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(54) Title: NOVEL THERMOSENSITIVE LIPOSOMES CONTAINING THERAPEUTIC AGENTS

(57) Abstract: A thermosensitive liposome for the delivery of active agents and a composition thereof are disclosed, wherein the liposome comprises at least one phosphatidylcholine, at least one phosphatidylglycerol and at least one lysolipid, and the gel to liquid phase transition temperature of said liposome is 39.0 °C to 45 °C.

NOVEL THERMOSENSITIVE LIPOSOMES CONTAINING THERAPEUTIC AGENTS

BACKGROUND

[0001] Liposomes have been used to deliver a wide variety of therapeutic agents. For example, antitumor agents such as actinomycin (US patent no. 3,993,754), anthracyclins (US patent no. 4,863,739), and vinca alkaloids (US patent no. 4,952,408) have been encapsulated in liposomes. More recently, thermosensitive liposomes containing active agents have been prepared and used to deliver the active agent to specific targets in a subject (US patent nos. 6,200,598 and 6,726,925, and Yatvin et al., Science 204:188 (1979). In use, thermosensitive liposomes are delivered to a subject and a target area in the subject is heated. When the thermosensitive liposome reaches the heated area, it undergoes a gel to liquid phase transition and releases the active agent. The success of this technique requires a liposome with a gel to liquid phase transition temperature within the range of temperatures that are obtainable in the subject.

[0002] There remains a need in the art for liposomes formulated to encapsulate a therapeutic agent such as an antitumor agent and undergo a gel to liquid phase transition at a temperature obtainable in a subject. This need and others are met by the present invention.

SUMMARY OF THE INVENTION

[0003] In one embodiment, the present invention provides a thermosensitive liposome. Thermosensitive liposomes of the invention typically comprise at least one phosphatidycholine, at least one phosphatidyglycerol, and at least one lysolipid. Thermosensitive liposomes of the invention will generally have a gel to liquid phase transition temperature of from about 39.0°C to about 45°C. Optionally, thermosensitive liposomes of the invention may comprise one or more additional lipid components, for example, may comprise a PEGylated phospholipid. A thermosensitive liposome according to the invention may also comprise one or more active agents, for example, therapeutic agents, imaging agents, and combinations thereof.

[0004] In a particular embodiment, the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is

distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC) and the thermosensitive liposome comprises a PEGylated phospholipid, for example, PEG-2000 modified distearoylphosphatidylethanolamine (DSPE-PEG2000). Thermosensitive liposomes of the invention may comprise a phosphatidycholine, a phosphatidyglycerol, a lysolipid and a PEGylated phospholipid in any ratio so long as the gel to liquid phase transition temperature is in the range of from about 39°C to about 45°C. One example of a suitable ratio is DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

[0005] Thermosensitive liposomes of the invention may comprise one or more active agents. Any active agent known to those skilled in the art may be used in combination with the thermosensitive liposomes of the invention to deliver the active agent to a selected site in a subject. As used herein, a subject is any mammal, in particular, humans cats and dogs. In one embodiment, thermosensitive liposomes of the invention comprise one or more anticancer agents. Examples of suitable anticancer agents include, but are not limited to, alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds. In a particular embodiment, thermosensitive liposomes of the invention may comprise an anthracycline antibiotic, for example, docetaxel.

[0006] The present invention also provides pharmaceutical compositions comprising thermosensitive liposomes of the invention comprising an active agent. In such pharmaceutical compositions, thermosensitive liposomes of the invention typically comprise at least one phosphatidycholine, at least one phosphatidyglycerol, at least one lysolipid, and have a gel to liquid phase transition temperature of from about 39.0°C to about 45°C. Thermosensitive liposomes for use in pharmaceutical compositions of the invention may further comprise a PEGylated phospholipid.

[0007] In one example of a suitable thermosensitive liposome for use in the pharmaceutical compositions of the invention the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC) and the thermosensitive liposome

comprises a PEGylated phospholipid, for example, PEG-2000 modified distearoylphosphatidylethanolamine (DSPE-PEG2000). Such thermosensitive liposomes of the invention may comprise a phosphatidycholine, a phosphatidyglycerol, a lysolipid and a PEGylated phospholipid in any ratio so long as the gel to liquid phase transition temperature is in the range of from about 39°C to about 45°C. One example of a suitable ratio is DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

[0008] Any active agent may be included in the pharmaceutical compositions of the invention, for example, therapeutic agents and/or imaging agents. In one embodiment, an active agent may be an anticancer agent. Examples of suitable anticancer agents include, but are not limited to, alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds. In a particular embodiment, thermosensitive liposomes of the invention may comprise an anthracycline antibiotic, for example, docetaxel. In a particular embodiment, thermosensitive liposomes of the invention may comprise a platinum containing compound, for example, carboplatin.

[0009] The present invention also provides methods of treating disease in a subject using thermosensitive liposomes of the invention. Such thermosensitive liposomes will typically comprise one or more active agents that can be used to treat the disease. A method of treating a disease in a subject in need thereof according to the invention may comprise administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a temperature sensitive liposome comprising an active agent, where in the liposome comprises at least one phosphatidycholine, at least one phosphatidyglycerol, at least one lysolipid, and has a gel to liquid phase transition temperature of from about 39.0°C to about 45°C. The portion of the subject comprising some or all of the diseased tissue is then heated to a temperature sufficient to cause the gel-liquid transition of the liposome thereby releasing the active agent in close proximity to the diseased tissue. Thermosensitive liposomes for use in the methods of the invention may also comprise a PEGylated phospholipid, for example, DSPE-PEG2000.

