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# DESCRIPTION

## *Technical field*

[0001] The present invention generally relates to the field of ultrasound contrast-agents (USCA). In particular, it relates to a freeze-dried composition according to claim 1, which may be reconstituted for preparing a suspension of gas-filled microvesicles with calibrated size, useful in diagnostic or therapeutic applications. It further relates to the method for the preparation of such freeze-dried composition.

## *Background of the invention*

[0002] Calibrated-size microvesicles (CMV) are a new generation of gaseous microbubbles having a narrow calibrated and controlled size distribution (mean diameter sizes between 3 and 8  $\mu\text{m}$ ) compared to commercially available polydisperse microbubble ultrasound contrast-agents (USCA). These calibrated-size microbubbles are designed to enhance imaging sensitivity and improve efficiency to deliver drug and gene to specific organs. Calibrated microvesicles can be produced using various techniques: decantation, mechanical filtration, centrifugation, bubble sorting and flow-focusing. Particularly, the flow-focusing technology allows the manufacturing of calibrated microvesicles (typically with a geometric standard deviation (GSD) values between 1.05 and 1.08) in a highly reproducible way at a reasonable production rate ( $\sim 60$  million bubbles per minute), with acceptable concentrations of suspended microvesicles (e.g. between  $3 \times 10^8$  CMV/mL and  $4 \times 10^8$  CMV/mL) for subsequent uses.

[0003] *Ref.1* [WO2018/041906 A1 - BRACCO SUISSE SA] and *Ref.2* [PCT application number PCT/EP2019/055325], both in the name of the Applicant, describe a method for preparing CMV, such as gas-filled microbubbles, in particular by using microfluidic technique. Notwithstanding aqueous suspensions of calibrated microvesicles are highly stable at room temperature for few weeks, this stability may pose some constraints for a pharmaceutical product development, for which longer shelf life is generally desirable. There is thus a need to develop a long-term storage procedure. Freeze-drying, also known as lyophilization, is a complex and challenging process, widely used in the pharmaceutical industry that has the advantage of preserving pharmaceutical products under a dry form over several months. In fact, freeze-dried products display greater storage stability and can be easily shipped. Freeze-drying is also used in the field of gas filled microvesicles for preparing freeze-dried dosage forms which are then reconstituted with an aqueous solvent in the presence of a gas, to form a suspension of gas-filled microvesicles.

[0004] *Ref.3* [US2017/080113 A1 - GE Healthcare] describes the preparation of a suspension of microbubbles with adjusted sizes and subsequently lyophilization with a sucrose solution thereof.

**[0005]** Ref.4 [WO97/29782 A1 - NICOMED IMAGING A/S] teaches that USCA precursors which are stable at room temperature (RT) can be prepared by lyophilization of C<sub>3</sub>F<sub>8</sub> microbubbles in the presence of a freeze-drying stabilizer, preferably sucrose.

**[0006]** Ref.5 [WO 2004/069284 A2 - BRACCO RESEARCH SA [CH]] discloses a method for preparing a lyophilized matrix starting from an emulsion prepared from an aqueous medium, a phospholipid and a water immiscible organic solvent. Upon reconstitution of said lyophilized matrix, a respective injectable contrast agent is obtained.

**[0007]** Up to now, according to Applicant's knowledge, such technique has not been applied yet for preparing a freeze-dried composition from a suspension of calibrated microvesicles.

**[0008]** As observed by the Applicant, one of the most challenging issue in preparing a freeze-dried composition of calibrated microvesicles is related to the need of avoiding substantial modification of the characteristics of the initial suspension of calibrated microvesicles, such as concentration, monodispersity or geometric standard deviation (GSD) and/or final mean diameter.

**[0009]** The Applicant has now found that such initial characteristics, can be preserved to an acceptable extent after the freeze-drying process by using a suitable freeze-drying protecting component at a suitable concentration.

### ***Summary of the invention***

**[0010]** In a first aspect, the present invention relates to a freeze-dried composition according to claim 1, which, upon reconstitution with a pharmaceutically acceptable solution in the presence of a biocompatible gas, provides a suspension of calibrated gas-filled microvesicles, wherein said reconstituted suspension of microvesicles have a geometric standard deviation (GSD) value of at least 1.22 or lower.

**[0011]** The freeze-drying protecting component is a polyethylene glycol (PEG).

**[0012]** The reconstituted suspension of calibrated microvesicles is characterized by a GSD of at least 1.22 or lower, preferably of at least 1.21, down to e.g. 1.10.

**[0013]** In an embodiment of the invention, said reconstituted suspension of calibrated microvesicles is characterized by a concentration of at least  $2.0 \times 10^8$  CMV/mL, preferably  $2.1 \times 10^8$  CMV/mL, more preferably  $2.3 \times 10^8$  CMV/mL, up to  $5.5 \times 10^8$  CMV/mL.

**[0014]** According to a further aspect, the invention relates to a method of preparing a freeze-dried composition for the preparation of a reconstituted suspension of calibrated gas-filled

microvesicles having a geometric standard deviation (GSD) of 1.22 or lower, according to claim 8.

**[0015]** A further aspect of the invention relates to a freeze-dried composition for preparing a suspension of calibrated gas-filled microvesicles having a geometric standard deviation (GSD) of 1.22 or lower, said freeze-dried composition being obtainable by a process according to claim 11.

**[0016]** A further aspect of the invention relates to a process for the preparation of an injectable contrast agent comprising a suspension of gas-filled microvesicles, wherein said process comprises reconstituting a freeze-dried composition as defined above, with a pharmaceutically acceptable solution in the presence of a biocompatible gas.

### ***Figures***

**[0017]**

**Figure 1:** Typical particle size distribution representation.

**Figure 2:** A schematic representation of the core portion of a microfluidic flow-focusing device.

**Figure 3:** An exemplary schematic drawing of a device useful for the process of the invention technology.

**Figure 4:** Influence of the concentration of PEG4000 and PEG8000 on the CMV yield after freeze-drying at two different freezing temperatures -40 °C and -60 °C.

