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(54) Title: LIPID COMPOSITION USED FOR CONSTRUCTION OF LIPOSOMAL GENETIC DRUG CARRIER TARGETED WITH ANTIBODIES, AND USE THEREOF

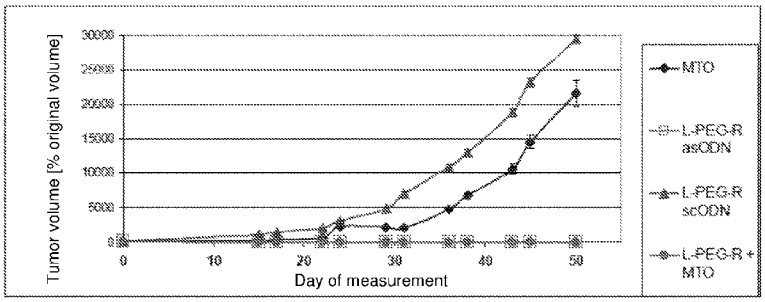


Fig. 12

(57) Abstract: The object of the present invention is a lipid composition used for creation of liposome carrier targeted with antibodies for genetic drugs in the form of as ODN, si RNA, mi RNA, ribozyme RNA, and DNAzyme, characterized in that it contains from 35.2% to 45.7% by weight of phosphatidylcholine (PC), from 12.3 to 9.1% by weight of dioleoylphosphatidylethanolamine (DOPE), from 4.9% to 6.4% by weight of 3β-[N-(Dimethylethane)carbamoyl]cholesterol (DC-CHOL), from 10.7% to 16.6% by weight of DSPE-PEG from 4.6% to 6.2% by mass of maleimide DSPE-PEG derivative (DSPE-PEG-Mal), and from 10.6% to 25.7% by mass of a trimethylammonium salt of 1,2-dioleoylpropane (DOTAP) and its use.

Lipid composition used for construction of liposomal genetic drug carrier targeted with antibodies, and use thereof

The present invention relates to a lipid composition used for formation of genetic drug liposome carrier targeted with antibodies being used in particular in treatment of chronic lymphocytic leukemia. Research was carried out at the University of Wroclaw, Faculty of Biotechnology, Department of Cytobiochemistry and at the Wroclaw Medical University in the Hematology Blood Neoplasms and Bone Marrow Transplantation Clinic.

Leukemia comprises a vast heterologous group of diseases of hematologic origin. During hematopoiesis, pluripotent bone marrow stem cells are subject to proliferation and differentiation into two main cell lines: myeloid and lymphoid. During further differentiation, myeloid cells mature into erythrocytes, monocytes, granulocytes, and blood platelets. Lymphoid cells, on the other hand, differentiate into B and T lymphocytes. Both the lymphoid and the myeloid line of stem cells can proliferate excessively on each stage of the differentiation process, resulting in an acute or chronic form of leukemia. Acute leukemia is a rapidly progressing disease, wherein the altered cells lose their natural properties. Chronic leukemia is characterized by slower development. Acute forms constitute ca. 50-60% of all leukemia cases, 20-30% are chronic lymphocytic leukemia forms, and 15-20% are chronic bone-marrow leukemia forms. Chronic lymphocytic leukemia is one of the most common leukemia forms in adults in Europe and North America. In Poland, the most common leukemia type is the acute bone-marrow leukemia (AML, about 1600 cases annually) and chronic lymphocytic leukemia CLL, appearing with a similar frequency, constituting about 1300 cases annually. The standard method of treatment for these leukemia types is chemotherapy, however, about 20% of cases remain resistant to the effects of cytostatics. Significant overexpression of *BCL-2* gene has been found in leukemia cells, and the relation between the high level of *BCL-2* gene expression and low effectiveness of chemotherapy was discovered. As the result of chromosome translocation t(14; 18), where the position of *BCL-2* gene (18q21) in the vicinity of IgH locus (14q32) results in *BCL-2* gene transcription disorders, increased level of mRNA, and Bcl-2 protein synthesis. Based on the above, we determine the chronic B-cell leukemia to be a disease of genetic origin. In the case of diseases of genetic origin, the most promising treatment method seems to be the gene therapy. Gene therapy consists of introduction of a functional gene into a diseased cell or in switching a function of a gene underlying the given disease on or off. Methods consisting in the elimination or at least limitation of the expression of the gene responsible for drug resistance or resistance to pro-apoptotic factors by means of antisense nucleotides (asODN), siRNA, miRNA and DNAzymes seem to be the most convenient for application in clinical practice. Therefore, a potential method to treat leukemia depending on overexpression of *BCL-2* is the application of a genetic drug in the form of antisense nucleotides (asODN) attachment of which to the complementary target mRNA induces its degradation by ribonucleases, thus reducing the level of the protein product in the cell. However, the largest problem relating to the effectiveness of the gene therapy is the effective delivery of the genetic drug to the target location. A genetic drug is a DNA or RNA molecule, that is, principally, not able to penetrate the cell membrane barrier. A significant problem is the sensitivity of "naked" nucleic acids molecules to nucleolytic enzymes present in the plasma and other tissue fluids. Due to its structure, a nucleic acid molecule also carries a high resultant negative charge promoting opsonization and removal from the bloodstream by

