58	cacagatgac attag	Seq. ID No. 124	of Bennett et al.
SEQ ID NO:	59	tgatatagag aaaca	Seq. ID No. 125	of Bennett et al.

SEQ ID NO:	60	ctcattatcc tttgg	Seq. ID No. 127	of Bennett et al.
SEQ ID NO:	61	ggttcagacc agg	Seq. ID No. 128	of Bennett et al.
SEQ ID NO:	62	tttatttagc cagta	Seq. ID No. 130	of Bennett et al.
SEQ ID NO:	63	agccccacgc actgg	Seq. ID No. 131	of Bennett et al.
SEQ ID NO:	64	tctcactcct atcccagt	Seq. ID No. 134	of Bennett et al.
SEQ ID NO:	65	attagtctga ctcgt	Seq. ID No. 138	of Bennett et al.
SEQ ID NO:	66	acattagtct gactc	Seq. ID No. 139	of Bennett et al.
SEQ ID NO:	67	cagatgacat tagtc	Seq. ID No. 142	of Bennett et al.
SEQ ID NO:	68	ctggactcac cacag	Seq. ID No. 143	of Bennett et al.
SEQ ID NO:	69	ggactcacca cagat	Seq. ID No. 144	of Bennett et al.
SEQ ID NO:	70	actcaccaca gatga	Seq. ID No. 145	of Bennett et al.
SEQ ID NO:	71	tcaccacaga tgaca	Seq. ID No. 146	of Bennett et al.
SEQ ID NO:	72	accacagatg acatt	Seq. ID No. 147	of Bennett et al.
SEQ ID NO:	73	agatgacatt ag	Seq. ID No. 153	of Bennett et al.
SEQ ID NO:	74	cagatgacat tag	Seq. ID No. 154	of Bennett et al.
SEQ ID NO:	75	acagatgaca ttag	Seq. ID No. 155	of Bennett et al.
SEQ ID NO:	76	ccacagatga cattag	Seq. ID No. 156	of Bennett et al.
SEQ ID NO:	77	accacagatg acattag	Seq. ID No. 157	of Bennett et al.
SEQ ID NO:	78	caccacagat gacattag	Seq. ID No. 158	of Bennett et al.
SEQ ID NO:	79	tcaccacaga tgacattag	Seq. ID No. 159	of Bennett et al.
SEQ ID NO:	80	ctcaccacag atgacattag	Seq. ID No. 160	of Bennett et al.

Oligonucleotides against rat CD40

Examples of rat antisense CD40 oligonucleotides are presented below. (See, Gao, Ph.D. thesis, 2003, University of Göttingen, Germany).

SEQ ID NO:	81	accgctgtcaacaagcagc	(rAS2 of Gao)
SEQ ID NO:	82	tcctagatggaccgctgt	(rAS3 of Gao)
SEQ ID NO:	83	taacacactgtcctag	(rAS4 of Gao)

Oligonucleotides against porcine CD40

Examples of porcine antisense CD40 oligonucleotides are presented below. See, Rushworth, *et al.*, Transplantation, 2002, 73(4), 635-642.

SEQ ID NO: 84	gctgatgacagtgtttct	(Aso3 of Rushworth et al.)
SEQ ID NO: 85	gcctcactctcgctcctg	(Aso8 of Rushworth et al.)
SEQ ID NO: 86	ggactgtatctggactgc	(Aso9 of Rushworth et al.)
SEQ ID NO: 87	gtggacagtcatgtatat	(Aso10 of Rushworth et al.)

The present invention therefore provides formulations of amphoteric liposomes that exhibit improved stability upon contact with mammalian serum, releasing less or no encapsulated drugs. Such liposomal formulations may be useful in the delivery of drugs after a systemic administration into the blood stream. The invention especially suits the delivery of oligonucleotides, a new class of drugs that is currently under development, and DNA plasmids, without being limited to such uses. The majority of such compounds have an intracellular site of action. Carrier systems are used to overcome the poor uptake of such substances and are sometimes an indispensable prerequisite.

Claims

1. A mixture of lipids capable of encapsulating an active agent to form a liposome, said mixture comprising phosphatidylcholine and phosphatidylethanolamine in a ratio of phosphatidylethanolamine to phosphatidylcholine in the range of about 0.5 to about 8.

2. A mixture as claimed in claim 1, wherein said ratio is in the range of about 0.75 to about 5.

3. A mixture as claimed in claim 1, wherein said ratio is in the range of about 1 to about 4.

4. A mixture as claimed in claim 1, claim 2 or claim 3, wherein said phosphatidylcholine is selected from DMPC, DPPC, DSPC, POPC, DOPC, soy bean PC or egg PC.

5. A mixture as claimed in any of claims 1 to 4, wherein said phosphatidylethanolamine is selected from DOPE or DMPE or DPPE.

6. A mixture as claimed in any preceding claim, wherein said mixture is neutral.

7. Neutral liposomes comprising a mixture of lipids as claimed in any preceding claim.

8. A mixture as claimed in any of claims 1 to 6, further comprising one or more charged amphiphiles.

9. A mixture as claimed in claim 8, wherein said one or more charged amphiphiles are amphoteric, being negatively charged or neutral at pH 7.4 and positively charged at pH 4.

10. A mixture as claimed in claim 9, wherein said mixture comprises a plurality of charged amphiphiles which in combination with one another have amphoteric character.

11. A mixture as claimed in claim 10, wherein said one or more charged amphiphiles comprise at least one pH sensitive anionic lipid and at least one pH sensitive cationic lipids.

12. A mixture as claimed in claim 11, wherein said anionic lipid is selected from DOGSucc, POGSucc, DMGSucc, DPGSucc and CHEMS.

13. A mixture as claimed in claim 11 or claim 12, wherein said cationic lipid is selected from MoChol, HisChol and CHIM.

14. A mixture as claimed in claim 11, claim 12 or claim 13, wherein the ratio between the cationic and the anionic lipids (the charge ratio) is in the range of 4:1 to 1:4.

15. A mixture as claimed in any of claims 11 to 14, wherein the ratio of cationic lipids to anionic lipids is in the range of 3:1 to 2:1, and said mixture comprises 5 to 95 mol.% charged lipids and 95 to 5 mol% phosphatidylcholine and phosphatidylethanolamine.