### ***Detailed description of the invention***

**[0018]** The expression "gas-filled microvesicles" generally refers to bubbles of gas bounded, at the gas/liquid interface, by a very thin envelope (film) involving a stabilizing amphiphilic material, typically a phospholipid, disposed at the gas to liquid interface. Said calibrated gas-filled microvesicles are suitable as contrast agents in ultrasound imaging techniques, known as Contrast-Enhanced Ultrasound (CEUS) Imaging, or in therapeutic applications, e.g. in combination with ultrasound mediated drug delivery.

**[0019]** These stabilized gas bubbles (dispersed in a suitable physiological solution) are generally referred to in the art with various terminologies, depending typically from the stabilizing material employed for their preparation; these terms include, for instance, "microspheres", "microbubbles", "microcapsules" or "microballoons", globally referred to here as "gas-filled microvesicles" (or "microvesicles" in short).

**[0020]** The term "calibrated" (when referred to gas-filled microvesicles) specifically refers to microvesicles suspensions with highly calibrated microvesicles (CMV), having different sizes between 3 and 8  $\mu\text{m}$ , characterized by a size distribution having a geometric standard deviation (GSD) of at least 1.2 or lower, preferably of at least 1.1, down to e.g. 1.05.

**[0021]** In this description and claims, the term "calibrated" is used interchangeably with "size-controlled", "monodispersed" or "monosize(d)" microvesicles.

**[0022]** In the present invention, calibrated gas filled microvesicles are preferably produced by using a microfluidic flow-focusing technology, where a gas thread is focused between two liquid flows in a flow-focusing device and phospholipid-stabilized calibrated microvesicles form and are collected in the outlet channel. Through this approach calibrated microvesicles are manufactured in a highly reproducible way at a reasonable production rate (~60 million bubbles per minute) (Fig 1 and Fig.2).

**[0023]** Depending on the parameters of the manufacturing process and device, the calibrated microvesicles may be obtained with relatively narrow size distribution around any desired mean diameter, e.g. from 3 to 8  $\mu\text{m}$ , preferably about 4  $\mu\text{m}$ .

**[0024]** The size distribution of said calibrated microvesicles is typically characterized by a geometric standard deviation (GSD) value of at least 1.20 or lower, preferably of at least 1.15, down to e.g. 1.05.

**[0025]** The calibrated microvesicles concentration (particularly upon production with microfluidic flow-focusing) is typically comprised between  $3 \times 10^8$  and  $4 \times 10^8$  CMV/mL, preferably close to  $4 \times 10^8$  CMV/mL, not lower than  $3 \times 10^8$  CMV/mL.

**[0026]** The "geometric standard deviation" (GSD) generally provides a suitable value for characterizing the breadth of the size distribution in a population of particles (gas-filled microvesicles in the specific case). A population of particles with a broad range of sizes will thus have a larger GSD value than one in which the particles sizes are narrowly distributed around a mean value (i.e. relatively similar in size).

**[0027]** **Figure 1** shows an example of a size distribution graph (by volume) of a population of gas filled microvesicles which can be obtained with a commercial particle analyser instrument (e.g. Coulter Counter Multisizer 3, equipped with the Multisizer 3 software), by determining the volume of gas for each of its channels, each channel corresponding to a predetermined diameter of the microvesicles (e.g. with increments of 0.1 microns). By the determination of the number of calibrated gas-filled microvesicles in the suspension, their respective diameter and volume distribution in a selected size range (e.g. between 3  $\mu\text{m}$  and 6  $\mu\text{m}$  for a 4.5  $\mu\text{m}$  CMV mean diameter), it is possible to calculate the GSD of a CMV distribution, by using the following **Equation 1**:

$$\sqrt{\frac{\sum n_i (\ln x_i - \ln \bar{x})^2}{\sum n_i}}$$

**Eq.1** 
$$GSD = e^{\sqrt{\frac{\sum n_i}{\sum n_i^3}}}$$

Where:

$n_i$  = percentage of volume of gas (with respect to the total one) entrapped in the microvesicles measured for the  $i$ th channel

$x_i$  = volume of the microvesicles in the  $i$ th channel, where

**Eq. 1.1.** 
$$x_i = d_i^3 \cdot \pi / 6$$

( $d_i$  = diameter of the microvesicle in the  $i$ th channel center)

$\bar{x}$  = geometric mean of the volume of the microvesicles in the selected range, where:

**Eq.1.2.** 
$$\bar{x} = 10^{\left[ \frac{\sum (n_i \cdot \log x_i)}{\sum n_i} \right]}$$

**[0028]** Among the various commercially available measuring devices, the Coulter Counter Multisizer 3, equipped with the Multisizer 3 software, is capable of calculating and providing such GSD value as defined above.

**[0029]** For instance, a GSD value of 1.2 indicates that about the 50% of CMV are calibrated between 2.5 and 5  $\mu\text{m}$ , for a mean diameter of 4  $\mu\text{m}$ ; a GSD of 1.05-1.08 (<1.1) indicates that about the 90-95% of CMV have sizes comprised between 2.5 and 5  $\mu\text{m}$ .

**[0030]** The expression "microvesicles concentration" as used herein refers to the number of CMV in a volume unit, determined using a Coulter Counter apparatus, i.e. number of CMV/mL.

**[0031]** As observed by the Applicant, while said calibrated microvesicles obtained through microfluidic flow-focusing can be stored at room temperature for few weeks without substantial impacts on their main characteristics, after said storage period the characteristics of said microvesicles (e.g. concentration and size distribution) may not be maintained and may progressively deteriorate.

**[0032]** The shelf-life of such suspensions of gas-filled microvesicles is thus relatively short for a pharmaceutical product and there is a need to develop a long-term storage procedure, able to preserve the CMV initial characteristics, such as concentration, GSD and final diameter for longer times, e.g. months or years.

**[0033]** The lyophilization process is a suitable approach to obtain a calibrated microvesicles dry form, with increased stability over time and with preserved initial characteristics.