reticuloendothelial system macrophages. Therefore, it is necessary to apply an appropriate carrier that will deliver the genetic drug into the target cell overcoming the above mentioned barriers. From international patent application WO 2009/031911, a lipid composition for producing liposomes is known, which contains natural and synthetic phospholipids, consisting of 38.7% by weight of egg yolk phosphatidylcholine, 13.5% by weight of dioleoylphosphatidylcholine, 5.4% by weight of DC-Cholesterol, 15.3% by weight of di-stearoylphosphoethanolamine derivative of polyethylene glycol (PEG2000), and 27% by weight of a trimethylammoniurn salt of 1,2-dioleoylpropane (DOTAP). However, the composition, due to the lack of an appropriate modification enabling ligand attachment, is not intended for selective delivery of genetic drug to the target cells. Furthermore, from international patent application WO 2008/120914, a lipid composition is known, which is intended to be used as an effective carrier of genetic drugs, containing EDOPC, DC-Cholesterol, DPhPE, and a liposome-nucleic acid complex. The composition referred to, however, demonstrates lower "capacity" of the genetic drug in relation to the present solution, being 2 to 20 µg of siRNA/ODN per 1 µmole of liposome casing lipids, compared to 11 to 32 µg of ODN per 1 µmole of lipids in the case of proposed below solution. Furthermore, the mentioned above carrier is characterized by a very broad range of particle diameter values, 30-450 nm, where particularly such a large particles as > 100 nm are not desired during intravenous application of the carrier. Additionally, there are no experiments confirming effective genetic drug delivery by means of this carrier to target cells. The authors of the report tested only the effectiveness of a non-targeted variation of the carrier upon direct injection into the tumor.. International patent application WO2009/120247 discloses a lipid composition as a genetic drug carrier that enables active delivery of the genetic drug to target cell by means of attaching holo-trasnsferrin to the surface of the nanoparticles referred to.. Using that type of ligand is not a favorable forecast regarding the selectivity and effectiveness of genetic drug delivery to the target cell in in vivo conditions. The author of the above patent application also uses monoclonal antibodies, including Rituximab anti-CD20 characterized with a very low dissociation constant of the magnitude of 1 nM, but he does not go beyond testing the targeted carrier at the in vitro level only. In *in vivo* conditions, using mouse model with subcutaneously implanted L1210 leukemia, he analyzes the accumulation of the targeted carrier in created tumors with large success. However, the driving force for such distribution is not the target delivery by means of a ligand but passive accumulation supported by EPR (Enhanced Penetration and Retention) phenomenon in the area of neoplastic tissues. In the state of art, there is still lack of developed, effective compositions for producing targeted liposome carriers enabling genetic drug delivery to neoplastic cells in order to modify expression of the gene responsible for the particular disease. The aim of the present invention is the creation of a targeted liposome carrier that could be successfully applied in genetic therapy using e.g. antisense oligonucleotides. The carrier must present protective properties towards the genetic drug encapsulated therein and at the same time, it must not change its therapeutic properties during long-term storage. The application of the invention will enable selective and effective delivery of drugs to target cells, will reduce the expression level of selected genes, e.g. anti-apoptotic ones, and will sensitize the cells changed by the disease to the effects of low doses of cytotoxic drugs. The object of the present invention is also obtaining a carrier of low nonspecific cytotoxic and hemolytic activity. Unexpectedly, the technical problems mentioned above have been solved by the present invention.

The first object of the present invention is a lipid composition used for construction of liposome carrier targeted with antibodies for genetic drug in the form of asODN, siRNA, miRNA, ribozyme RNA, or DNAzyme, characterized in that it contains from 35.2% to 45.7% by mass of phosphatidylcholine (PC), from 12.3 to 19.1% by mass of dioleoylphosphatidylethanolamine (DOPE), from 4.9% to 6.4% by mass of 3β-[N-(Dimethylethane)carbamoyl]cholesterol (DC-CHOL), from 10.7% to 16.6% by mass of DSPE-PEG from 4.6% to 6.2% by mass of maleimide DSPE-PEG derivative (DSPE-PEG-Mal), and from 10.6% to 25.7% by mass of a trimethylammonium salt of 1,2-dioleoylpropane (DOTAP). Equally preferably, the composition according to the present invention is characterized in that it contains 42.5% by mass of PC, 14.8% by mass of DOPE, 5.9% by mass of DC-CHOL, 11.95% by mass of DSPE-PEG, 5.05% by mass of DSPE-PEG-Mal, and 19.8% by mass of DOTAP.

The second object of the present invention is an use of the lipid composition specified in first object of the invention for production of a liposome carrier for genetic drugs for treating leukemia.

The liposome structure created this way provides protection to the genetic drug against degradation. Furthermore, the genetic drug carrier enables effective and selective drug delivery to the disease changed cell, which makes it possible to minimize the side effects of the therapy. This condition can be satisfied by attaching the ligand targeting the carrier to the target cell. The characteristic contents of maleimide groups of the modified DSPE-PEG-Mal lipid enables attachment of thiolated proteins and peptides, here - antibodies providing guiding the genetic drug to the target cells. Effective attachment of antibodies to the surface of liposomes of the composition according to the present invention is subject to the presence of thiol groups in the antibody molecule (obtained in amine group thiolation with Traut's reagent) and the appropriate mole ratio of IgG to maleimide groups in the bi-layer of the carrier. What is also significant, is the fact that the obtained liposomes do not form aggregates in the suspension. The obtained liposome preparations do not show hemolytic activity towards human erythrocytes. The targeted liposome carrier of the lipid composition according to the present invention demonstrates protective properties towards the genetic drug encapsulated therein.

Exemplary embodiments of the invention have been presented in the illustrations, where fig. 1 represents a graph illustrating the liposome molecules diameter size distribution in the original preparation and after 12 months of storage in the form of suspension at the temperature of 4°C, fig. 2 represents a graph illustrating the liposomes zeta potential value distribution after 12 months of storage in the form of suspension at the temperature of +4°C, fig. 3 represents a diagram illustrating fluctuations in the nucleic acid content in liposomes during 12 months of storage in the form of suspension at the temperature of +4°C, fig. 4 represents a diagram illustrating the hemolytic properties of liposomes towards human erythrocytes, fig. 5 represents the results of electrophoretic analysis of the plasmid DNA encapsulated in the carrier, fig. 6 is a diagram illustrating the nonspecific cytotoxicity of the liposomes, fig. 7 represents a diagram illustrating the effectiveness of attaching antibodies to liposomes, fig. 8 represents graphs illustrating the selectivity and effectiveness of immuno-liposomes towards target and nontarget selected cell lines: L1210, Jurkat T and Daudi, fig. 9 illustrates the penetration route and selectivity of immuno-liposomes, fig. 10 illustrates the effectiveness of *BCL-2* gene expression silencing by immuno-liposomes in Raji (CD20+) leukemic cell line, fig. 11 illustrates Daudi (CD20+) cell survival curves in a function of

concentration of added immuno-liposomes and mitoxantrone, fig. 12 represents a chart illustrating the effectiveness of treatment with immuno-liposomes containing asODN against *BCL-2* gene in SCID mouse with tumors induced via subcutaneous implantation of Daudi CD20+ cells.