16. A mixture as claimed in any of claims 11 to 14, wherein the ratio of cationic lipids to anionic lipids is about 1:1, and said mixture comprises 5 to 75 mol.% charged lipids and 95 to 25 mol% phosphatidylcholine and phosphatidylethanolamine.

17. A mixture as claimed in any of claims 11 to 14, wherein the ratio of cationic lipids to anionic lipids is in the range of 1:3 to 1:2, and said mixture comprises 40 to 75 mol.% charged lipids and 60 to 25 mol% phosphatidylcholine and phosphatidylethanolamine.

18. A mixture as claimed in any of claims 10 to 14, wherein said mixture comprises:

70 to 20 mol.% of POPC and DOPE in a ratio in the range of 1:1 to 1:4; and

30 and 80 mol.% of an amphoteric pair of charged lipids, said pair being selected from MoChol and CHEMS, MoChol and

DMGSucc, MoChol and DOGSucc, CHIM and CHEMS or CHIM and DMGSucc, the ratio of cationic to anionic lipid being in the range of 3:1 to 1:1.

19. A mixture as claimed in claim 18, wherein said mixture consists of a formulation selected from:

POPC/DOPE/MoChol/CHEMS	6:24:47:23 (mol.%)
POPC/DOPE/MoChol/CHEMS	15:45:20:20 (mol.%)
POPC/DOPE/MoChol/CHEMS	10:30:30:30 (mol.%)
POPC/DOPE/MoChol/DMGSucc	6:24:47:23 (mol.%)
POPC/DOPE/MoChol/DMGSucc	16:24:30:30 (mol.%)

20. A mixture as claimed in any of claims 11 to 14 or 17, wherein said mixture comprises:

70 to 20 mol.% of POPC and DOPE in a ratio in the range of 1:1 to 1:4; and

30 and 80 mol.% of MoChol and DMGSucc or DOGSucc, wherein the molar amount of DMGSucc or DOGSucc exceeds the molar amount of MoChol.

21. A mixture as claimed in claim 20, wherein the ratio of cationic to anionic lipid is in the range of 1:3 to 1:2, and said mixture comprises 30 to 50 mol.% POPC and DOPE and 70 to 50 mol.% charged lipids.

22. A mixture as claimed in claim 21, wherein said mixture consists of a formulation selected from:

POPC/DOPE/MoChol/DMGSucc	6:24:23:47 (mol.%)
POPC/DOPE/MoChol/DMGSucc	10:30:20:40 (mol.%)

23. Amphoteric liposomes comprising a mixture of lipids as claimed in any of claims 9 to 22.

24. Amphoteric liposomes as claimed in claim 23, wherein said liposomes have a size in the range of 50 to 500nm.

25. Amphoteric liposomes as claimed in claim 23 or claim 24, wherein said liposomes encapsulate at least one active agent.

26. Amphoteric liposomes as claimed in claim 25, wherein said active agent comprises a nucleic acid that is capable of being transcribed in a vertebrate cell into one or more RNAs, said RNAs being mRNAs, shRNAs, miRNAs or ribozymes, said mRNAs coding for one or more proteins or polypeptides.

27. Amphoteric liposomes as claimed in claim 26, wherein said nucleic acid is a circular DNA plasmid, a linear DNA construct or an mRNA.

28. Amphoteric liposomes as claimed in claim 25, wherein said active agent is an oligonucleotide.

29. Amphoteric liposomes as claimed in claim 28, wherein said oligonucleotide is a decoy oligonucleotide, an antisense oligonucleotide, a siRNA, an agent influencing transcription, an agent influencing splicing, Ribozymes, DNAzymes or Aptamers.

30. Amphoteric liposomes as claimed in claim 28 or claim 29, wherein said oligonucleotides comprise modified nucleosides such as DNA, RNA, locked nucleic acids (LNA), peptide nucleic acids (PNA), 2'O-methyl RNA (2'Ome), 2' O-methoxyethyl RNA (2'MOE) in their phosphate or phosphothioate forms.

31. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is an antisense oligonucleotide of 15 to 30 basepairs length.

32. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is a siRNA of 15 to 30 basepairs length.

33. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is a decoy oligonucleotide of 15 to 3C basepairs length.

34. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is an agent influencing the transcription of 15 to 30 basepairs length.

35. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is a DNAzyme of 25 to 50 basepairs length.

36. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is a Ribozyme of 25 to 50 basepairs length.

37. Amphoteric liposomes as claimed in claim 28, claim 29 or claim 30, wherein said oligonucleotide is a Aptamer of 15 to 60 basepairs length.

38. Amphoteric liposomes as claimed in any of claims 28 to 37, wherein said oligonucleotide is adapted to target a nucleic acid encoding CD40 gene, its sense or antisense strand, any exons or introns or untranslated regions thereof thereby to modulate expression of CD40 in mammalian cells.

39. Amphoteric liposomes as claimed in claim 38, wherein said oligonucleotide is directed against any mRNA of CD40, wherein such mRNAs include pre-mRNA and their subsequently matured forms.

40. Amphoteric liposomes as claimed in claim 38 or claim 39, wherein said mixture of lipids consists of a formulation selected from:

POPC/DOPE/MoChol/CHEMS	6:24:47:23 (mol.%)
POPC/DOPE/MoChol/CHEMS	15:45:20:20 (mol.%)

POPC/DOPE/MoChol/CHEMS	10:30:30:30 (mol.%)
POPC/DOPE/MoChol/DMGSucc	6:24:47:23 (mol.%)
POPC/DOPE/MoChol/DMGSucc	16:24:30:30 (mol.%)
POPC/DOPE/MoChol/DMGSucc	6:24:23:47 (mol.%)
POPC/DOPE/MoChol/DMGSucc	10:30:20:40 (mol.%)

41. Amphoteric liposomes as claimed in any of claims 28 to 40, wherein at least 80 wt.% of said oligonucleotide is disposed inside said liposomes.

42. Amphoteric liposomes as claimed in any of claims 28 to 41, wherein said liposomes comprise non-encapsulated oligonucleotides.