**[0034]** It has now been surprisingly found that CMV initial characteristics, such as concentration, GSD and final diameter, can be substantially preserved after the freeze-drying process by using suitable freeze-drying protecting components.

[0035] In a first aspect, the present invention provides a freeze-dried composition according to claim 1, which upon reconstitution with a suitable aqueous solution in the presence of biocompatible gas, provides a suspension of calibrated gas-filled microvesicles, wherein said microvesicles have a GSD value of at least 1.22 or lower, preferably of at least 1.21, down to e.g. 1.10.

[0036] In the present description and claims, the term "freeze-drying" and "lyophilization" are used interchangeably, as well the terms "freeze-dried" and "lyophilized".

#### **Freeze-dried composition**

[0037] As used herein, the expression "freeze-dried composition" indicates any dry dosage form for long-term storage of gas-filled microvesicles formulations obtained through a lyophilization process. Said freeze-dried composition can comprise one or more active ingredient and a freeze-drying protecting component.

[0038] The expression "active ingredient" as used herein indicates the microvesicle-stabilizing materials, e.g. the amphiphilic materials, which are comprised in the freeze-dried composition with the freeze-drying protecting components.

#### **Freeze-drying protecting component**

[0039] As used herein, the expression "freeze-drying protecting component" refers to a component suitable for freeze drying, which is included in the microvesicles suspension before the lyophilization process thereof.

[0040] The term "freeze-drying protecting component" designates any compound added to protect the active ingredient during any phase of the freeze-drying process. Examples of suitable freeze-drying protecting components are polyethylene glycols (PEG), polyols, saccharides, surfactants, buffers, amino acids, chelating complexes, and inorganic salts.

[0041] According to an embodiment of the invention, said freeze-drying protecting component is selected among the group of polymers, polyols and saccharides.

[0042] In a preferred embodiment of the invention said freeze-drying protecting component is a polymer, preferably a hydrophilic polymer, more preferably a polyglycol.

[0043] In a still more preferred embodiment, said polymer is a polyethylene glycol (PEG).

[0044] Polyethylene glycol (PEG) has its standard chemical meaning. The chemical formula of PEG is  $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_m\text{CH}_2\text{OH}$  where  $m$  represents the average number of oxyethylene



groups. Typical polyethylene glycols are available in a wide range of average molecular weights, starting from 190-210 g/mol (PEG 200;  $m=4.2$ ) to 7000-9000 g/mol (PEG 8000;  $m=181.4$ ).

**[0045]** According to this description, the expression "molecular weight" indicates the average length of the PEG polymer chains, with a variability of  $\pm 10\%$  on the indicated molecular weight.

**[0046]** The freeze-drying protecting component is a PEG having a molecular weight comprised between 2000 and 10000 g/mol, preferably between 4000 and 8000 g/mol.

**[0047]** According to an embodiment, the freeze-drying protecting component is PEG having a molecular weight of 8000 g/mol ( $\pm 10\%$ ). According to another embodiment, the freeze-drying protecting component is PEG having a molecular weight close to 4000 g/mol ( $\pm 10\%$ ).

**[0048]** In an embodiment of the invention, the freeze-drying protecting component is added to the suspension of CMV as a solution having a concentration comprised between 100 mg/mL and 300 mg/mL, preferably 120 mg/mL and 250 mg/mL, more preferred close to 200 mg/mL.

**[0049]** In a still more preferred embodiment, the Applicant has found that it is preferred adding a PEG to the suspension of CMV as a solution having a concentration of 200 mg/mL, to improve the preservation of initial characteristics of the CMV suspension reconstituted after the freeze-drying process.

**[0050]** Another parameter which may be considered in the selection of a freeze-drying protecting component is the viscosity.

**[0051]** As used herein, the term viscosity refers to dynamic viscosity ( $\mu$ , Pa·s units) which is characterized by the resistance to laminar flow of an incompressible fluid (e.g. the CMV suspension either before the freeze-drying step or reconstituted from the freeze-dried product).

**[0052]** The viscosity of a CMV suspension comprising a freeze-drying protecting component can affect its processing e.g. during its manufacturing, or the administration of the reconstituted suspension of CMV from the freeze-dried composition.

**[0053]** Generally, during the manufacturing process, lower viscosity CMV suspensions are preferred, e.g. to avoid excessive overpressure in the microfluidic flow-focusing device.

**[0054]** From another point of view, reconstituted freeze-dried composition having a lower viscosity can facilitate its administration, especially through the injection routes (e.g. parenteral and intradermal).

**[0055]** The viscosity of a suspension depends on the concentration and molecular weight of its components; in particular, in the present case, on the PEG used as freeze-drying protecting

component. Precisely, higher concentrations and/or higher molecular weights of the PEG component increase the viscosity of the CMV suspension.

**[0056]** For instance, solutions comprising PEG4000 typically possesses relatively lower viscosity values (in general, from about one half and one third) with respect to solutions comprising an equal amount of PEG8000.

**[0057]** CMV suspension comprising PEG4000, either at 10% or at 20%, may thus provide some advantages in terms of either processing or administration of the suspension.

**[0058]** In this description and claims, the term "polyol" has its conventional chemical meaning; it indicates any organic compound with more than two hydroxyl functional groups, characterized by the general formula  $\text{HOCH}_2(\text{CHOH})_n\text{CH}_2\text{OH}$ , where  $n$  is an integer from 1 to 6, preferably from 2 to 4. Suitable polyols include erythritol, xylitol, sorbitol, lactitol and mannitol.

**[0059]** In this invention, said polyol is preferably selected from the group of polyols having a carbon chain length from four to six carbons atoms.

**[0060]** The term "saccharide" has its standard meaning in the field of chemistry. Saccharides, also called carbohydrates, are molecular compounds made from just three elements: carbon, hydrogen and oxygen. The simplest saccharides are called monosaccharides and they are the building units for bigger saccharides, such as disaccharides, trisaccharide and polysaccharides.