[0010] In one example of thermosensitive liposomes for use in the methods of the invention, the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC). Such thermosensitive liposomes for use in the methods of the invention may comprise a phosphatidycholine, a phosphatidyglycerol, a lysolipid and a PEGylated phospholipid in any ratio so long as the gel to liquid phase transition temperature is in the range of from about 39°C to about 45°C. One example of a suitable ratio is DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

[0011] In one embodiment, the present invention comprises a method of treating cancer in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a temperature sensitive liposome comprising an anticancer agent, wherein the liposome comprises at least one phosphatidycholine, at least one phosphatidyglycerol, at least one lysolipid, and has a gel to liquid phase transition temperature of from about 39.0°C to about 45°C. Examples of suitable anticancer agents include, but are not limited to, alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds. In one embodiment, the anticancer agent may be an anthracycline antibiotic, for example, docetaxel. In a particular embodiment, thermosensitive liposomes of the invention may comprise a platinum containing compound, for example, carboplatin.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 is a DSC trace showing the gel-liquid phase transition of an exemplary thermosensitive liposome of the invention.

[0013] Figure 2 is a graph of particle size as a function of the amount of cryoprotectant in the liposome preparation lyophilized.

[0014] Figure 3 is a graph of particle size upon rehydration of lyophilized liposomes of the invention as a function of water content of the liposomes at various rates of temperature during freezing.

[0015] Figure 4A is a schematic of the protocol used to test the effects of standing on particle size of rehydrated liposomes of the invention. Figure 4B is a graph showing the particle size distribution of rehydrated liposomes of the invention over a one hour time period.

DETAILED DESCRIPTION OF THE INVENTION

[0016] Thermosensitive liposomes of the invention typically comprise one or more phosphatidycholines. Suitable examples of phosphatidylcholines that can be used in the practice of the invention include, but are not limited to, 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).

[0017] Thermosensitive liposomes of the invention typically comprise one or more phosphatidylglycerols. Suitable examples of phosphatidylglycerols include, but are not limited to, 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG), and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG).

[0018] Thermosensitive liposomes of the invention typically comprise one or more lysolipids. As used herein "lysolipid" refers to any derivative of phosphatidic acid (1,2-diacyl-*sn* glycero-3-phosphate) that contains only one acyl chain covalently linked to the glycerol moiety. Derivatives of phosphatidic acid include, but are not limited to, phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine. Any lysolipid known to those skilled in the art may be used in the practice of the invention.

[0019] Active agents

[0020] Thermosensitive liposomes of the invention may be formulated to comprise one or more active agent. As used herein, "active agent" includes any compound desired to be delivered to a specific site in a subject. Any active agent may be used in the practice of the invention.

[0021] Anticancer agents may be used as the active agents in the thermosensitive liposomes of the invention. Suitable examples of anticancer agents include:

[0022] alkylating agents, for example, nitrogen mustards (e.g., Chlorambucil, Chlormethine, Cyclophosphamide, Ifosfamide, Melphalan, nitrosoureas (e.g., Carmustine, Fotemustine, Lomustine, Streptozocin), platinum containing compounds (e.g., Carboplatin, Cisplatin, Oxaliplatin, BBR3464), Busulfan, Dacarbazine, Mechlorethamine, Procarbazine, Temozolomide, ThioTEPA, and Uramustine;

[0023] antimetabolites that target, for example, folic acid (e.g., aminopterin, methotrexate, pemetrexed, raltitrexed), purine metabolism (e.g., cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, thioguanine), pyrimidine metabolism (e.g., capecitabine, cytarabine, fluorouracil, floxuridine, gemcitabine);

[0024] spindle poison plant alkaloids, for example, taxanes (e.g., docetaxel, paclitaxel) and vinca (e.g., vinblastine, vincristine, vindesine, vinorelbine);

[0025] cytotoxic/antitumor antibiotics, for example, anthracycline antibiotics (e.g., daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, valrubicin, carinomycin, nacetyladriamycin, rubidazone, 5-imidodaunomycin, N30 acetyldaunomycin, and epirubicin), bleomycin, hydroxyurea, mitomycin, and actinomycin;

[0026] topoisomerase inhibitors, for example, camptothecines (e.g., camptothecin, topotecan, irinotecan), podophyllum (e.g., etoposide, teniposide).

[0027] monoclonal antibodies, for example, Alemtuzumab, Bevacizumab, Cetuximab, Gemtuzumab, Panitumumab, Rituximab, Tositumomab, and Trastuzumab;

[0028] photosensitizers, for example, aminolevulinic acid, methyl aminolevulinate, porfimer sodium, and verteporfin;

[0029] kinase inhibitors, for example, Dasatinib, Erlotinib, Gefitinib, Imatinib, Lapatinib, Nilotinib, Sorafenib, Sunitinib, and Vandetanib.

[0030] In additional embodiments, the thermosensitive liposomes of the invention can comprise more than one antineoplastic agent, or more than one thermosensitive liposome can be used in the methods of the invention, each of which comprises different active agents, for example, different anticancer agents.

[0031] Additional active agents that can be used in the practice of the present invention include, but are not limited to antibiotics, antifungals, anti-inflammatory agents, immunosuppressive agents, anti-infective agents, antivirals, anthelminthic, and antiparasitic compounds.

[0032] Methods of use

[0033] Thermosensitive liposomes of the invention can be administered to a subject using any suitable route, for example, intravenous administration, intraarterial administration, intramuscular administration, peritoneal administration, as well as other suitable routes. Tissues which can be treating using the methods of the present invention include, but are not limited to, liver, kidney, bone, soft tissue, muscle, adrenal tissue and breast. Tissues that can be treated include both cancerous tissue, otherwise diseased or compromised tissue, as well as healthy tissue if so desired.

[0034] The dose of active agent administered to the subject using the thermosensitive liposomes of the invention is readily determined by those of skill in the art, and suitably is administered intravenously over an extended time period, for example over about 15 minutes to about 1 hour.

[0035] The dose of active agent may be adjusted as is known in the art depending upon the active agent comprised in the carrier.

[0036] The target tissue of the subject may be heated before and/or during and/or after administration of the thermosensitive liposomes of the invention. In one embodiment, the target tissue is heated first (for example, for 10 to 30 minutes) and the liposomes of the invention are delivered into the subject as soon after heating as practicable. In another embodiment, thermosensitive liposomes of the invention are delivered to the subject and the target tissue is heated as soon as practicable after the administration.