Example

In order to obtain 1 ml of lipid composition in the form of liposomal suspension, 100-300 µg of oligonucleotides (asODN, scODN, miRNA) were suspended in 150 µl of deionized water, ddH2O. This solution was supplemented with 300 µl of chloroform. In order to obtain one phase, 300 µl of methanol were added, and then it was titrated by drop, adding methanol until obtaining clear solution(<100 µl additionally). 150 µl of DOTAP chloroform solution containing 2 mg of the lipid was added to the clear mixture. It was stirred gently to obtain a single phase. The mixture was left to rest for 30-40 minutes in room temperature. After that time 600 µl of chloroform was added to the mixture, it was stirred, and then the same volume of ddH2O water was added. The mixture was left for 1 minute for to separate the organic and inorganic phases. Next, it was centrifuged in 800 x g conditions in room temperature for 10 minutes. After centrifugation, the upper phase was removed, and the lower phase was added to the chloroform mixture of lipid solutions: 4.3 mg PC, 1.5 mg DOPE, 0.6 mg DC-CHOL, 1.19 mg DSPE-PEG, 0.51 mg DSPE-PEG-MAL, after which 250 µl of PBS buffer was added (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The mixture was subjected to sonication for 1 minute with concurrent cooling the sample in the ice bath. After obtaining milky-white emulsion in the result of sonication, organic solvent was evaporated in nitrogen stream and then additionally in a vacuum evaporator for 2 h until the residual organic solvent was removed. The resultant gel was hydrated by supplementing with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) to the volume of 1 ml. The suspension was calibrated in a high pressure extruder (the value of pressure applied: 1.2-2.0 kPa) through polycarbonate filters (MilliPore, Whatman) with the pores diameter of 400, 200 and 100 nm, at least ten times through each filter.

In order to attach the antibodies to the liposome carrier, they were prepared as follows: the thiolation reaction of free amine groups was carried out using 1.95 mg of antibodies (here: RituximabR) dissolved in 195 µl of citrate buffer ($C_6H_5O_7Na_3 \cdot 2H_2O$, NaCl, NaOH, Polysorbate80 (Polyoxyethylene sorbitan monolaurate), and 0.036 mg of Traut's reagent in the presence of EDTA (20 mM) at pH 7.4, and then the whole reaction mixture was incubated for 4h in temperature of +4°C, stirring gently. After that time, the excess of Traut's reagent was removed in dialysis against PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Such prepared antibody solution was mixed with 1 ml of earlier prepared liposome preparation as described above. The reaction of attaching the antibodies to the liposomes was carried out at the temperature of +4°C for 24h hours. Unbound antibodies were separated from the immuno-liposomes by gel filtration on Sepharose 4B (column measuring 25 x 1,2 cm equilibrated with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) at the temperature of +4°C. The content of antibodies in the liposome fraction was determined by means of ELISA test.

Animal model experiment:

NOD/SCID mice were subcutaneously implanted with Daudi (CD20+) line leukemic cells. Suspension of cells coming from *in vitro* culture was used for subcutaneous transplantation, in the amount of $1*10^6$/mouse. After formation of subcutaneous tumor, the mice were treated with drug solutions in the form of liposome suspension according to the invention and water solution of mitoxantrone. Groups of 8 animals each were used for the experiment. Among the analyzed groups, 3 were control groups in order to exclude the therapeutic effects of separately acting low mitoxantrone dose of 0.1 mg/kg body mass, immuno-liposomes according to the invention, but containing the "scrambled" sequence, and PBS buffer. In the other 2 groups, the therapeutic effect of the drug proposed herein, in the form of immuno-liposomes according to the invention targeted with Rituximab antibodies, containing anti-*BCL-2* asODN separately and in combined therapy with a low dose of mitoxantrone (0.1 mg/kg body mass) was analyzed.

The solutions of the drugs were prepared before use. The preparations were administered in the volumes of 200-300 µl (10 µl/1g body mass), wherein the dose each time contained, for immuno-liposome preparation: 800 µg/kg body mass of ODN, 88 mg/kg body mass of lipids, 650 µg/kg body mass Rituximab antibodies or 0.1 mg/kg body mass of mitoxantrone. The solutions were injected into the lateral tail vein after immobilizing the animal. The effectiveness of the treatment is presented in the chart illustrating the dependency of tumor growth from the time of treatment for each preparation administered as shown in fig. 12, where the remission of neoplastic tumors is clearly visible in the group treated with immuno-liposomes according to the present invention, containing anti- *BCL-2* asODN, and with the same immuno-liposomes in combination with a low dose of mitoxantrone. The experiment demonstrated high effectiveness of the immuno-liposome preparation according to the present invention, containing anti-*BCL-2* asODN in tumor (CD20+) therapy, both when used separately and in combination with a low cytostatic dose.

The application of the herein presented lipid composition for production of liposome carrier for genetic drug in the form of asODN, siRNA or miRNA guarantees obtaining liposomes of stable size, with the diameter of about 110+/-5 nm, low growth dynamics during a year of storage in the form of suspension or freeze-dried, as illustrated by the chart presented in fig. 1, size of which was measured by dynamic light scattering technique. Of major importance is the fact that the obtained liposomes do not form aggregates in the suspension. Furthermore, the obtained liposome preparation demonstrates stability in zeta potential of ca. 23 mV (fig. 2) measured by means of combined electrophoresis and LDV (Laser Doppler Velocimetry) techniques, and the content of nucleic acid in the range of 95-98% (fig. 3). The obtained liposome preparations do not show hemolytic activity towards human erythrocytes. Obtained hemolysis values of up to 7% remain within the error limits of the method (Fig. 4). The hemolytic activity of the carrier was evaluated by examining the effects of hemoglobin from erythrocytes isolated from human blood after incubation with liposomes. Erythrocytes in the amount of $7*10^7$ were incubated with the increasing amount of 0.5 – 4 µg pDNA/ml encapsulated in the liposome carrier according to the present invention. The blind sample consisted of erythrocytes suspended in PBS and incubated exactly asexamined samples. The total hemolysis was assumed to be $7*10^7$ of cells suspended in deionized water and incubated as above. Hemoglobin leakage from liposome erythrocytes was the indicator of

liposomes' hemolytic activity. Hemoglobin absorbance was measured at the wave length of 570 nm after 2 hours of incubation at 37°C.

The targeted liposome carrier of the lipid composition according to the present invention demonstrates protective properties towards the genetic drug encased therein. Liposome carrier containing plasmid DNA was incubated with DNAse for 2 h at the temperature of +37°C, after which the protective properties of the liposomes were examined by means of electrophoretic analysis of the plasmid DNA encapsulated in the carrier (fig. 5). A broad range of polyethylene glycol lipid derivative, DSPE, in the range of 10.7-16.6% by mass, was tested in the embodiment, which significantly lowered the non-specific cytotoxic activity of liposomes (Fig. 6). In order to check the nonspecific cytotoxic activity of liposome preparations, the latter were tested against a broad spectrum of cell lines (Jurkat T lymphoblastic leukemia model, HL 60 myeloid leukemia, and HEL cells belonging to the erythroid line). The liposome preparations of composition according to the present invention demonstrate low nonspecific cytotoxic activity (Fig. 6). The liposomes cytotoxic activity was measured by means of MTT test (MTT Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich). The method uses the redox activity of mitochondria. In the effect of mitochondrial dehydrogenase, the yellow, water soluble tetrazolium salt is transformed into purple, insoluble in water crystals of formazan. The amount of produced formazan crystals is proportional to the count of living cells.