### **Amphiphilic material**

**[0061]** Materials suitable for forming the stabilizing layer of the gas-filled microvesicle (i.e. microvesicle-stabilizing materials) are those known in the art. These preferably include amphiphilic materials.

**[0062]** The term "amphiphilic material" as used herein includes compounds having a molecule with a hydrophilic polar head portion (e. g. a polar or ionic group), capable of interacting with an aqueous medium, and a hydrophobic organic tail portion (e. g. a hydrocarbon chain), capable of interacting with e. g. an organic solvent. These compounds thus generally act as "surface active agents", i.e. compounds which are capable of stabilizing mixtures of otherwise generally immiscible materials, such as mixtures of two immiscible liquids (e. g. water and oil), mixtures of liquids with gases (e. g. gas microbubbles in water) or mixtures of liquids with insoluble particles (e.g. metal nanoparticles in water).

**[0063]** Suitable amphiphilic materials comprise, for instance, phospholipids; lysophospholipids; fatty acids, such as palmitic acid, stearic acid, arachidonic acid or oleic acid; lipids bearing polymers, such as chitin, hyaluronic acid, polyvinylpyrrolidone or polyethylene glycol (PEG),

also referred as "pegylated lipids"; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; cholesterol, cholesterol sulfate or cholesterol hemisuccinate; tocopherol hemisuccinate; lipids with ether or ester-linked fatty acids; polymerized lipids; diacetyl phosphate; dicetyl phosphate; ceramides; polyoxyethylene fatty acid esters (such as polyoxyethylene fatty acid stearates), polyoxyethylene fatty alcohols, polyoxyethylene fatty alcohol ethers, polyoxyethylated sorbitan fatty acid esters, glycerol polyethylene glycol ricinoleate, ethoxylated soybean sterols, ethoxylated castor oil or ethylene oxide (EO) and propylene oxide (PO) block copolymers; sterol esters of sugar acids including cholesterol glucuronides, lanosterol glucuronides, 7-dehydrocholesterol glucuronide, ergosterol glucuronide, cholesterol gluconate, lanosterol gluconate, or ergosterol gluconate; esters of sugar acids and alcohols including lauryl glucuronide, stearyl glucuronide, myristoyl glucuronide, lauryl gluconate, myristoyl gluconate, or stearyl gluconate; esters of sugars with aliphatic acids including sucrose laurate, fructose laurate, sucrose palmitate, sucrose stearate, glucuronic acid, gluconic acid or polyuronic acid; saponins including sarsasapogenin, smilagenin, hederagenin, oleanolic acid, or digitoxigenin; glycerol or glycerol monoesters with fatty acids, including glycerol monopalmitate, glycerol monostearate, , glycerol monomyristate or glycerol monolaurate,; long chain alcohols including n-decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol, or n-octadecyl alcohol; 6-(5-cholesten-3(3-yloxy)-1-thio-  $\beta$  -D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3  $\beta$  -yloxy)hexyl-6-amino-6-deoxy-1-thio-  $\beta$  -D-galactopyranoside; 6-(5-cholesten-3  $\beta$  -yloxy)hexyl-6-amino-6-deoxy-1-thio-  $\beta$  -D-mannopyranoside; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methyl-amino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)-octadecanoyl]-2-aminopalmitic acid; N-succinyldioleoylphosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine or palmitoylhomocysteine; alkylamines or alkylammonium salts, comprising at least one (C<sub>10</sub>-C<sub>20</sub>), preferably (C<sub>14</sub>-C<sub>18</sub>), alkyl chain, such as, for instance, N-stearylamine, N,N'-distearylamine, N-hexadecylamine, N,N'-dihexadecylamine, N-stearylammmonium chloride, N,N'-distearylammmonium chloride, N-hexadecylammmonium chloride, N,N'-dihexadecylammmonium chloride, dimethyldioctadecylammmonium bromide (DDAB), hexadecyltrimethylammmonium bromide (CTAB); tertiary or quaternary ammonium salts comprising one or preferably two (C<sub>10</sub>-C<sub>20</sub>), preferably (C<sub>14</sub>-C<sub>18</sub>), acyl chain linked to the N-atom through a (C<sub>3</sub>-C<sub>6</sub>) alkylene bridge, such as, for instance, 1,2-distearoyl-3-trimethylammmonium-propane (DSTAP), 1,2-dipalmitoyl-3-trimethylammmonium-propane (DPTAP), 1,2-oleoyl-3-trimethylammmonium-propane (DOTAP), 1,2-distearoyl-3-dimethylammmonium-propane (DSDAP); and mixtures or combinations thereof.

**[0064]** According to this invention, the amphiphilic material is a phospholipid.

**[0065]** The term "phospholipid" is intended to encompass any amphiphilic phospholipidic compound, the molecules of which are capable of forming a stabilizing film of material (typically in the form of a mono-molecular layer) at the gas-water boundary interface in the final microbubbles suspension. Accordingly, these materials are also referred to in the art as "film-forming phospholipids".

**[0066]** Examples of suitable phospholipids include esters of glycerol with one or preferably two (equal or different) residues of fatty acids and with phosphoric acid, wherein the phosphoric acid residue is in turn bound to a hydrophilic group, such as, for instance, choline (phosphatidylcholines - PC), serine (phosphatidylserines - PS), glycerol (phosphatidylglycerols - PG), ethanolamine (phosphatidylethanolamines - PE), inositol (phosphatidylinositol). Esters of phospholipids with only one residue of fatty acid are generally referred to in the art as the "lyso" forms of the phospholipid or "lysophospholipids". Fatty acids residues present in the phospholipids are in general long chain aliphatic acids, typically containing from 12 to 24 carbon atoms, preferably from 14 to 22; the aliphatic chain may contain one or more unsaturations or is preferably completely saturated. Examples of suitable fatty acids included in the phospholipids are, for instance, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, oleic acid, linoleic acid, and linolenic acid. Preferably, saturated fatty acids such as myristic acid, palmitic acid, stearic acid and arachidic acid are employed.