[0037] Any suitable means of heating the target tissue may be used, for example, application of radiofrequency radiation, application of ultrasound which may be high intensity focused ultrasound, application of microwave radiation, and application of warmed material (e.g., water bath).

[0038] It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein can be made without departing from the scope of the invention or any

embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

EXAMPLE 1

[0039] Preparation and characterization of a thermosensitive taxotere liposome

[0040] The following materials were used in the preparation of the liposomes of the invention: dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylglycerol (DSPG), monostearoylphosphatidylcholine (MSPC), PEGylated distearoylphosphatidylethanolamine (DSPE-PEG2000), NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, lactose, Na_2CO_3, sodium citrate$\cdot H_2O$, citric acid, $CHCl_3$, methanol, ethanol, and distilled water.

[0041] The following equipment was used in the preparation of the liposomes of the invention: water bath, rotary evaporator, homogenizer-extruder, freeze dryer, laser light scattering particle sizer (Smypatec Nanophox), and thermometer.

[0042] Method for preparing a 20 ml batch of docetaxel containing liposomes

[0043] Measure out the following components in the amounts indicated.

Component	DPPC	DSPG	MSPC	DSPE-PEG2000	Taxotere
Quantity	669mg	75mg	75mg	75mg	37.5mg

[0044] Liposomes of the invention may comprise a phosphatidyl choline at an amount from about 70% to about 80% of the total weight of lipid. Liposomes of the invention may comprise a phosphatidyl glycerol at an amount from about 5% to about 10% of the total weight of lipid. Liposomes of the invention may comprise a lysolipid at an amount from about 5% to about 10% of the total weight of lipid. Liposomes of the invention may comprise a PEGylated lipid at an amount from about 5% to about 10% of the total weight of lipid. One suitable example is DPPC:DSPG:MSPG:DSPE-PEG200 at a weight percent ratio 75:8:8:8. Liposomes of the invention may be loaded with active agent at an active agent to lipid weight ratio of from about 0.01 to about 2.0, from about 0.02 to about 1.0, from about 0.02 to about 0.5, from about 0.02

to about 0.25, from about 0.02 to about 0.1, from about 0.02 to about 0.09, from about 0.02 to about 0.08, from about 0.02 to about 0.07, from about 0.02 to about 0.06, from about 0.02 to about 0.05, from about 0.02 to about 0.04, or from about 0.02 to about 0.03.

[0045] Dissolve above materials with $CHCl_3$/Methanol (3:1) at 55 °C. Then remove organic solvent with rotary evaporator. This may be accomplished by rotary evaporation at 55°C for 1 hour. After drying, nitrogen may be blown over the dried material for a suitable period of time, for example, 5 minutes.

[0046] The dried material is then rehydrated. A suitable rehydration solution is phosphate buffered saline (PBS) to which lactose or other stabilizing materials (e.g., sugars) may be added. A suitable protocol for rehydration is to add 20 ml of PBS-5% lactose solution (pH 7.3 ± 0.2) and rotating on the rotary evaporator at atmospheric pressure for 1 hour at 50°C. After rehydration the solution can be degassed under reduced pressure to remove bubbles.

[0047] After hydration, the particle size of the liposomes may be adjusted to the desired range , for example, 100±15 nm. A suitable extrusion protocol is to use a C3 homogenizer/extruder with a 200 nm filter and extrude three times. Change to a 100 nm filter and extrude three times. Finally, change to an 80 nm filter and extrude three times. The particle size distribution of the liposomes can be measured using any suitable technique, for example, using Photon Crosscorrelation Spectroscopy (PCCS) and a Nanophox sensor (Sympatec GmbH). After extrusion, sterilize by 0.22 μm filtration.

[0048] After sterilization, the liposome is filled into vial and lyophilized. The lyophilization program is as follows: -50°C 2h, -45°C 1h, -35°C 10h, -15°C 5h, 0°C 2h, 10°C 2h, 20°C 6h.

[0049] Another suitable method for preparation of the liposomes of the invention is as follows:

[0050] Dissolve the same components as above in $CHCl_3$/Methanol (3:1) at 55 °C. Remove organic solvent with rotary evaporator as above. Rehydrate with 20 ml of PBS-5% lactose solution at 50 °C as above. Place the rehydrated material in a homogenizer and process at 15,000 psi for 5 minutes to reduce the particle size. Take the homogenized material and use an extruder with a 100 nm filter and extrude six

times to reduce particle size to 100±15 nm (100 nm×6) and then sterilize by 0.22 μm filtration. After sterilization, the liposome is filled into vial and lyophilized. The lyophilization program is as follows: -50°C 2h, -45°C 1h, -35°C 10h, -15°C 5h, 0°C 2h, 10°C 2h, 20°C 6h.

[0051] Analytical methods

[0052] Morphology of the liposomes can be analyzed by electron microscopy. Liposomes were negative stained with phosphotungstic acid and transferred to copper mesh. The water was allowed to evaporate and the samples were observed under the electronic microscope. Liposomes prepared by the methods of the invention were homogeneous when viewed under the electron microscope.

[0053] The percentage of drug encapsulated (Encapsulation%) was measured for the liposomes prepared as described above. Encapsulation % = Encapsulated drug/Total drug X 100%. The Encapsulation% was determined as follows: 1 ml of the liposome was centrifuged at 6000 rpm for 5 min. The docetaxel in the supernatant was measured by HPLC. The docetaxel content of the liposomes was determined by extracting the docetaxel from the liposomes and measuring the extracted docetaxel by HPLC. For extraction, 0.1 ml of liposome was diluted with water:acetonitrile (45:55) to 0.5ml. 4 ml tert-butyl methyl ether was added and mixed for 30 seconds. The mixture was centrifuge the mixture at 300 g for 15 min. 3 ml of the organic layer was removed and dried by rotary evaporation. The dried material was resuspended in 200μl water:acetonitrile (45:55) and 5-10μl was inject on the HPLC for analysis.