The obtained immuno-liposome preparation (targeted with antibodies) demonstrates high selectivity towards target cells exposing surface CD20 antigen. The selectivity of immuno-liposomes prepared according to the present invention was tested in vitro against L1210 (CD20), Jurkat T (CD20-), HeLa (CD20-), Daudi (CD20+), and RPMI 8226 (CD20+) cells, based on MTT toxicity tests (Fig. 8), and fluorescence imaging coming from liposomes marked with DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, Invitrogen) (Fig. 9a), cell nuclei colored with DAPI. By means of the MTT toxicity test (performed as above), It was demonstrated that immuno-liposomes containing the genetic drug, the anti- *BCL-2* asODN are selective and effective only against cells exposing CD20 antigen (Fig. 8), wherein the toxicity of the construct only against the target CD20+ cells was assumed as the selectivity measure. Additionally, experiments concerning the selectivity of the carrier according to the present invention were carried out by using immuno-liposomes marked with DiD fluorescent dye, against HeLa cells (adherent of characteristic shape, CD20-) and Daudi (of regular shape, CD20+) cells. The immuno-liposomes selectively reach only CD20+ cells, where the accumulation of immuno-liposomes fluorescently stained with DiD is visible in the area of the cell membrane of CD20+ cells (Fig. 9b).

The lipid composition of immuno-liposomes according to the present invention effectively reduces *BCL-2* gene expression in Raji (CD20+) cells, which was evaluated on the basis of Bcl-2 protein level analysis in control cells and in those treated with immuno-liposomes, with Western Blot technique (Fig. 10).

The liposome preparation of the composition according to the present invention is also intended for application in genetic therapy combined with cytostatics, and therefore the effect of *BCL-2* gene expression reduction upon survivability of Daud CD20+ cells and upon the effectiveness of combined therapy was also examined. Reduction of *BCL-2* gene expression by means of asODN in the immuno-

liposomes according to the present invention significantly improves the effectiveness of the cytostatic, here: mitoxantrone, in the doses corresponding to IC 50 1.25 nM (Fig. 11).

Application of an appropriate lipid composition, particularly increasing the content of DSPE-PEG, using maleimide-modified DSPE-PEG, selection of charges ratio between the cationic lipid and the genetic drug in the asODN form, and attachment of "targeting" antibodies, which in case of the tested system were humanized RituximabR (MabThera®) antibodies, enables selective and effective delivery of a genetic drug to leukemic cells containing CD20 surface marker.

Claims

1. Lipid composition used for construction of liposome carrier targeted with antibodies for genetic drugs in the form of asODN, siRNA, miRNA, ribozyme RNA, and DNAzyme, characterized in that it contains from 35.2% to 45.7% by weight of phosphatidylcholine (PC), from 12.3 to 19.1% by weight of dioleoylphosphatidylethanolamine (DOPE), from 4.9% to 6.4% by weight of 3β-[N-(Dimethylethane)carbamoyl]cholesterol (DC-CHOL), from 10.7% to 16.6% by weight of DSPE-PEG from 4.6% to 6.2% by mass of maleimide DSPE-PEG derivative (DSPE-PEG-Mal), and from 10.6% to 25.7% by mass of a trimethylammonium salt of 1,2-dioleoylpropane (DOTAP).

2. The composition of claim 1, characterized in that it contains 42.5% by mass of PC, 14.8% by mass of DOPE, 5.9% by mass of DC-CHOL, 11.95% by mass of DSPE-PEG, 5.05 % by mass of DSPE-PEG-Mal, and 19.8% by mass of DOTAP.

3. An use of the composition specified in claim 1 for production of a liposome carrier for genetic drugs for treating leukemia.

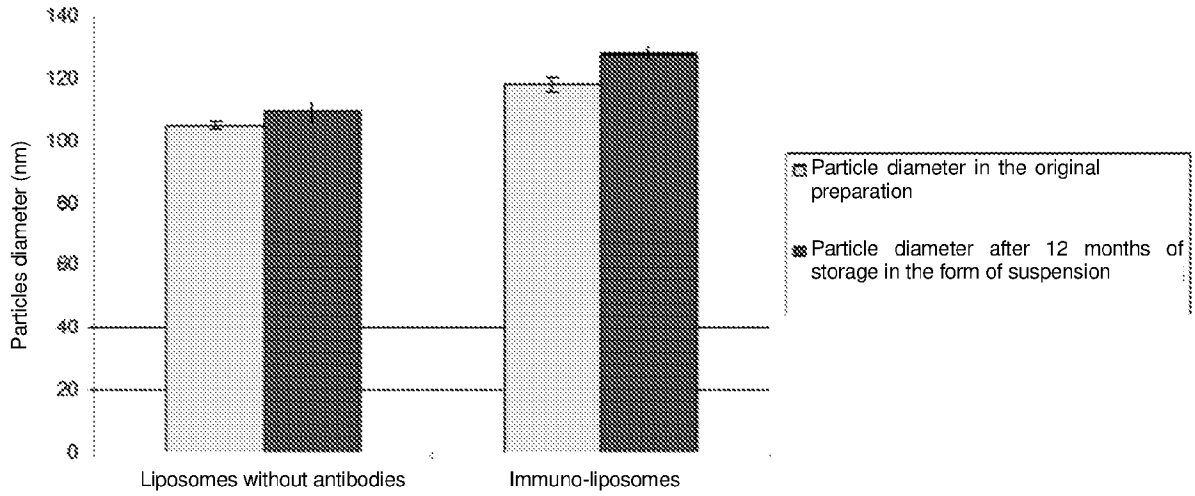
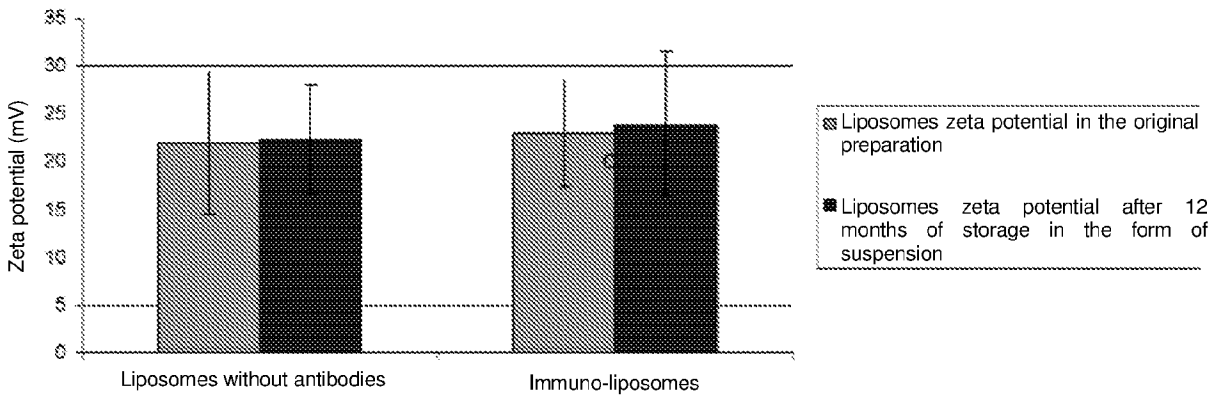