**[0067]** Further examples of phospholipids are phosphatidic acids, i.e. the diesters of glycerol-phosphoric acid with fatty acids; sphingolipids such as sphingomyelins, i.e. those phosphatidylcholine analogues where the residue of glycerol diester with fatty acids is replaced by a ceramide chain; cardiolipins, i.e. the esters of 1,3-diphosphatidylglycerol with a fatty acid; glycolipids such as gangliosides GM1 (or GM2) or cerebrosides; glucolipids; sulfatides and glycosphingolipids.

**[0068]** As used herein, the term "phospholipids" include either naturally occurring, semisynthetic or synthetically prepared products that can be employed either singularly or as mixtures.

**[0069]** Examples of naturally occurring phospholipids are natural lecithins (phosphatidylcholine (PC) derivatives) such as, typically, soya bean or egg yolk lecithins.

**[0070]** Examples of semisynthetic phospholipids are the partially or fully hydrogenated derivatives of the naturally occurring lecithins. Preferred phospholipids are fatty acids diesters of phosphatidylcholine, ethylphosphatidylcholine, phosphatidylglycerol, phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol or of sphingomyelin.

**[0071]** Examples of preferred phospholipids are, for instance, dilauroyl-phosphatidylcholine (DLPC), dimyristoyl-phosphatidylcholine (DMPC), dipalmitoyl-phosphatidylcholine (DPPC), diarachidoyl-phosphatidylcholine (DAPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), 1,2 Distearoyl-*sn*-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dipentadecanoyl-phosphatidylcholine (DPDPC), 1-myristoyl-2-palmitoyl-phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl-phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl-phosphatidylcholine (PSPC), 1-stearoyl-2-palmitoyl-phosphatidylcholine (SPPC), 1-palmitoyl-2-oleylphosphatidylcholine (POPC), 1-oleyl-2-palmitoyl-phosphatidylcholine (OPPC), dilauroyl-phosphatidylglycerol (DLPG) and its alkali metal salts, diarachidoylphosphatidyl-glycerol (DAPG) and its alkali metal salts, dimyristoylphosphatidylglycerol (DMPG) and its alkali metal salts, dipalmitoylphosphatidylglycerol (DPPG) and its alkali metal salts,

distearoylphosphatidylglycerol (DSPG) and its alkali metal salts, dioleoyl-phosphatidylglycerol (DOPG) and its alkali metal salts, dimyristoyl phosphatidic acid (DMPA) and its alkali metal salts, dipalmitoyl phosphatidic acid (DPPA) and its alkali metal salts, distearoyl phosphatidic acid (DSPA), diarachidoylphosphatidic acid (DAPA) and its alkali metal salts, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), distearoyl phosphatidyl-ethanolamine (DSPE), dioleoylphosphatidyl-ethanolamine (DOPE), diarachidoyl-phosphatidylethanolamine (DAPE), dilinoleylphosphatidylethanolamine (DLPE), dimyristoyl phosphatidylserine (DMPS), diarachidoyl phosphatidylserine (DAPS), dipalmitoyl phosphatidylserine (DPPS), distearoylphosphatidylserine (DSPS), dioleoylphosphatidylserine (DOPS), dipalmitoyl sphingomyelin (DPSP), and distearoylsphingomyelin (DSSP), dilauroyl-phosphatidylinositol (DLPI), diarachidoylphosphatidylinositol (DAPI), dimyristoylphosphatidylinositol (DMPI), dipalmitoylphosphatidylinositol (DPPI), distearoylphosphatidylinositol (DSPI), dioleoyl-phosphatidylinositol (DOPI).

**[0072]** Suitable phospholipids further include phospholipids modified by linking a hydrophilic polymer, such as polyethyleneglycol (PEG) or polypropyleneglycol (PPG), thereto. Preferred polymer-modified phospholipids include "pegylated phospholipids", i.e. phospholipids bound to a PEG polymer. Examples of pegylated phospholipids are pegylated phosphatidylethanolamines ("PE-PEGs" in brief) i.e. phosphatidylethanolamines where the hydrophilic ethanolamine moiety is linked to a PEG molecule of variable molecular weight (e.g. from 300 to 20000 daltons, preferably from 500 to 5000 daltons), such as DPPE-PEG (or DSPE-PEG, DMPE-PEG, DAPE-PEG or DOPE-PEG). For example, DPPE-PEG2000 refers to DPPE having attached thereto a PEG polymer having a mean average molecular weight of about 2000.

**[0073]** Particularly preferred phospholipids are DAPC, DSPC, DPPC, DMPA, DPPA, DSPA, DMPG, DPPG, DSPG, DMPS, DPPS, DSPS and Ethyl-DSPC. Most preferred are DPPG, DPPS and DSPC.

**[0074]** Mixtures of phospholipids can also be used, such as, for instance, mixtures of DPPE and/or DSPE (including pegylated derivatives), DPPC, DSPC and/or DAPC with DSPS, DPPS, DSPA, DPPA, DSPG, DPPG, Ethyl-DSPC and/or Ethyl-DPPC.

**[0075]** For instance, a mixture of phospholipids may include phosphatidylcholine derivatives, phosphatidic acid derivatives and pegylated phosphatidylethanolamine, e.g. DSPC/DPPA/DPPE-PEG, DPPC/DPPA/DPPE-PEG, DSPC/DPPA/DSPE-PEG, DPPC/DPPA/DSPE-PEG, DAPC/DPPA/DPPE-PEG, DAPC/DPPA/DSPE-PEG, DSPC/DSPA/DPPE-PEG, DPPC/DSPA/DSPE-PEG, DSPC/DSPG/DPPE-PEG, DPPC/DSPG/DSPE-PEG.