43. A pharmaceutical composition comprising active agent-loaded amphoteric liposomes as claimed in any of claims 25 to 42 and a pharmaceutically acceptable vehicle therefor.

44. A pharmaceutical composition as claimed in claim 43, wherein said liposomes have a size of greater than about 150 nm.

45. A pharmaceutical composition as claimed in claim 43, wherein said liposomes have a size of less than about 150 nm.

46. A pharmaceutical composition as claimed in claim 43, claim 44 or claim 45, said composition further comprising empty liposomes having a similar composition and size to said active agent-loaded amphoteric liposomes.

47. Use of amphoteric liposomes as claimed in any of claims 38, or 39 to 42 when dependent upon claim38, for the prevention or treatment of an inflammatory, immune or autoimmune disorder of a human or non-human animal.

48. Use of amphoteric liposomes as claimed in any of claims 38, or 39 to 42 when dependent upon claim 38, for the prevention or treatment of graft rejection, graft-versus-host disease,

diabetes type I, multiple sclerosis, systemic lupus erythematosous, rheumatoid arthritis, asthma, inflammatory bowel disease, psoriasis or thyroiditis, wherein said amphoteric liposomes are formulated for systemic administration.

49. Use of amphoteric liposomes as claimed in any of claims38, or 39 to 42 when dependent upon claim 38, for the prevention or treatment for the prevention or treatment of graft rejection, graft-versus-host disease, inflammatory bowel disease, asthma, Crohn's disease or ulcerative colitis, wherein said amphoteric liposomes is formulated for local administration.

50. A method of treating a human or non-human animal by administering systemically thereto at a low lipid dose a pharmaceutical composition as claimed in any of claims 43 to46, wherein said liposomes have a size of greater than 150 nm, thereby targeting said active agent to the liver.

51. A method of treating a human or non-human animal by administering systemically thereto at a high lipid dose a pharmaceutical composition as claimed in any of claims 43 to46, wherein said liposomes have a size of greater than 150 nm, thereby targeting said active agent to the spleen, sites of infections and inflammations or solid tumours.

52. A method of treating a human or non-human animal by administering systemically thereto at a low lipid dose a pharmaceutical composition as claimed in any of claims 43 to46, wherein said liposomes have a size of less than 150 nm, thereby targeting the active agent to the liver.

53. A method of treating a human or non-human animal by administering systemically thereto at a high lipid dose a pharmaceutical composition as claimed in any of claims 43 to46, wherein said liposomes have a size of less than 150 nm, thereby

targeting the active agent to sites of infections and inflammations or solid tumours, excluding the spleen

54. A method as claimed in claim 51 or claim 53, further comprising lowering the drug / lipid ratio to the desired lipid concentration.

55. A method as claimed in claim 51 or claim 53, further comprising including in said composition empty liposomes having a similar size and composition to said active agent-loaded liposomes.

Figure 1:

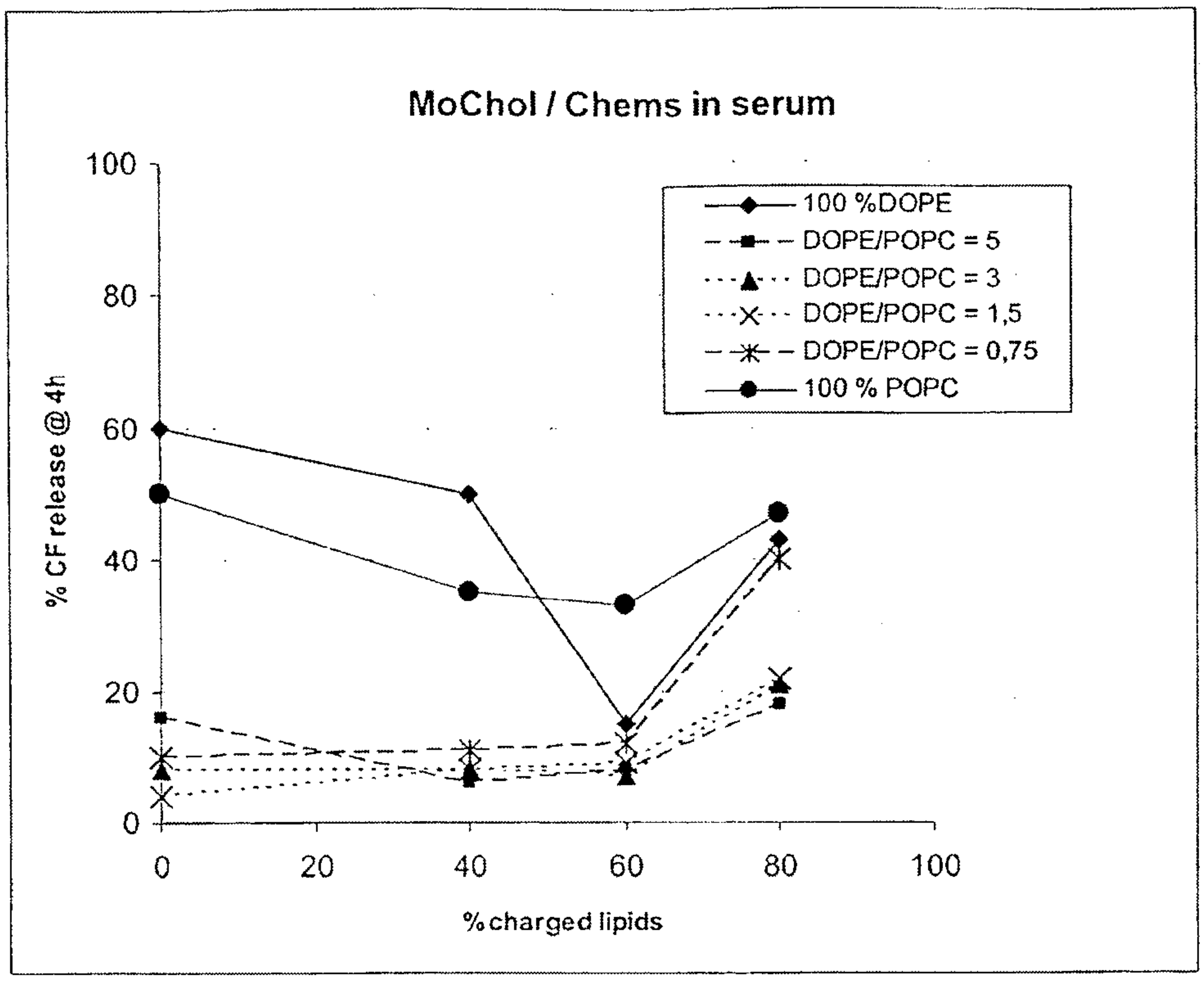


Figure 2:

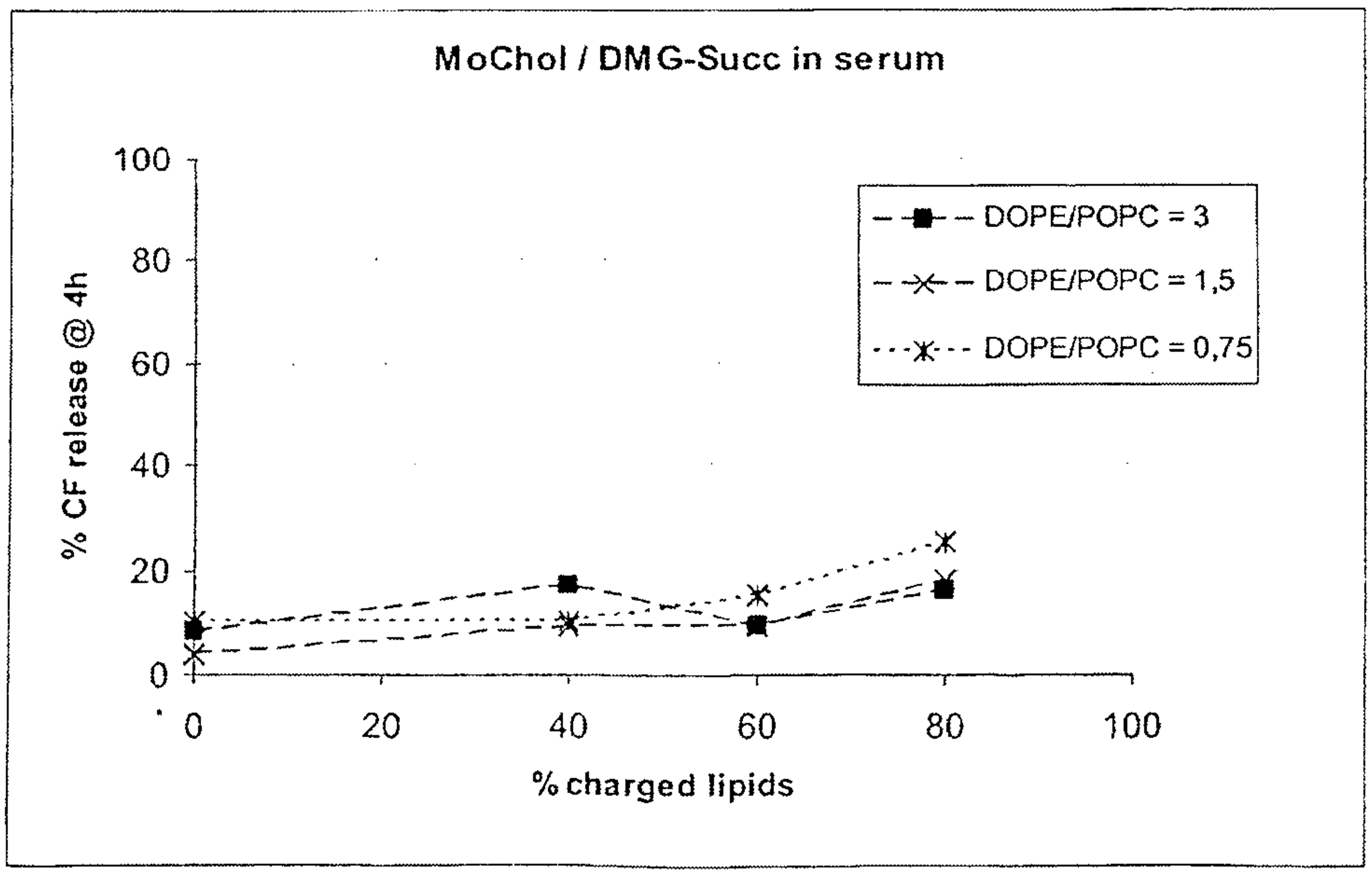
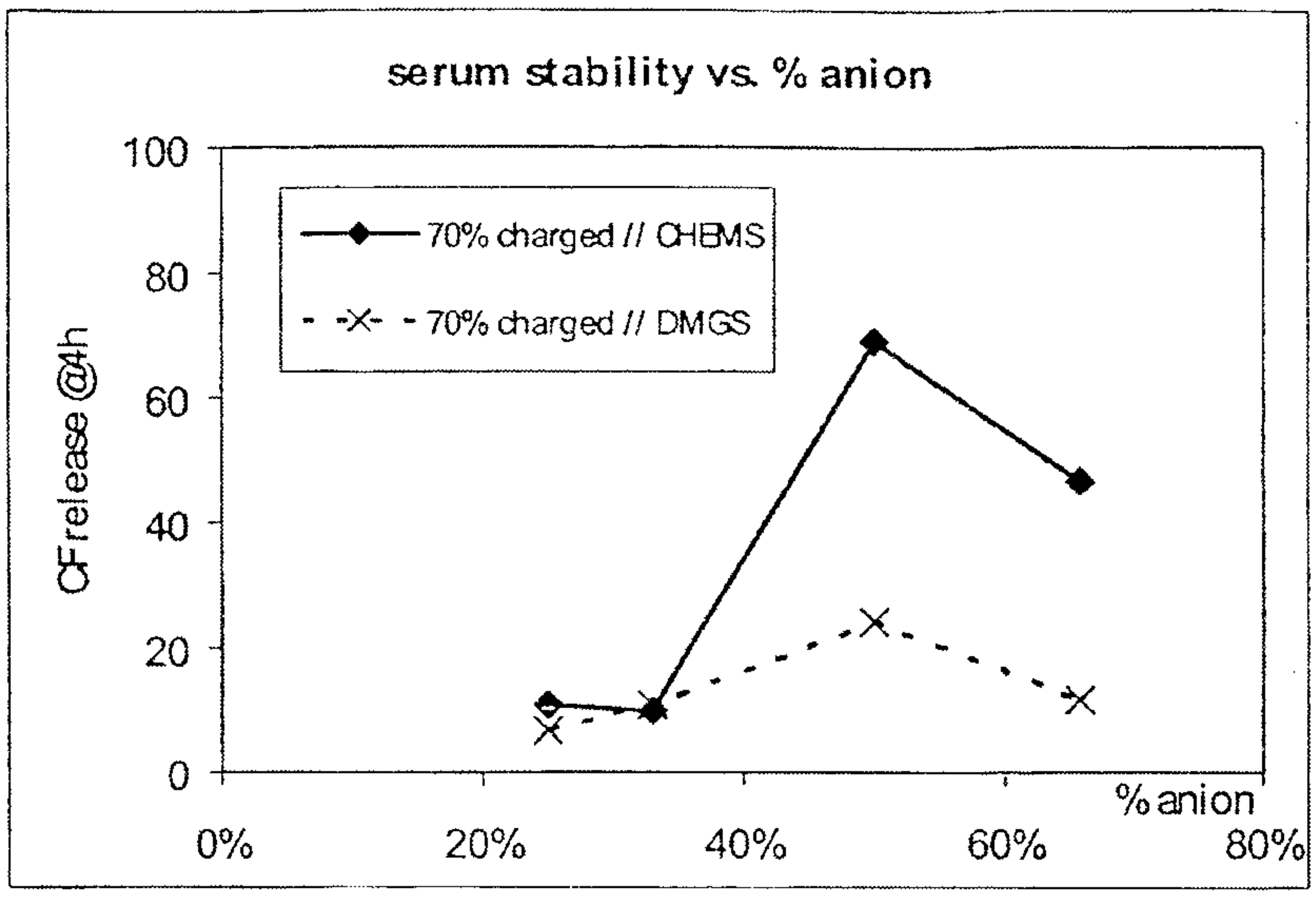