Particles diameter (nm)
140
120
100
80
60
40
20
0
Liposomes without antibodies
Immuno-liposomes
Particle diameter in the original preparation
Particle diameter after 12 months of storage in the form of suspension

Fig. 1


Zeta potential (mV)
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30
25
20
15
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5
0
Liposomes without antibodies
Immuno-liposomes
Liposomes zeta potential in the original preparation
Liposomes zeta potential after 12 months of storage in the form of suspension

Fig. 2

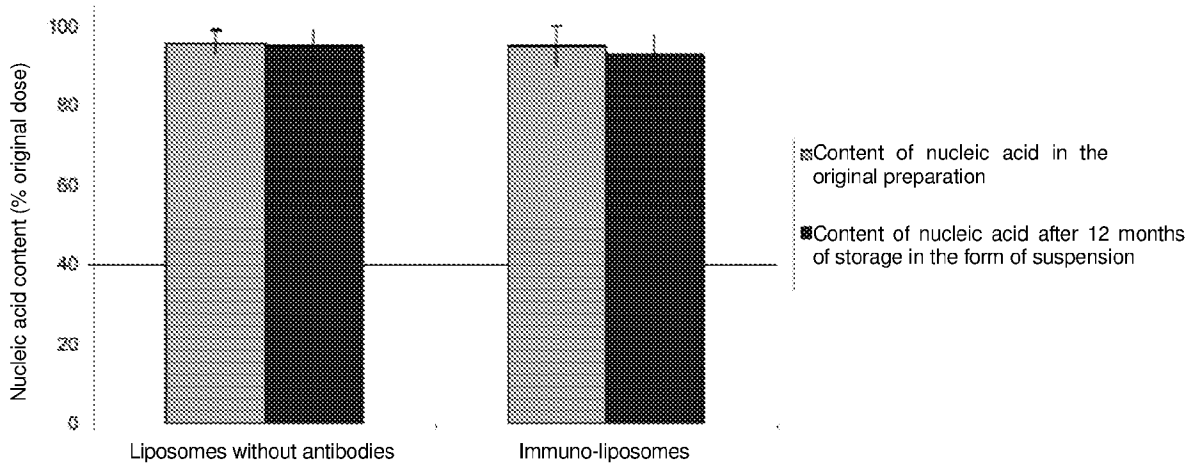


Fig. 3

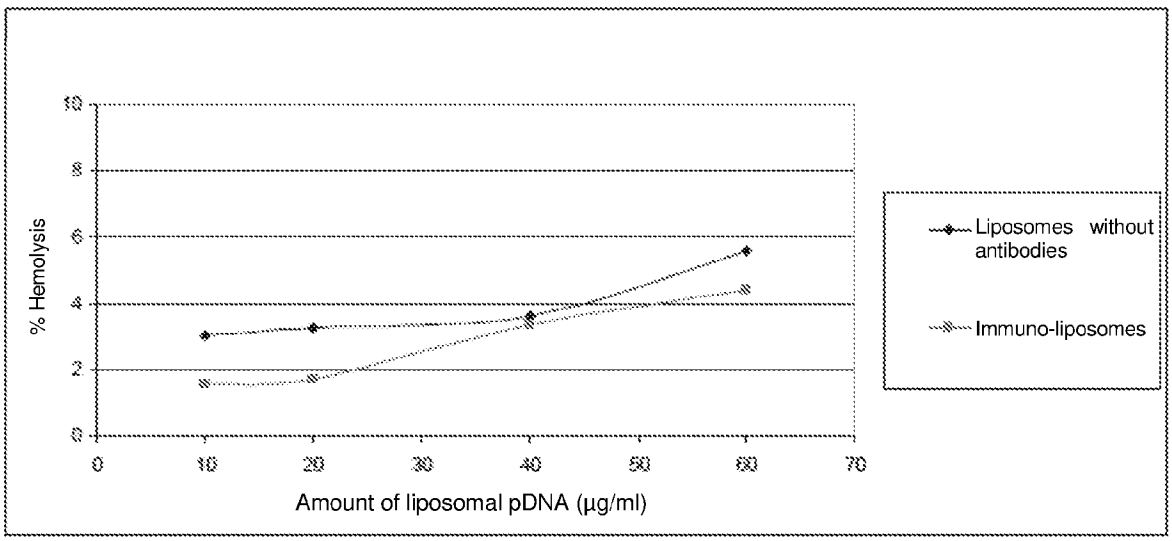


Fig. 4

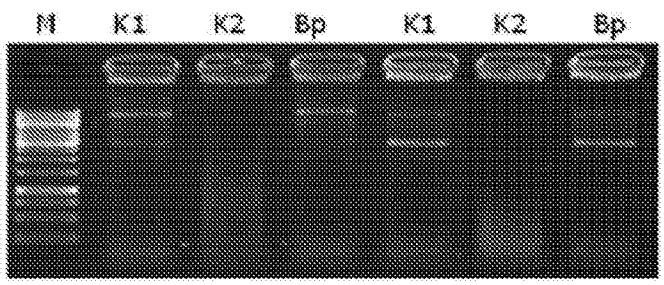


Fig. 5

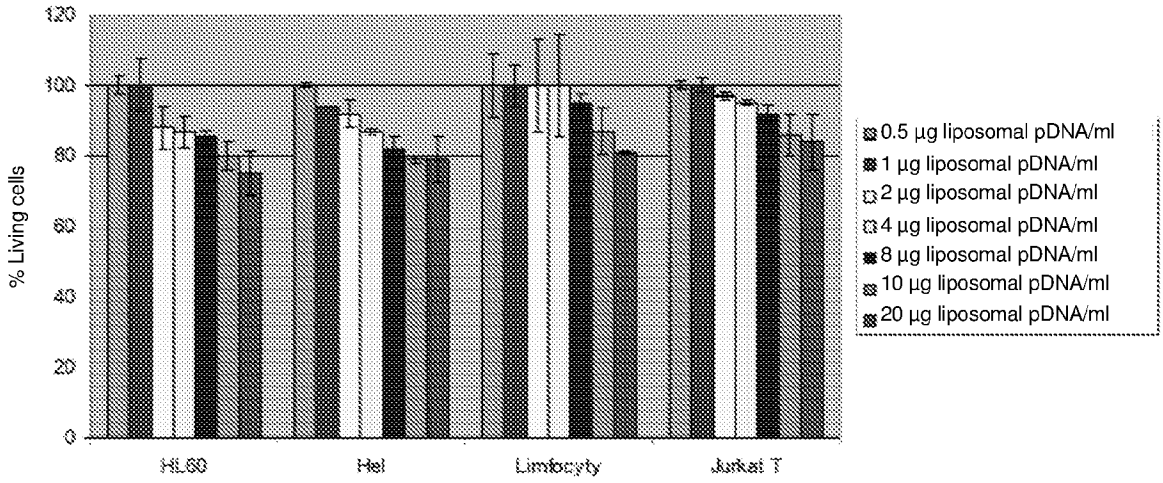

120
100
80
60
40
20
0
% Living cells
HL60
Hel
Limfocyty
Jurkat T
0.5 µg liposomal pDNA/ml
1 µg liposomal pDNA/ml
2 µg liposomal pDNA/ml
4 µg liposomal pDNA/ml
8 µg liposomal pDNA/ml
10 µg liposomal pDNA/ml
20 µg liposomal pDNA/ml

Fig. 6

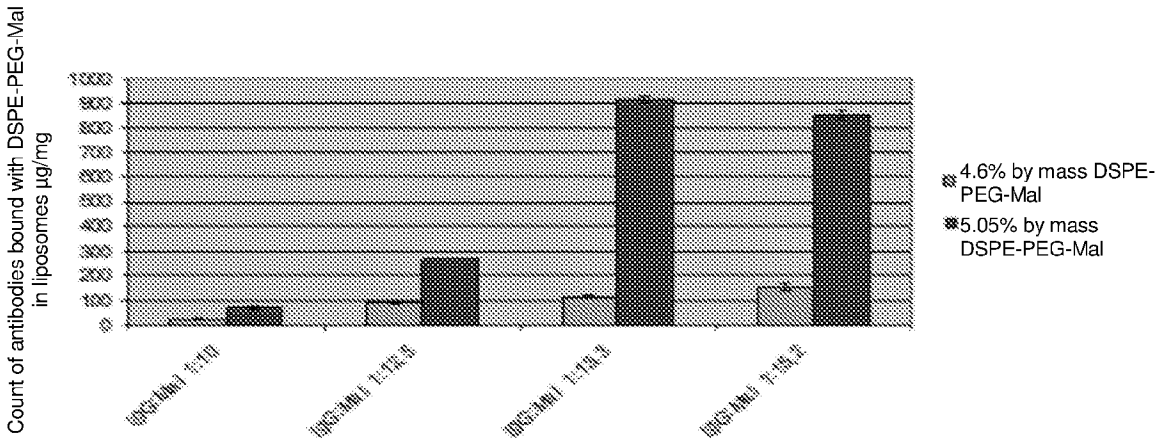
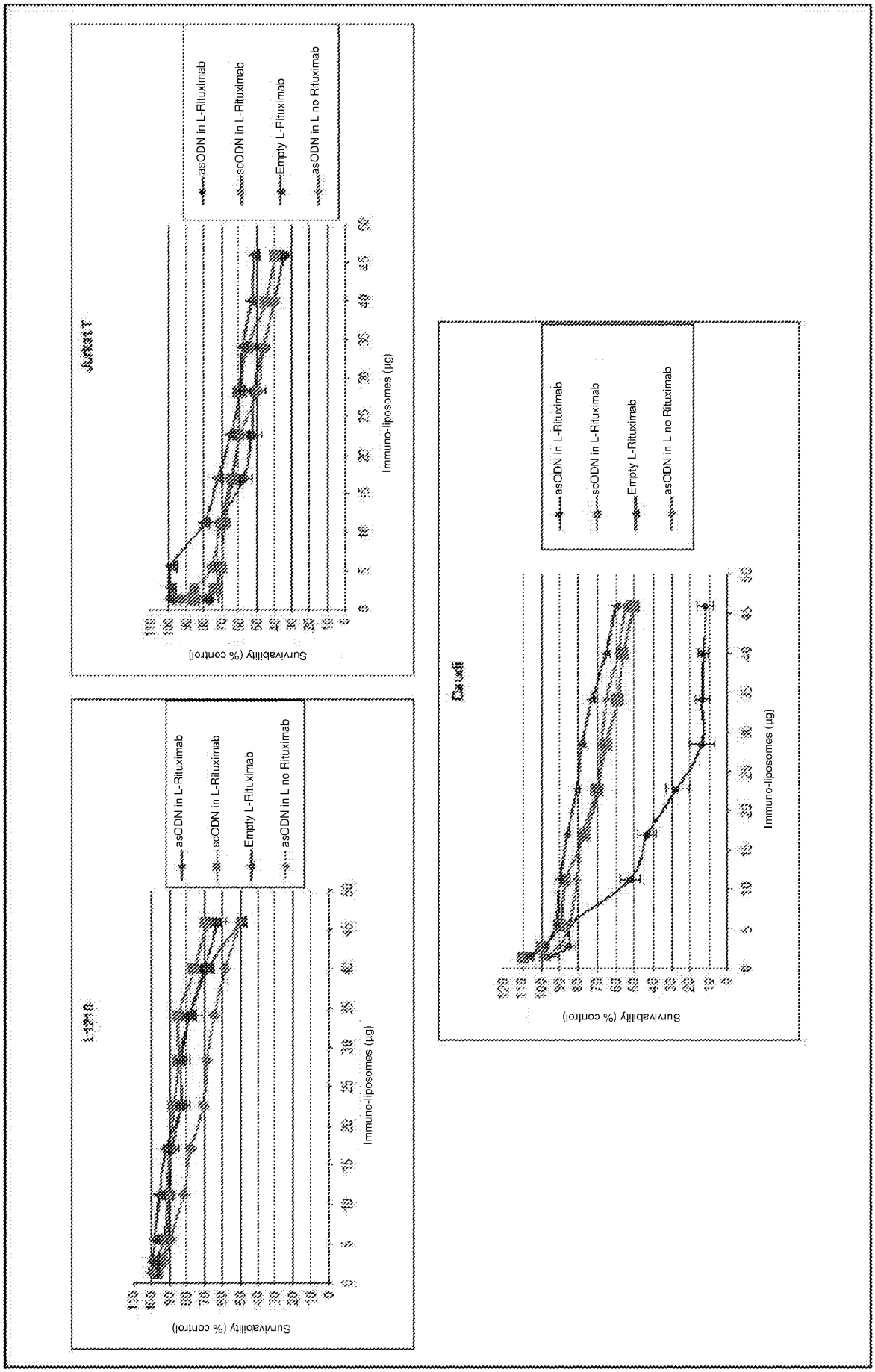
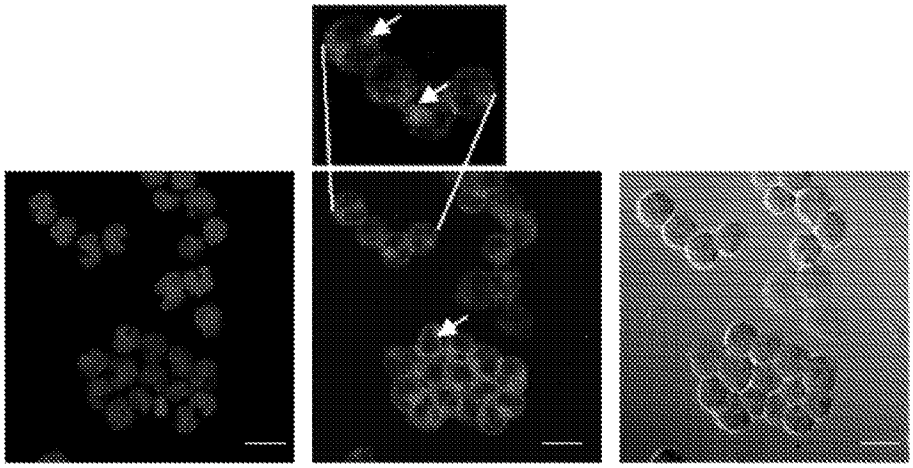