**[0076]** According to the present invention, the phospholipid can conveniently be used in admixtures with any of the above listed amphiphilic compounds. Thus, for instance, lipids such as cholesterol, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propyl gallate or ascorbyl palmitate, fatty acids such as myristic acid, palmitic acid, stearic acid, arachidic acid

and derivatives thereof or butylated hydroxytoluene and/or other non-phospholipid compounds can optionally be added to one or more of the foregoing phospholipids, e.g. in proportions preferably ranging from zero to 50% by weight, more preferably up to 25%. For instance, mixtures of amphiphilic materials comprising phospholipids and fatty acids can advantageously be used, including DSPC/DPPG/palmitic acid, DSPC/DPPE-PEG/palmitic acid, DPPC/DPPE-PEG/palmitic acid, DSPC/DSPE-PEG/palmitic acid, DPPC/DSPE-PEG/palmitic acid, DSPC/DPPE-PEG/stearic acid, DPPC/DPPE-PEG/stearic acid, DSPC/DSPE-PEG/stearic acid or DPPC/DSPE-PEG/stearic acid.

**[0077]** The microvesicles prepared according to the invention may optionally comprise a targeting ligand.

**[0078]** The term "targeting ligand" includes within its meaning any compound, moiety or residue having, or being capable to promote, a targeting activity (e.g. including a selective binding) of the microvesicles of a composition of the invention towards any biological or pathological site within a living body. Targets with which targeting ligand may be associated include tissues such as, for instance, myocardial tissue (including myocardial cells and cardiomyocytes), membranous tissues (including endothelium and epithelium), laminae, connective tissue (including interstitial tissue) or tumors; blood clots; and receptors such as, for instance, cell-surface receptors for peptide hormones, neurotransmitters, antigens, complement fragments, and immunoglobulins and cytoplasmic receptors for steroid hormones.

**[0079]** The targeting ligand may be synthetic, semi-synthetic, or naturally occurring. Materials or substances which may serve as targeting ligands include, for example, but are not limited to proteins, including antibodies, antibody fragments, receptor molecules, receptor binding molecules, glycoproteins and lectins; peptides, including oligopeptides and polypeptides; peptidomimetics; saccharides, including mono and polysaccharides; vitamins; steroids, steroid analogs, hormones, cofactors, bioactive agents and genetic material, including nucleosides, nucleotides and polynucleotides.

**[0080]** The targeting ligand may be an amphiphilic compound per se (which is admixed with the other components of the microvesicle) or a compound bound to an amphiphilic molecule (e.g. a phospholipid) employed for the formation of the microvesicles.

### **Gas**

**[0081]** Suitable gases comprise biocompatible fluorinated gases, preferably perfluorinated gases. Fluorinated gases include materials which contain at least one fluorine atom such as, for instance, fluorinated hydrocarbons (organic compounds containing one or more carbon atoms and fluorine); sulfur hexafluoride; fluorinated, preferably perfluorinated, ketones such as perfluoroacetone; and fluorinated, preferably perfluorinated, ethers such as perfluorodiethyl ether. Preferred compounds are perfluorinated gases, such as SF<sub>6</sub> or perfluorocarbons (perfluorinated hydrocarbons), i.e. hydrocarbons where all the hydrogen atoms are replaced

by fluorine atoms, which are known to form particularly stable gas-filled microvesicles suspensions.

**[0082]** The term "perfluorocarbon" includes saturated, unsaturated, and cyclic perfluorocarbons. Examples of biocompatible, physiologically acceptable perfluorocarbons are: perfluoroalkanes, such as perfluoromethane, perfluoroethane, perfluoropropanes, perfluorobutanes (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-isobutane), perfluoropentanes, perfluorohexanes or perfluoroheptanes; perfluoroalkenes, such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2ene) or perfluorobutadiene; perfluoroalkynes (e.g. perfluorobut-2-yne); and perfluorocycloalkanes (e.g. perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethylcyclobutanes, perfluorocyclopentane, perfluoromethylcyclopentane, perfluorodimethylcyclopentanes, perfluorocyclohexane, perfluoromethylcyclohexane and perfluorocycloheptane). Preferred saturated perfluorocarbons include, for example,  $\text{CF}_4$ ,  $\text{C}_2\text{F}_6$ ,  $\text{C}_3\text{F}_8$ ,  $\text{C}_4\text{F}_8$ ,  $\text{C}_4\text{F}_{10}$ ,  $\text{C}_5\text{F}_{12}$  and  $\text{C}_6\text{F}_{14}$ . Particularly preferred gases are those which are in gaseous form at room temperature, including  $\text{SF}_6$ ,  $\text{C}_3\text{F}_8$  and  $\text{C}_4\text{F}_{10}$ .

**[0083]** An embodiment thus relates to a method of preparing a freeze-dried composition for long term storage of calibrated gas-filled microvesicles, comprising the steps of:

1. a. preparing a suspension of calibrated gas-filled microvesicles comprising a polyglycol as a freeze-drying protecting component;
2. b. freeze-drying the calibrated microvesicles suspension.

**[0084]** Preferably, the method of preparation of step a) is the microfluidic flow-focusing technique, as illustrated in *Ref.1* [WO2018/041906 A1 - BRACCO SUISSE SA] and *Ref.2* [PCT application number PCT/EP2019/055325].

**[0085]** **Figure 2** shows a schematic representation of the core portion **200** of a flow-focusing device ("microfluidic chip") useful in the process of the invention. The chip comprises a first feed channel **201** for feeding the gaseous flow **201'** and two additional orthogonal feed channels **202a** and **202b** for supplying the liquid flow comprising the amphiphilic material.

**[0086]** The gas flow and the two liquid flows are directed towards the contact zone **203** and then through the calibrated orifice **204**, shown as a dotted line in **Figure 2**. The calibrated orifice is connected to a calibrated channel **204'** having preferably the same cross-section as the orifice, which is in turn connected to an initial portion **205** of the outlet channel **206**. In an alternative embodiment (not shown) the calibrated orifice **204** may be a nozzle directly connected to the initial portion **205** of outlet channel **206** i.e. without the calibrated channel in-between. The microvesicles **203'** are formed in the calibrated orifice and directed, through calibrated channel **204'**, to the initial portion **205** of the outlet channel **206**. The hydraulic diameter of the outlet channel is generally larger than the hydraulic diameter of the calibrated

orifice and typically increases from the initial diameter of the calibrated orifice to the final diameter of the outlet channel **206**, corresponding substantially to the hydraulic diameter of a collecting tube (not shown), connecting the flow-focusing device to a container, e.g. a sealed vial for collecting the suspension of microvesicles.