Figure 3:



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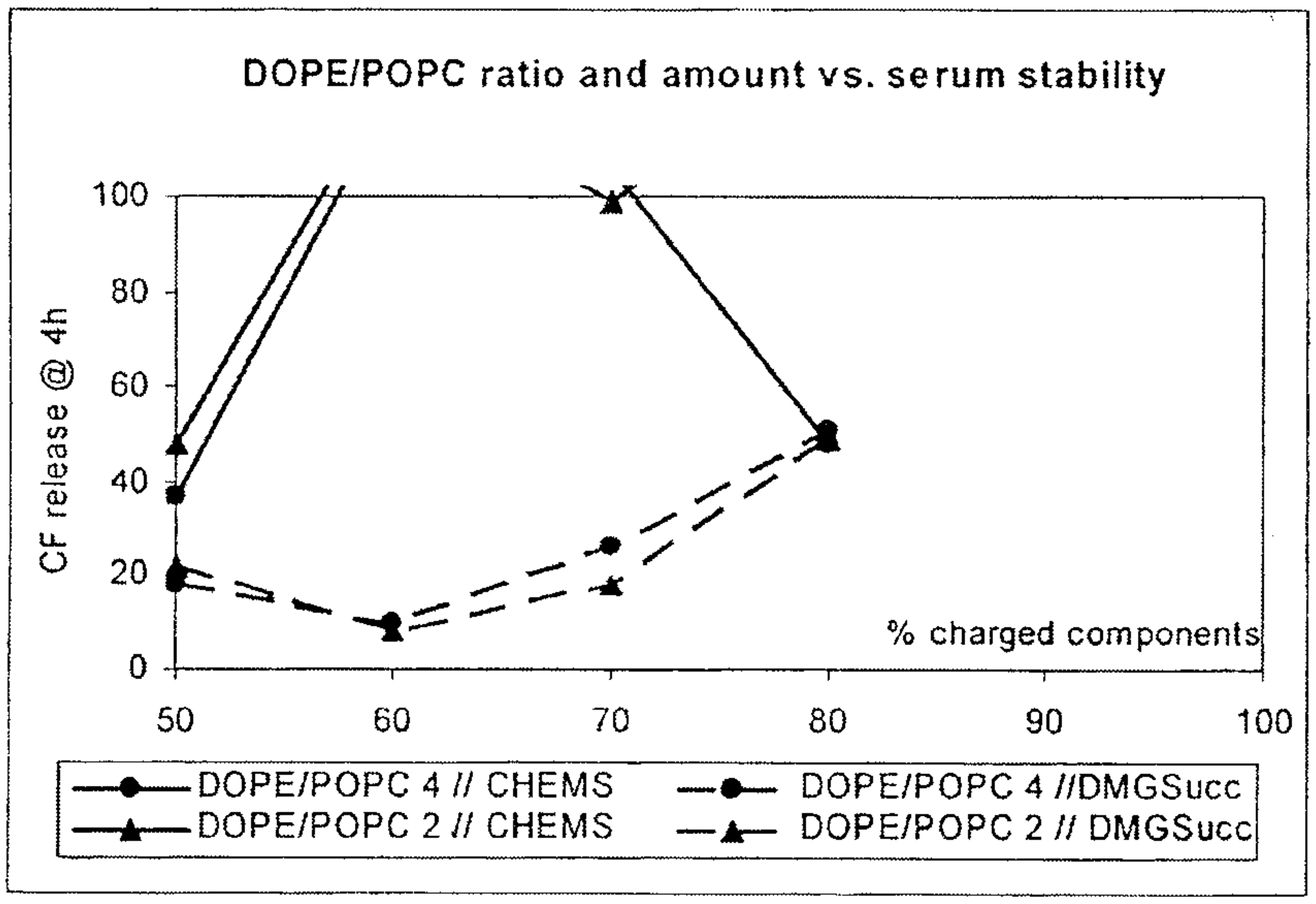