[0054] The HPLC analysis was conducted under the following conditions: a Venusil C 18 column (Reverse phase C 18 column) was used with a mobile phase of water:acetonitrile (45:55) at lml/min. Column temperature was 30 °C. UV detection was set at 230mn. Under these conditions, the drug detection limit is between 20 - 800ng.

[0055] The ability of the above protocol to recover docetaxel in a sample was determined. To 0.lml of liposomes prepared as described above 0.1 ml of docetaxel standard solution was added. The sample was diluted with water:acetonitrile (45:55) to 0.5ml. 4 ml tert-butyl methyl ether was added and the sample mixed for 30 seconds. The sample is then centrifuged at 300 g for 15 min. 3 ml of the organic layer is dried by rotary evaporation. 200μl of water:acetonitrile (45:55) is added to

the residue and 5-10μl is then injected on the HPLC. The following table provides the recovery rate at various concentrations of docetaxel.

Drug conc.	Recovery %	Recovery %	Recovery %	mean
80 μg/ml	100.34	99.97	99.41	99.91
100 μg/ml	99.15	96.63	98.08	97.95
120 μg/ml	97.68	99.01	99.41	98.70

[0056] The phase transition temperature of the liposomes prepared according to the invention was determined. Differential Scanning Calorimetry (DSC) measurements were performed using a Q100 (TA Instruments, Inc. New Castle DE) with empty hermetically sealed aluminum pans as reference. The lipid concentration was made 20 mg/ml and 10 μl of liposome suspension was carefully placed and sealed in the aluminum hermetic pans. The scan rate was set at 2°C per minute. Figure 1 shows a DSC trace obtained with the liposomes of the invention. DSC spectrum show that the taxotere thermosensitive liposome phase transition temperature is at about 42℃.

[0057] The stability of the liposomes prepared by the above methods was assessed by periodically measuring the particle size during storage. The results in the table below show that liposomes prepared as above are stable for at least 3 months.

[0058] Particle size of the liposome

Time	Before lyo	After lyo	1 month	2 month	3 month
Size	97nm	101nm	107nm	106nm	106nm

[0059] 3 months stability test of Lot 060322

[0060] The drug content was monitored as well. The results showed that after lyophilization, the liposome is stable at 2-8°C for at least 3 months.

[0061] Drug content of the liposome

Time	After lyo	1 month	2 months	3 months
Taxotere (mg/ml)	1.132	1.131	1.132	1.130

[0062] The drug encapsulation rate was monitored as well. The results showed that after lyophilization, the amount of drug encapsulated by liposome is stable at 2-8°C for at least 3 months.

[0063] Drug encapsulation

Time	After lyo	1 month	2 months	3 months
Encapsulation %	99.0	98.6	98.1	98.3

[0064] Different cryoprotectants were tested for their effect on particle size during lyophilization. Lactose, trehalose, sucrose and mannitol were tested. The results showed that lactose and sucrose are more effective than mannitol and trehalose. Figure 2 shows a graph of particle size as a function of the % by weight of cryoprotectant present in the solution lyophilized.

[0065] The rate at which the liposomes are frozen for lyophilization and the water content of the liposomes has an effect on the particle size. Figure 3 shows a graph of particle size as a function of the water content of the liposomes at three different freezing rates. has an effect on the particle size.

[0066] Rehydration media has also impact on the liposome particle size. Water, D5W and 0.9% NaCl were tested. 0.9% NaCl and 5% dextrose in water maintain the liposome particle size. The following table shows the results of two different liposome formations with three independent measurements. The average diameter of the liposomes is provided in nanometers (nm). Formulation F4-1 had the following components DPPC : DSPG : DSPE-PEG : MSPC : Docetaxel at the following weight% 71.56 : 8.15 : 8.24 : 8.02 : 4.00 and F4-2 had the same components at 71.78 : 8.06 : 8.10 : 8.07 : 3.98 weight %.

Rehydration Media	Formulation	1(nm)	2(nm)	3(nm)
Water	F4-1	136	133	139
	F4-2	132	128	141
D5W	F4-1	97	101	103
	F4-2	102	104	105
0.9% NaCl	F4-1	101	105	102
	F4-2	106	103	101

[0067] The stability of the liposomes after rehydration was examined. The lyophilized liposome was rehydrated with 0.9% NaCl and tested as shown schematically in Figure 4A. The particle size distribution was monitored with dynamic light scattering apparatus by repeated scans over a period of 1 hour. The results show that the particle distribution of rehydrated liposome is stable in 1 hour (Figure 4B).

[0068] The lyophilized liposome was stored at various temperatures for 9 months. The liposome was tested at 0, 1, 3, 6 and 9 months. The encapsulation % and average particle size were tested. The results in the following table show that liposome was stable up to 9 months at 4°C.

Storage time(month)	Storage temperature -20°C	Storage temperature 4°C	Storage temperature 20°C
0	92.1 %(98nm)	92.1 %(98nm)	92.1 %(98nm)
1	91.8%(101nm)	91.5%(103nm)	91.7%(107nm)
2	91.6%(102nm)	91.4%(105nm)	91.1%(106nm)
3	91.5%(105nm)	91.2%(106nm)	90.8%(109nm)
6	91.4%(108nm)	91.3%(107nm)	90.9%(111nm)
9	90.8%(112nm)	90.5%(110nm)	90.2%(116nm)

EXAMPLE 2

[0069] In vivo drug distribution obtained with the liposomes of the invention compared to that obtained with free docetaxel.