**[0087]** In the initial portion **205** of the outlet channel of the device and preferably also in the contact zone **203** and in the calibrated orifice **204** the temperature of the microvesicles is controlled, as described in *Ref.1* [WO2018/041906 A1 - BRACCO SUISSE SA] and *Ref.2* [PCT application number PCT/EP2019/055325].

### **Liquid flow**

**[0088]** The aqueous liquid flow for preparing the calibrated gas-filled microvesicles according to the method of the invention comprises an amphiphilic material (as above defined) at a concentration of e.g. from 5.0 to 20 mg/mL, preferably from 7.5 to 15 mg/mL, dispersed in an aqueous carrier.

**[0089]** Suitable aqueous carriers, which are preferably physiologically acceptable, comprise water (preferably sterile water), aqueous solutions such as saline (which may advantageously be balanced so that the final product for injection is not hypotonic), or solutions of one or more tonicity adjusting substances. Tonicity adjusting substances comprise salts or sugars, sugar alcohols, glycols or other non-ionic polyol materials (e.g. glucose, sucrose, sorbitol, mannitol, glycerol, polyethylene glycols, propylene glycols and the like), chitosan derivatives, such as carboxymethyl chitosan, trimethyl chitosan or gelifying compounds, such as carboxymethylcellulose, hydroxyethyl starch or dextran.

**[0090]** In an alternative embodiment, an additional oil phase may be added for incorporating therapeutic hydrophobic substances into the microvesicles. To this end, two additional conduits may be provided in the device for supplying the desired oil phase, as described for instance by *Ref.1* [WO2018/041906 A1 - BRACCO SUISSE SA] and *Ref.2* [PCT application number PCT/EP2019/055325]. The formed gas-filled microvesicles will thus have a film of oil disposed at the interface between gas and the stabilizing layer of amphiphilic material, which can be loaded with a desired therapeutic agent. Suitable oils may include any biocompatible oil which is liquid at room temperature including, for instance, mono-, di- or tri-esters of glycerol with saturated or unsaturated (C<sub>2</sub>-C<sub>18</sub>) alkyl chains (including homo- or hetero-alkylesters), such as glycerol monobutylin, glycerol monolinoleate, 1,2-dihexanoyl glycerol, 1,2 dioctanoyl glycerol, 1,2-dioleoyl-sn-glycerol, triacetin, tributyrin, tricaproin, tricaprylin, tricaprillin, and mixtures thereof; or natural oils such as soya oil, olive oil, safflower seed oil, sunflower seed oil, peanut oil and mixtures thereof.

### **Mixed gas flow**



[0091] The freshly formed microvesicles comprise a gas selected among those previously indicated. Preferably the gas is a mixture of a gas highly soluble in water ("HS gas") and of a gas with low solubility in water ("LS gas"), as anticipated in *Ref. 2* [PCT application number PCT/EP2019/055325].

[0092] During the stabilization phase, the major amount of the highly soluble gas rapidly dissolves in water while the poorly soluble one remains entrapped into the densely packed layer of amphiphilic compounds, typically with some residual amount of HS solubility gas dispersed therein.

[0093] Examples of HS gases include nitrogen, air, and carbon dioxide, this latter being particularly preferred because of its higher solubility in water.

[0094] Suitable LS gases are fluorinated gases, preferably perfluorinated gases. Fluorinated gases those previously described in the present description.

[0095] In a preferred embodiment of the invention, gas-filled microvesicles comprising CO<sub>2</sub>/C<sub>4</sub>F<sub>10</sub> in a volume ratio of from 80/20 to 90/10, e.g. 85/15 can be prepared with a gas-mixing device similar to the one schematically illustrated in **Figure 3**.

[0096] **Figure 3** shows an example of a microfluidic flow-focusing device used to produce calibrated microvesicles. A gas flow **302** (comprising e.g. a mixture of C<sub>4</sub>F<sub>10</sub> and CO<sub>2</sub>) and a liquid flow **301** (comprising an amphiphilic material, e.g. a phospholipid, fatty acid or mixtures thereof), are supplied to microfluidic chip **303** to produce microvesicles through orifice **304**. The microvesicles suspension is collected in a vial **305**, which is preferably prefilled with a gas (e.g. C<sub>4</sub>F<sub>10</sub>) at ambient pressure. A venting device (e.g. a needle **306**) is preferably used to equalize the overpressure generated by the liquid filling of the vial. At the end of the collection of the microvesicles suspension, the venting device is preferably removed and the container is preferably sealed to avoid further gaseous exchange with the external atmosphere.

[0097] According to a preferred embodiment of this invention, after the collection phase, the calibrated microvesicles obtained through the microfluidic flow-focusing method, are treated using suitable washing techniques, in order to remove not-assembled amphiphilic material and possible residue compounds.

[0098] In the present description, the term "washing" indicates any operation carried out on the freshly prepared microvesicles suspension, finalized to remove (or substantially reduce the amount of) not-assembled amphiphilic material and residue compounds.

[0099] According to this description, suitable washing techniques comprise centrifugation, filtration, bubble sorting and decantation.

[0100] In the present description, the expression "not-assembled amphiphilic material"

indicates amphiphilic molecules that, at the end of the preparation process, are present in the calibrated microvesicles suspension, but are not forming the stabilizing layer of the gas-filled microvesicles.

**[0101]** In the present description "residues compounds" indicate any possible additive substance that is added to the amphiphilic material solution during the microvesicles preparation, such as tonicity adjusters as described before.

**[0102]** In a preferred embodiment of this invention, after the washing operation, a freeze-drying protecting component is added to the calibrated microvesicles suspension.