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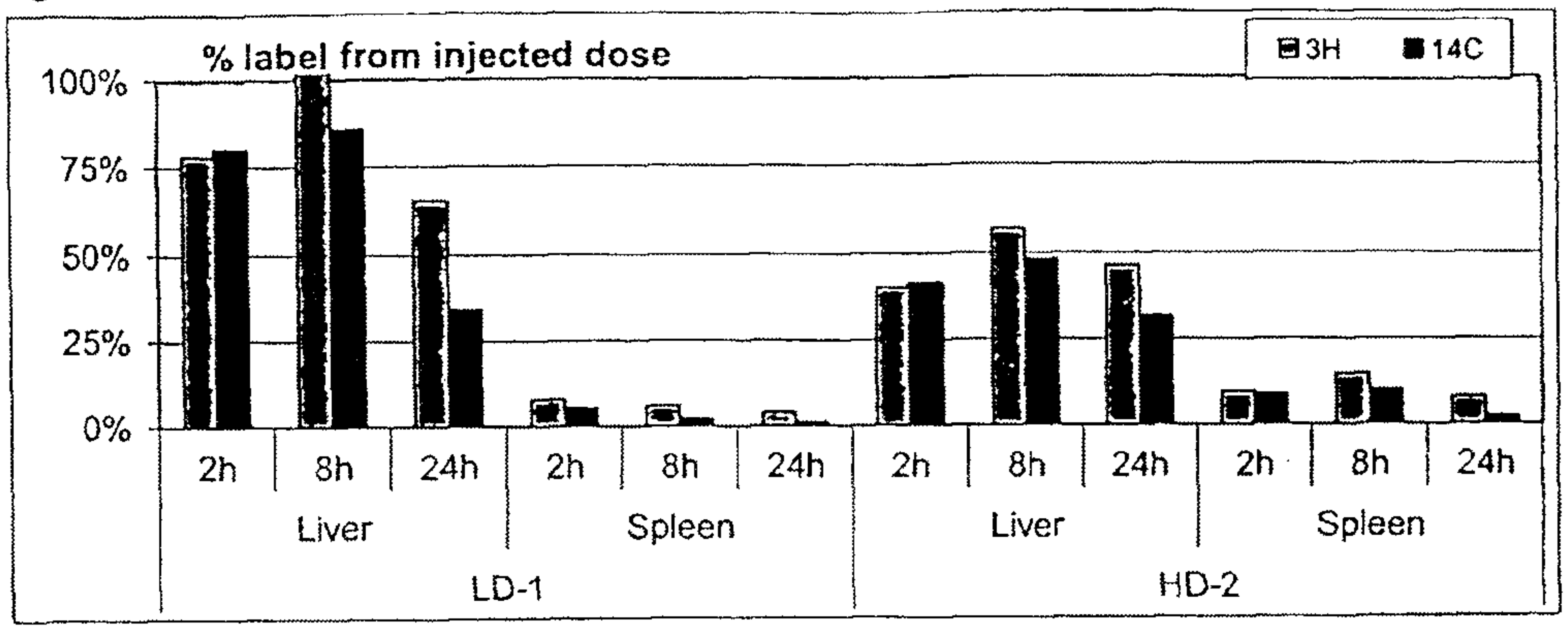

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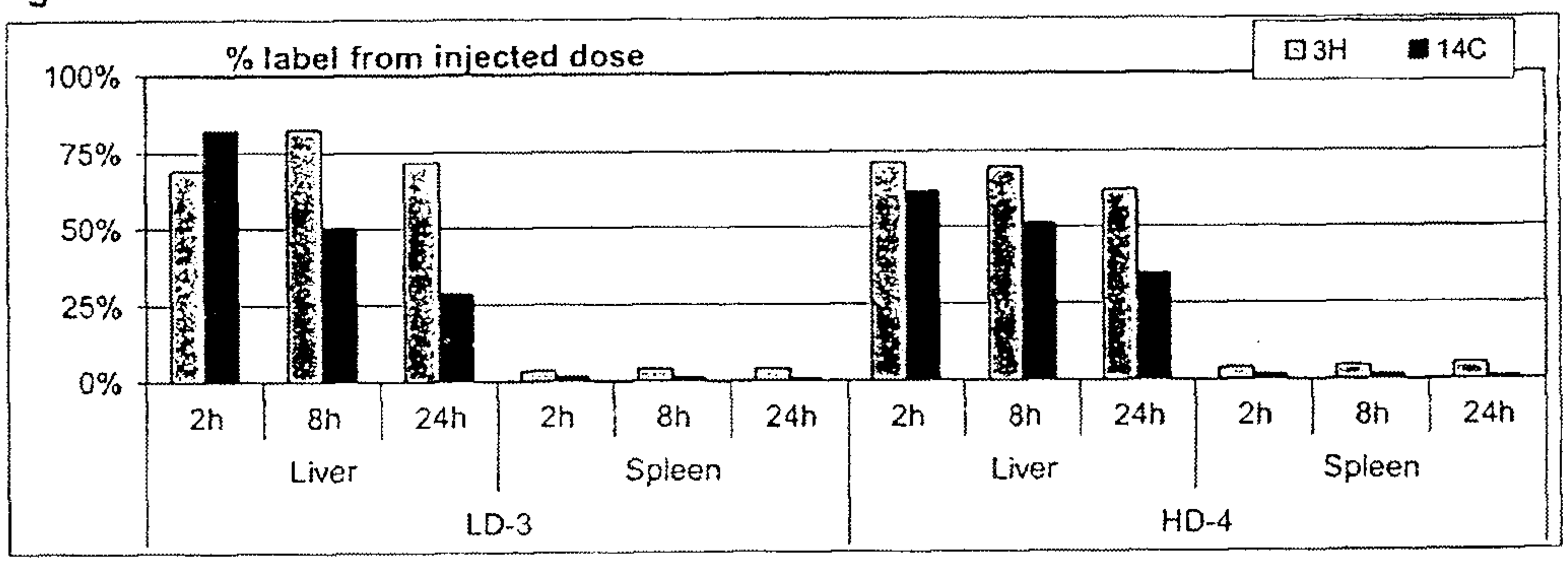
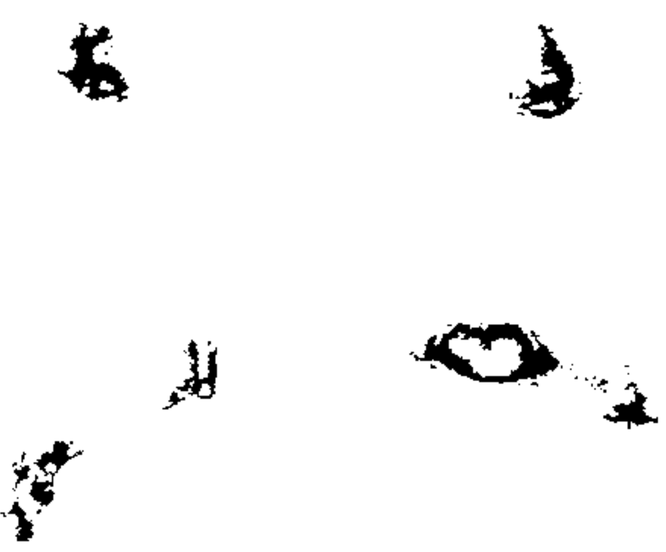