[0070] Six female BALB/c mice (20±2 g) were tested with liposomes at a dose of 10mg/kg administered by tail vein injection. The mice were randomly separated into 3 groups. The mice were anesthetized and put on a Styrofoam board with a hole in it. One leg of the mouse was pulled through the hole to other side of the board. The board was placed in a water bath to heat the leg at 43.5±0.5℃ for 15 min. The drug was then injected (Taxotere-containing liposome of the invention or Taxotere as control). The mice were heated for 30min. after injection. Muscles from the heated and non heated legs were excised. The drug content in the muscles was analyzed by HPLC. The results are showed in following table.

	Docetaxel Liposome		Docetaxel	
	A_{drug}/A_{ref} % (heated leg)	A_{drug}/A_{ref} % (non-heated leg)	A_{drug}/A_{ref} % (heated leg)	A_{drug}/A_{ref} % (non-heated leg)
Group 1	13.3	4.9	5.46	5.48
Group 2	14.3	5.60	6.53	6.29
Group 3	11.1	5.69	5.48	5.32

[0071] The data show that the temperature sensitive liposome delivered more that twice as much docetaxel to the heated leg than to the non-heated leg.

EXAMPLE 3

[0072] In vivo drug distribution obtained with the liposomes of the invention compared to that obtained with non-thermosensitive docetaxel-containing liposome

[0073] The thermosensitive liposome and non thermosensitive liposome were made as the formula showed the following table:

Composition	Docetaxel	DPPC	DSPG	PEG-DSPE	MSPC
Thermosensitive	25mg	450mg	50mg	50mg	50mg
Non- thermosensitive	25mg	450mg	50mg	50mg	0

[0074] Six female BALB/c mice (20±2 g) were tested with liposomes at a dose of 10mg/kg administered by tail vein injection. The mice were randomly separated into 2 groups. The mice were anesthetized and put on a Styrofoam board with a hole in it. One leg of the mouse was pulled through the hole to other side of the board. The board was placed in a water bath to heat the leg at 43.5±0.5℃ for 15 min. The drug was then injected (Taxotere-containing liposome of the invention or non-thermosensitive Taxotere-containing liposomes as control). The mice were heated for 30min. after injection. Muscles from the heated and non heated legs were excised. The drug content in the muscles was analyzed by HPLC. The results are showed in following table.

Group	Thermosensitive Liposome		Non thermosensitive liposome	
Heat/No heat	Heated	Non heated	Heated	Non heated
A_{drug}/A_{ref} %	0.704	0.428	0.443	0.444

[0075] In the thermosensitive liposome group, drug concentration in heated tissue is about 2 times higher than non heated tissue. In docetaxel injection (Example 2) and non thermosensitive liposome groups, the drug concentration is the same between heated and non heated tissue. These results showed that thermosensitive liposome did release the drug at experimental conditions.

EXAMPLE 4

[0076] In vivo efficacy of docetaxel delivered using the liposomes of the invention compared to that of free docetaxel in mice bearing Lewis lung cancer.

[0077] Twelve female Kunming mice, between 7-9 weeks old weighing 20±2g were used. Docetaxel-containing thermosensitive liposomes of the invention were prepared as described above. Treated animals were injected with 75mg/m^2 docetaxel [

1gS(cm2)=0.8762+0.6931g W(g)] either in a thermosensitive liposome of the invention or as free docetaxel which were stored at 2-8°C until administration.

[0078] Kunming mice were purchased from the Animal Center of China Medical Science Institute. Animals were housed in appropriate isolated caging with sterile rodent food and a 12-h light/dark cycle. The mouse Lewis lung carcinoma cell was used in this study. The right lower leg of each mouse was implanted s.c. with 3 x 10^6 cells in 0.1 ml of PBS. Tumors were allowed to grow to 4-6mm in diameter before starting treatment. Mice were carefully monitored for general well-being, weight, and tumor volume. Mice with weight loss of 15% of the initial weight or tumor volume 1000 mm^3 would be scheduled to be euthanized.

[0079] The 12 mice were stratified by tumor volume and randomized to 3 treatment groups: saline, free docetaxel and the thermosensitive liposomes of the invention.

Group	Animals /Group	Treatment	Dose m m^2	Time of Heat Mins
1	2	Saline	0	30
2	4	Docetaxel injection	75	30
3	6	Docetaxel Thermosensitive Liposome	75	30

[0080] The treatment was started at day 8 of the tumor implantation. Mice in all treatment groups were anesthetized with an IP injection of pentobarbital (80 mg/kg); treatment was administered in a volume of 0.2 ml via tail vein injection. This dose of anesthesia provided adequate immobilization for the 1-h treatment period.

[0081] Except for the saline group, all treatment groups were given an equivalent dose of 75 mg/m^2 of docetaxel. Immediately after injection, the mice were positioned in specially designed holders that allowed the isolated leg tumor to be placed in a water bath for 30 minutes. The water bath temperature was set at 43°C. This water bath temperature has been calibrated previously to give tumor temperatures of 42°C. The treatment was repeated at day 12 and 16. All the mice were sacrificed at day 18. The tumors were surgically excised and the tumor weights were recorded. The tumor growth inhibition was calculated as follows:

[0082] Tumor Inhibit Ratio = (Vs-Vx)/ Vs

[0083] Where: Vs is tumor volume of saline group, Vx is the tumor volume of treatment group.

[0084] The results are shown in the following table.

Group	Mice No	Tumor Weight	Average	Inhibition%
Saline	1	5.874	4.537	0
	2	3.199		
Docetaxel Injection	3	0.500	1.002	77.91
	4	0.118		
	5	1.380		
	6	2.010		
Docetaxel Thermosensitive Liposome	7	0.031	0.0785	98.27
	8	0.009		
	9	0.078		
	10	0.152		
	11	0.151		
	12	0.050		

[0085] Delivering docetaxel in a thermosensitive liposome formulation and local heating of the tumor resulted in greater tumor inhibition than delivery of docetaxel alone. In two mice treated with thermosensitive liposome, the tumors almost disappeared.

[0086] All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A thermosensitive liposome comprising at least one phosphatidycholine, at least one phosphatidyglycerol, and at least one lysolipid, wherein the liposome has a gel to liquid phase transition temperature of from about 39.0°C to about 45°C.