Count of antibodies bound with DSPE-PEG-Mal in liposomes µg/mg
4.6% by mass DSPE-PEG-Mal
5.05% by mass DSPE-PEG-Mal

Fig. 7


Jurkat
Daudi
asODN in L-Rituximab
scODN in L-Rituximab
Empty L-Rituximab
asODN in L no Rituximab
Survivability (% control)
Immuno-liposomes (µg)

a)



b)

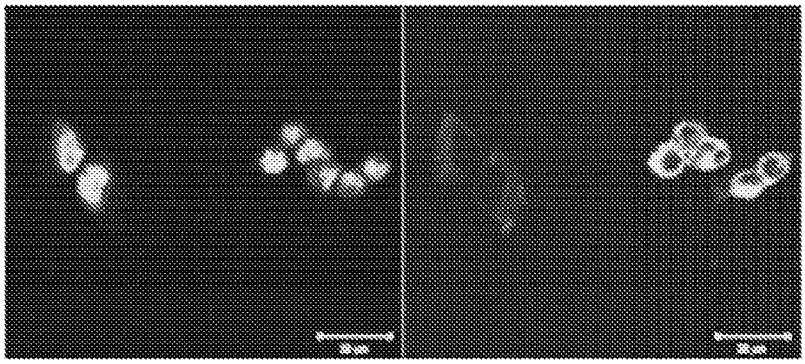


Fig. 9

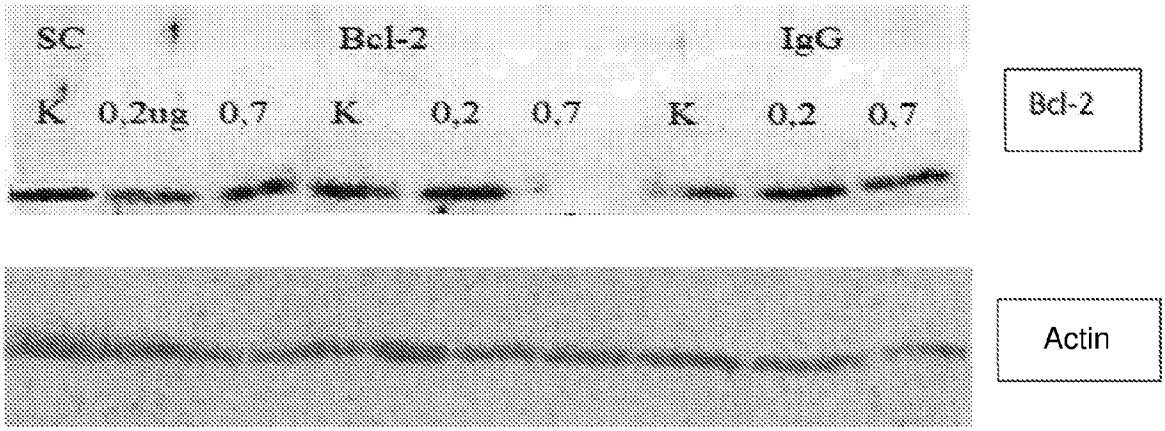


Fig. 10

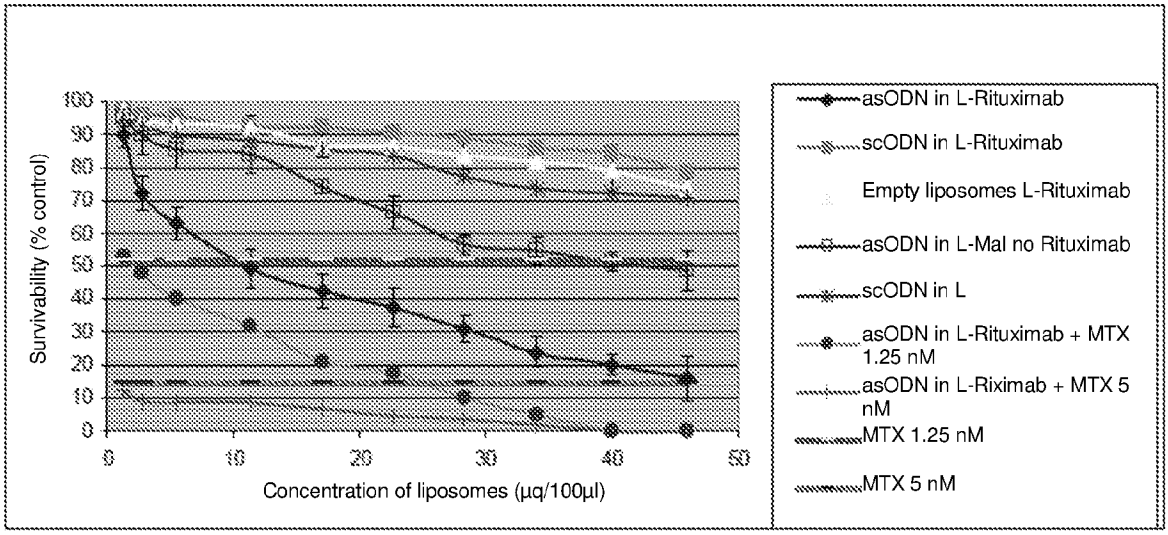

Survivability (% control)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Concentration of liposomes (µq/100µl)
asODN in L-Rituximab
scODN in L-Rituximab
Empty liposomes L-Rituximab
asODN in L-Mal no Rituximab
scODN in L
asODN in L-Rituximab + MTX 1.25 nM
asODN in L-Riximab + MTX 5 nM
MTX 1.25 nM
MTX 5 nM

Fig. 11

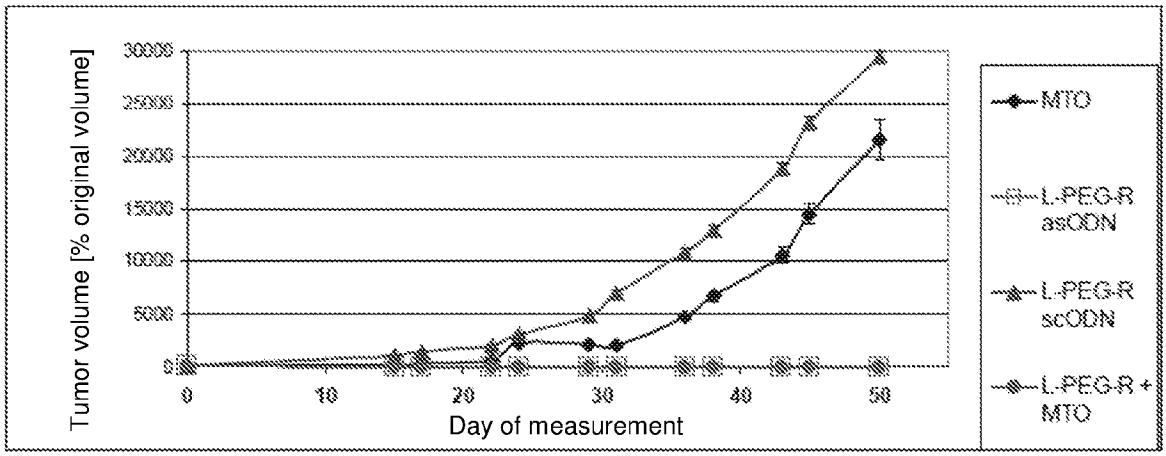

Tumor volume [% original volume]
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
Day of measurement
MTO
L-PEG-R asODN
L-PEG-R scODN
L-PEG-R + MTO

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2015/050011

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 A61K9/127 A61K48/00 C12N15/88 A61P35/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, COMPENDEX, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009/031911 A1 (UNIV WROCLAWSKI [PL]; AKADEMIA MEDYCZNA IM PIASTOW S [PL]; SIKORSKI AL) 12 March 2009 (2009-03-12) cited in the application page 1, paragraphs 1,2 page 2, last line - page 3, paragraph 2 example 1 claims	1-3
Y	WO 2009/120247 A2 (UNIV OHIO STATE RES FOUND [US]; LEE ROBERT J [US]; YU BO [US]; LEE L J) 1 October 2009 (2009-10-01) cited in the application paragraphs [0323] - [0326], [0329], [0345] - [0347], [0370], [0371] figures 30,31A,31B	1-3

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 August 2015	15/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Villard, Anne-Laure

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/202687 A1 (PERRI PATRIZIA [IT] ET AL) 8 August 2013 (2013-08-08) paragraphs [0062], [0303], [0304]	1-3