Figure 7:

front left	2142
front right	**8907**
back left	2785
back right	2829

animal 71
back right paw is arthritic

front left	1719
front right	1885
back left	2176
back right	**7016**



healthy animal without Cy5.5

front left	2404
front right	3794
back left	2709
back right	3258

Figure 8:

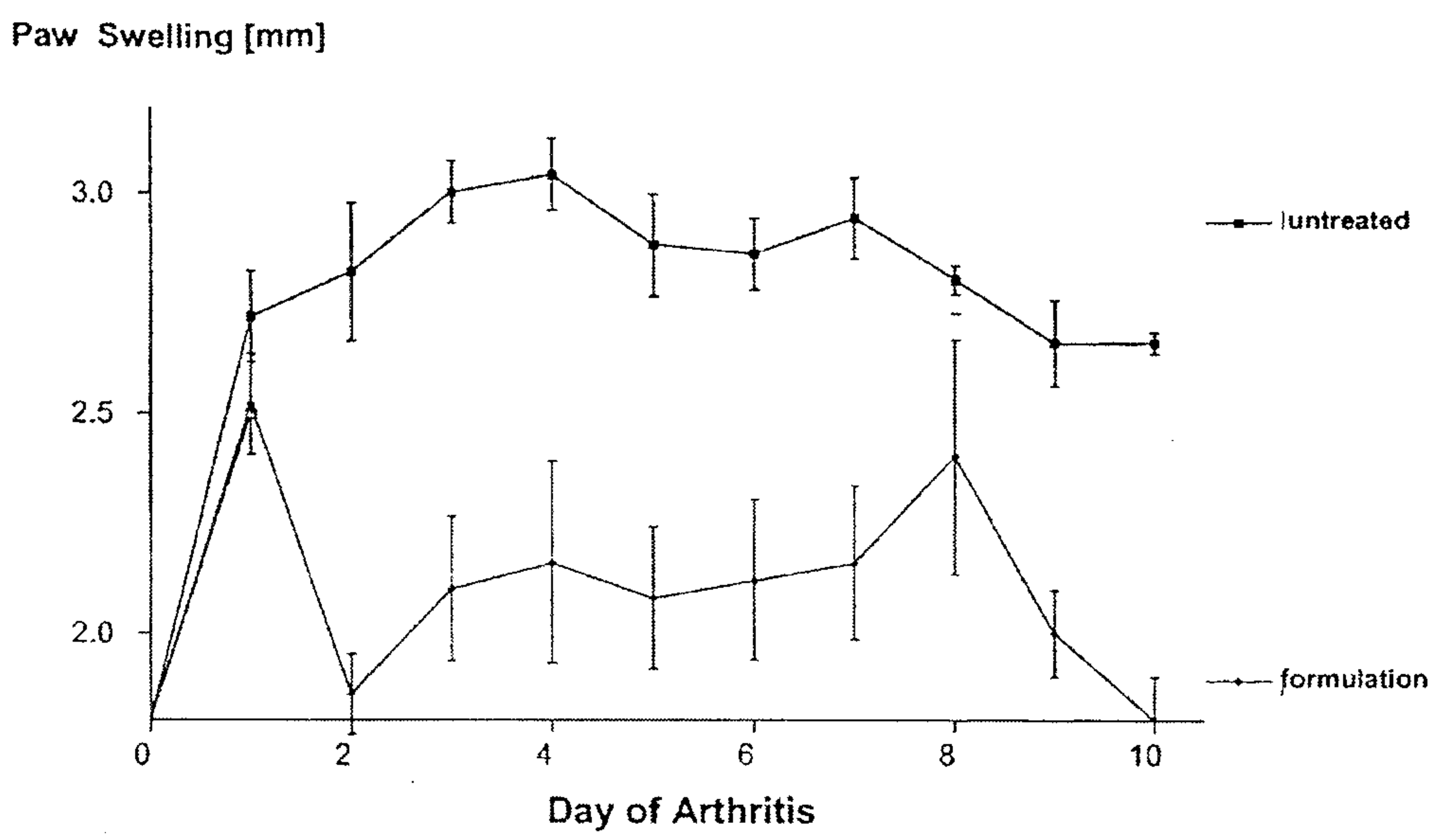
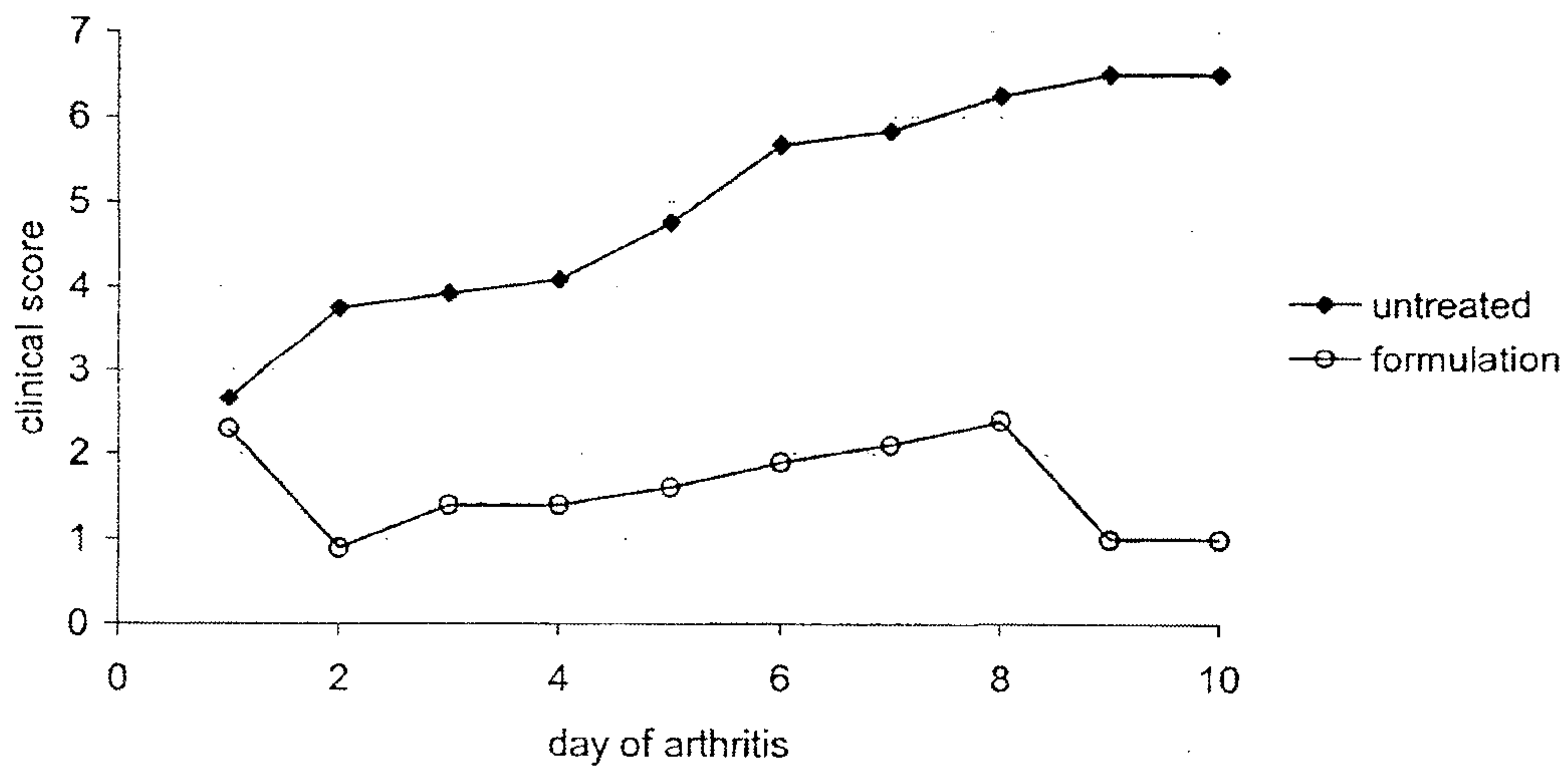