2. A thermosensitive liposome according to claim 1, further comprising a PEGylated phospholipid.

3. A thermosensitive liposome according to claim 1, further comprising an active agent.

4. A thermosensitive liposome according to claim 1, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC).

5. A thermosensitive liposome according to claim 4, further comprising a PEGylated phospholipid.

6. A thermosensitive liposome according to claim 5, wherein the PEGylated lipid is PEG-2000 modified distearoylphosphatidylethanolamine (DSPE-PEG2000).

7. A thermosensitive liposome according to claim 1, comprising DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

8. A thermosensitive liposome according to claim 3, wherein the active agent is an anticancer agent.

9. A thermosensitive liposome according to claim 8, wherein the anticancer agent is selected from the group consisting of alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds.

10. A thermosensitive liposome according to claim 9, wherein the anticancer agent is an anthracycline antibiotic.

11. A thermosensitive liposome according to claim 9, wherein the anticancer agent is docetaxel.

12. A thermosensitive liposome according to claim 9, wherein the anticancer agent is a platinum containing compound.

13. A thermosensitive liposome according to claim 9, wherein the anticancer agent is carboplatin.

14. A pharmaceutical composition comprising a thermosensitive liposome comprising an active agent, where in the liposome comprises at least one phosphatidycholine, at least one phosphatidyglycerol, at least one lysolipid, and has a gel to liquid phase transition temperature of from about 39.0°C to about 45°C.

15. A pharmaceutical composition according to claim 14, wherein the liposome further comprises a PEGylated phospholipid.

16. A pharmaceutical composition according to claim 14, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC).

17. A pharmaceutical composition according to claim 16, wherein the liposome further comprises a PEGylated phospholipid.

18. A pharmaceutical composition according to claim 17, wherein the PEGylated lipid is PEG-2000 modified distearoylphosphatidylethanolamine (DSPE-PEG2000).

19. A pharmaceutical composition according to claim 14, wherein the liposome comprises DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

20. A pharmaceutical composition according to claim 19, wherein the active agent is an anticancer agent.

21. A pharmaceutical composition according to claim 20, wherein the anticancer agent is selected from the group consisting of alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds.

22. A pharmaceutical composition according to claim 20, wherein the anticancer agent is an anthracycline antibiotic.

23. A pharmaceutical composition according to claim 20, wherein the anticancer agent is docetaxel.

24. A pharmaceutical composition according to claim 20, wherein the anticancer agent is a platinum containing compound.

25. A pharmaceutical composition according to claim 20, wherein the anticancer agent is carboplatin.

26. A method of treating a disease in a subject in need thereof, comprising:

administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a temperature sensitive liposome comprising an active agent, where in the liposome comprises at least one phosphatidycholine, at least one phosphatidyglycerol, at least one lysolipid, and has a gel to liquid phase transition temperature of from about 39.0°C to about 45°C; and

heating an area of the subject comprising all or a portion of the disease.

27. A method according to claim 26, wherein the liposome further comprises a PEGylated phospholipid.

28. A method according to claim 26, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine (DPPC), the phosphatidylglycerol is distearoylphosphatidylglycerol (DSPG), and the lysolipid is monostearoylphosphatidylcholine (MSPC).

29. A method according to claim 28, wherein the liposome further comprises a PEGylated phospholipid.

30. A method according to claim 29, wherein the PEGylated lipid is PEG-2000 modified distearoylphosphatidylethanolamine (DSPE-PEG2000).

31. A method according to claim 26, wherein the liposome comprises DPPC : DSPG : MSPC : DSPE-PEG2000 : active agent in the ratio of about 72 : 8 : 8 : 8 : 4 on a weight percentage basis.

32. A method according to claim 26, wherein the disease is cancer and the active agent is an anticancer agent.

33. A method according to claim 32, wherein the anticancer agent is selected from the group consisting of alkylating agents, antimetabolites, antitumor antibiotics, anthracycline antibiotics, plant alkaloids, taxol derivatives, topoisomerase inhibitors, monoclonal antibodies, photosensitizers, kinase inhibitors, and platinum containing compounds.