Y	YU BO ET AL: "Targeted nanoparticle delivery overcomes off-target immunostimulatory effects of oligonucleotides and improves therapeutic efficacy in chronic lymphocytic leukemia", BLOOD, vol. 121, no. 1, January 2013 (2013-01), pages 136-147, XP002743337, abstract page 137, section "Preparation of rituximab-conjugated liposomal ODN nanoparticles" pages 141-143, section "In vivo evaluation of RIT-INP-G3139 in a preclinical model" page page 146, right-hand column, last paragraph	1-3
Y	-& YU BO ET AL.: "Supplementary Material for Targeted nanoparticle delivery overcomes off-target immunostimulatory effects of oligonucleotides and improves therapeutic efficacy in chronic lymphocytic leukemia", BLOOD, January 2013 (2013-01), pages 1-21, XP002743506, page 2, "Preparation of Rituximab conjugated ODN nanoparticles"	1-3
Y	ROTHDIENER M ET AL: "Targeted delivery of SiRNA to CD33-positive tumor cells with liposomal carrier systems", JOURNAL OF CONTROLLED RELEASE, vol. 144, no. 2, June 2010 (2010-06), pages 251-258, XP002743334, ELSEVIER NLD DOI: 10.1016/J.JCONREL.2010.02.020 abstract pages 252, sections 2.4., 2.5.	1-3
Y	DENG LI ET AL: "Comparison of anti-EGFR-Fab' conjugated immunoliposomes modified with two different conjugation linkers for siRNA delivery in SMMC-7721 cells", INTERNATIONAL JOURNAL OF NANOMEDICINE, vol. 8, 2013, pages 3271-3283, XP002743335, abstract	1-3

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIM K S ET AL: "Targeted gene therapy of LS174T human colon carcinoma by anti-TAG-72 immunoliposomes", CANCER GENE THERAPY, vol. 15, no. 5, May 2008 (2008-05), pages 331-340, XP002743336, ISSN: 0929-1903 page 333, section "Pegylated liposome synthesis and plasmid DNA encapsulation" abstract -----	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/PL2015/050011

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 2009031911 A1	12-03-2009
WO 2009120247 A2	01-10-2009	CA 2710983 A1	01-10-2009
		US 2011038941 A1	17-02-2011
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		WO 2012052898 A1	26-04-2012