Figure 9



(SEQ ID NO:4)

gcctcgccATGGTTCGTCTGCCTCTGAAGTGTCTCCTCTGGGGCTGCTTTTTGACCGCCGTCCACCCAGAACCACCCACTTCATGCAAAGAAAACCAATACCCAACAAACAGCCGG
ASO 1

TGCTGTAATTTGTGCCCGCCAGGACAGAAACTGGTGAACCACTGCACAGAGGTCACTGAAACAGAATGCCTTCCTTGCAGTTCCAGCGAATTCCTAGCCACCTGGAATAGAGAGAA
ASO 2

ACACTGTCATCAGCACAAATACTGCGACCCCAACCTAGGTCTCCAGGTCCAGAGGGAGGGCACCTCGAAAACAGACACCACTTGTGTGTGCAGTGAAGGCCATCACTGTACCAACA
ASO 3

GCGCCTGTGAAAGTTGCACCTTGCACAGCTTGTGCTTCCCTGGCCTCGGGGTCAAGCAGATGGCGACAGAGGTTTCTGACACTATCTGTGAACCCTGCCCAGTTGGCTTCTTCTCC
ASO 4

AATGTATCATCTGCTTCAGAAAAGTGTCAGCCTTGG[illegible]CAAGCTGCGAGAGCAAAGGCCTGGTGGAACAACGTGCGGGGACTAACAAGACCGATGTTGTCTGTGGTTTCCAGAGTCG
ASO 5

GATGAGAGCCCTGGTGGTTATCCCCATCACGCTGGGGATCCTGTTTGCCGTCCTGTTGGTATTTCTCTGTATCAGAAAGGTGACCAAGGAGCAGGAGACTAAGGCCCTGCACCCTA
ASO 6

AGACTGAAAGGCAGGATCCCGTGGAGACGATTGATCTGGAGGATTTTCCCGACTCCACCGCTCCGGTGCAGGAGACCTTACATTGGTGCCAGCCCGTCACCCAGGAGGATGGCAAA
ASO 7

GAGAGCCGCATCTCCGTGCAGGACCGACAGTGAggctgtgcgtggccaggagcgtggaggcacgggcacaggggcatgtgactgcagagcccggggcggctgctgctgctgtggcg
ASO 8

Gtggtgagagggtggtgctgggcacagccccttctgcctgcacccctgcagtccagatacagtccacctcgaggagcttctcaccccagccctggagcccattcaatctcagtttg
ASO 9

cttttaaagatggagacaaaactttggggagtcacagccacagtaataaccaccagagcttccaacccagaggttcagtacctgcagatgcaagggatggcgtctaggagcccagg

aggcatatacatgactgtccaccactgcattgttcgtgacagtgagtgactggaaactgcttaactgtccatcaacaggggactggctaaataaaattgtaacatgtttatgcaaa
ASO 10

aaaaaaaa

FIG. 10