34. A method according to claim 32, wherein the anticancer agent is an anthracycline antibiotic.

35. A method according to claim 32 wherein the anticancer agent is docetaxel.

36. A method according to claim 32, wherein the anticancer agent is aplatinum containing compound.

37. A method according to claim 32 wherein the anticancer agent is carboplatin.

FIGURE 1

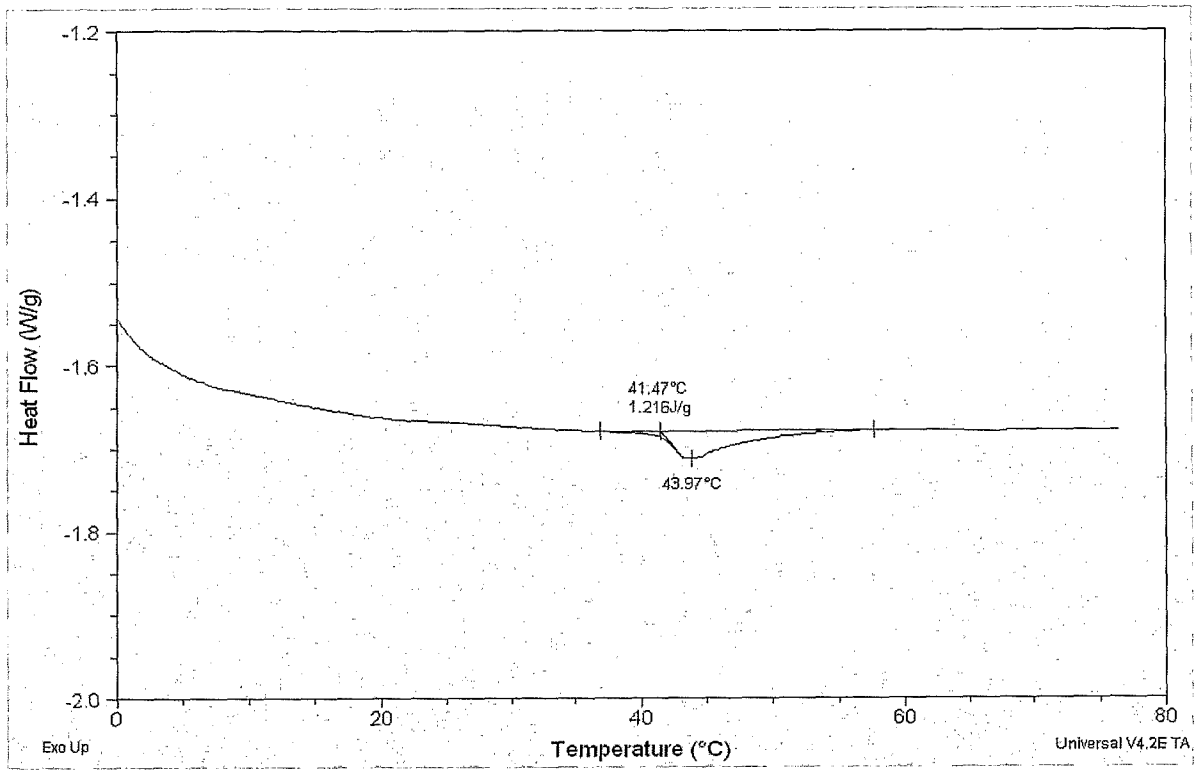

-1.2
-1.4
-1.6
-1.8
-2.0
Heat Flow (W/g)
41.47°C
1.216J/g
43.97°C
0
20
40
60
80
Exo Up
Temperature (°C)
Universal V4.2E TA

FIGURE 2

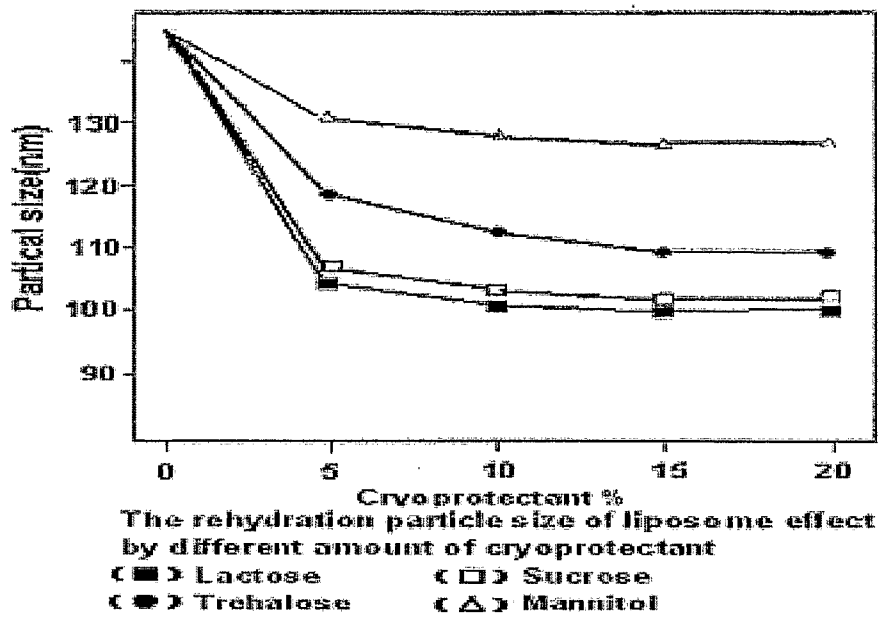
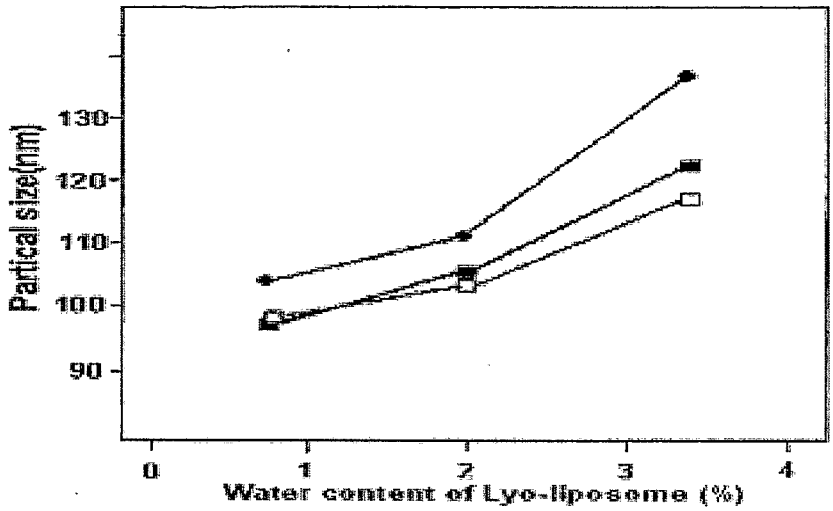

Partical size(nm)
130
120
110
100
90
0
5
10
15
20
Cryoprotectant %
The rehydration particle size of liposome effect by different amount of cryoprotectant
Lactose
Sucrose
Trehalose
Mannitol

FIGURE 3



The rehydration particle size of liposome effect by the different mode of freeze and water content of lyo-liposome
(■) 0.5°C/min (□)1.5°C/min (●) >20°C/min

FIGURE 4A

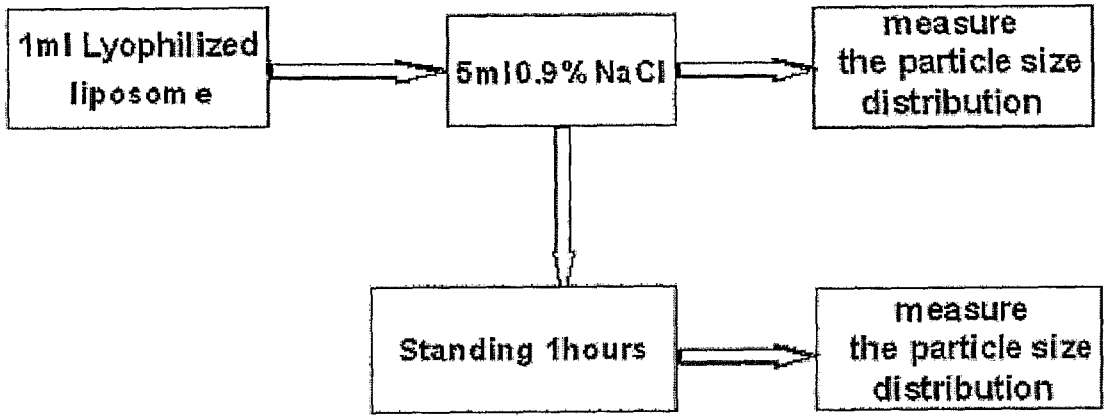
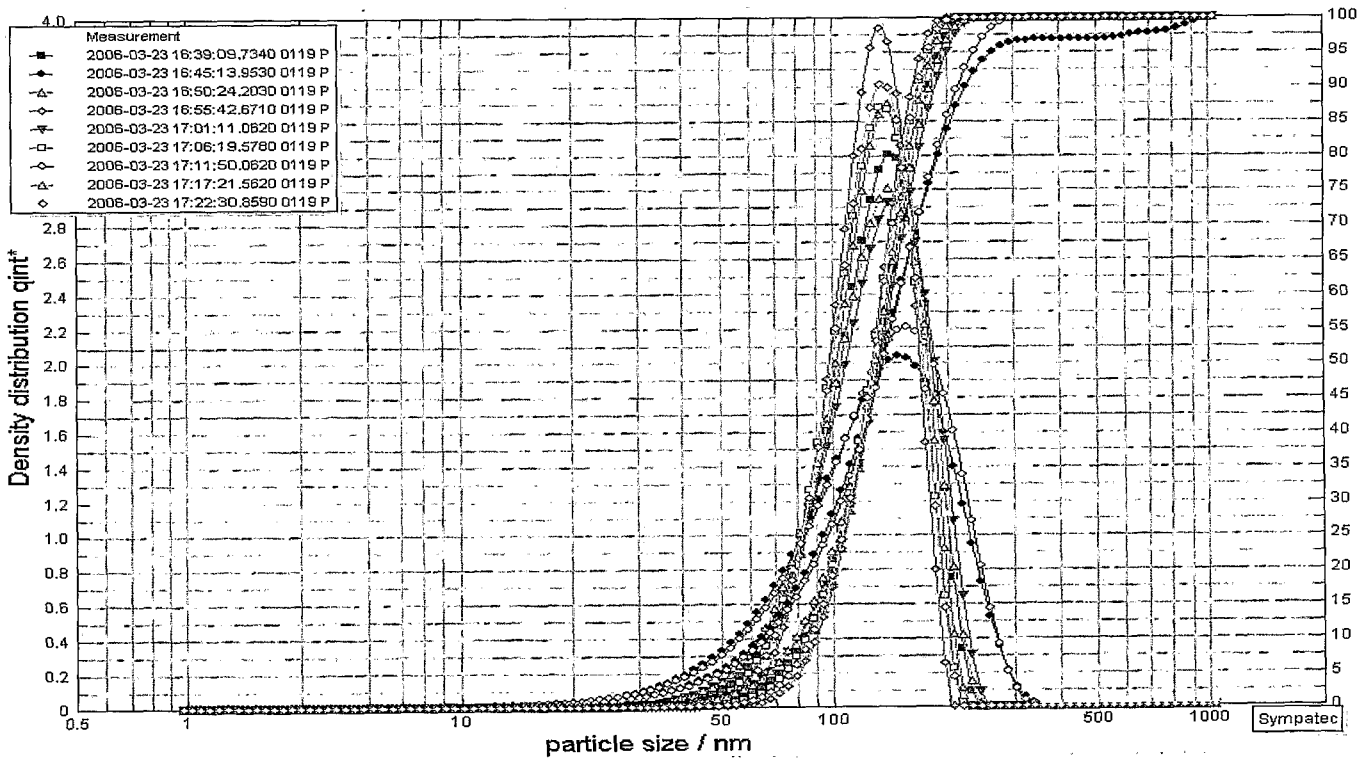


FIGURE 4B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2007/003128

A. CLASSIFICATION OF SUBJECT MATTER

A61K9/127 (2006.01) i

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)

IPC: A61K

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)

EPODOC, WPI, PAJ, CA, MEDLINE, CPRS, CNKI: liposome, thermosensitive, temperature sensitive, phase transition temperature, phosphatidylcholine, phosphatidylglycerol, lysolipid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6726925 B1 (Duke University), 27 Apr. 2004 (27.04.2004), see example 5, claims 1, 6, 12, 13, 17, 18	1-25

☐ Further documents are listed in the continuation of Box C. ☒ 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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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
4 Aug. 2008 (04.08.2008)	**21 Aug. 2008 (21.08.2008)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer LIU,Qiming Telephone No. (86-10)62411122

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2007/003128

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-37
because they relate to subject matter not required to be searched by this Authority, namely:
the subject-matter of claims 26-37 is directed to a method of therapeutical treatment.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2007/003128

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
US 6726925 B1	27.04.2004	WO9965466 A	23.12.1999
		CA2335250 A	23.12.1999
		AU4556499 A	05.01.2000
		US6200598 B	13.03.2001
		EP1089713 AB	11.04.2001
		EP19990928513	09.06.1999
		JP2002518317T T	25.06.2002
		AU749806B B	04.07.2002
		US2002102298 A	01.08.2002