**[0103]** Alternatively, the freeze-drying protecting component can be added to the liquid flow comprising the amphiphilic compounds, described above, during the preparation of the microvesicles by microfluidic technique.

**[0104]** Initial CMV characteristics are particularly preserved when using a polyethyleneglycol as freeze-drying protecting component characterized in that said polyethyleneglycol is added to the suspension of CMV as a solution having a total concentration comprised between 100 mg/ml and 300 mg/mL, preferably between 120 mg/ml and 250 mg/mL, more preferably the total concentration of PEG is 200 mg/mL.

**[0105]** In a preferred embodiment of the invention, before the freeze-drying process, the CMV suspension comprises a polyethyleneglycol as freeze-drying protecting component at a concentration between 12 and 25%, preferably between 14 and 24% and still more preferred between 18-22% (w/v%).

**[0106]** The freeze-drying protecting component represents the larger amount of the final freeze-dried preparation, wherein it is typically at least 90%, preferably between 94% and 99.7%, more preferably 99,5%, up to 99.9% (w/w).

**[0107]** Said freeze-drying protecting components are those described above in the present description. Using polyethylene glycols as freeze-drying protecting components has shown advantageous results when used in the freeze-drying process of the calibrated microvesicles suspensions, to prepare a freeze-dried composition which can then be reconstituted to obtain suspensions of calibrated microvesicles having acceptable characteristics in terms of concentration and size distribution, as compared to those of the initial suspension (before freeze-drying).

**[0108]** For instance, the addition of a polyethylene glycol as protecting component results to be the best strategy to substantially preserve the GSD value after the freeze-drying process when compared with the addition of a polyol or a saccharide. According to an embodiment, GSD values comprised between 1.20 and 1.22 can be obtained when using a polyethylene glycol, in comparison with the use of a polyol or a saccharide which gives calibrated microvesicles characterized by a higher GSD value, up to 1.34.

**[0109]** In a preferred embodiment, the use of a polyethylene glycol as freeze-drying protecting component is able to significantly improve the yield of calibrated microvesicles after reconstitution of the freeze-dried product with values higher than 60%, e.g. 68%, higher than those obtained with the use of a polyol or a saccharide.

**[0110]** In this description and claims, the term freeze-drying has its standard meaning in the pharmaceutical technology field. Freeze-drying process consists of drying a pre-frozen liquid product under low pressure or vacuum and at low temperature. The main objective is to remove liquid from the product in order to provide a freeze-dried product suitable for long term storage.

**[0111]** As observed by the Applicant, freeze-drying parameters may be selected to further optimize the characteristics of the reconstituted suspension of microvesicles (e.g. yield, size, GSD).

**[0112]** In a preferred embodiment of the invention, the freezing temperature of said method for long term storage of calibrated gas-filled microvesicles, ranges between -30 °C and - 70 °C, preferably between -30 °C and -60 °C.

**[0113]** In a still preferred embodiment, the negative pressure applied during the freeze-drying process is preferably of 0.5 mbar or lower, preferably 0.2 or lower, e.g. close to 0.1 mbar.

**[0114]** A further aspect of the invention relates to a freeze-dried composition for preparing a suspension of calibrated gas-filled microvesicles having a geometric standard deviation (GSD) of 1.22 or lower, said freeze-dried composition being obtainable by a process according to claim 11.

**[0115]** A further aspect of the invention relates to a process for the preparation of an injectable contrast agent comprising a suspension of gas-filled microvesicles, wherein said process comprises reconstituting a freeze-dried composition, obtained as described above, comprising an amphiphilic material and a freeze-drying protecting component, with a pharmaceutically acceptable solution in the presence of biocompatible gas.

**[0116]** The freeze-dried composition can then be reconstituted with a suitable pharmaceutically acceptable (aqueous) solution in the presence of biocompatible gas, thus providing a suspension of calibrated gas-filled microvesicles, wherein said microvesicles have a GSD of at least 1.22 or lower, preferably of at least 1.21, down to e.g. 1.10.

**[0117]** In this invention, pharmaceutically acceptable (aqueous) solutions are water, typically sterile, pyrogen free water (to prevent as much as possible contamination in the final reconstituted product), aqueous solutions such as saline (which may advantageously be balanced so that the final product for injection is not hypotonic), or aqueous solutions of one or more tonicity adjusting substances such as salts or sugars, sugar alcohols, glycols or other

non-ionic polyol materials.

**[0118]** The freeze-dried composition is typically reconstituted with a volume of aqueous solution similar to the volume of suspension which underwent the freeze-drying process. Accordingly, the concentration of the freeze-drying protecting components in the reconstituted suspension is substantially the same as the one in the initial suspension.

**[0119]** Another embodiment of the invention thus relates to a reconstituted CMV suspension having a GSD of at least 1.22 and a polyethylene glycol at a concentration between 12 and 25%, preferably between 14 and 24% and still more preferred between 18-22%.

**[0120]** These amounts of freeze-drying protecting components are typically higher than the amounts in prior art preparations of gas-filled microvesicles, which are generally lower than 10% (w/w).

**[0121]** Surprisingly, the reconstituted suspensions of calibrated microvesicles were found to have substantially maintained the initial characteristics of calibrated microvesicles, as characterized before the lyophilization process, and thus suitable for subsequent pharmaceutical uses.

**[0122]** In an embodiment of this invention, said reconstituted suspension of calibrated microvesicles is characterized by a concentration of at least  $2.0 \times 10^8$  CMV/mL, preferably  $2.1 \times 10^8$  CMV/mL, more preferably  $2.3 \times 10^8$  CMV/mL, up to  $5.5 \times 10^8$  CMV/mL.

**[0123]** As indicated above, the expression "microvesicles concentration" refers to the number of microvesicles in a volume unit, determined using a Coulter Counter apparatus, i.e. number of CMV/mL.

**[0124]** Typically, the concentration of calibrated microvesicles (%), measured after the reconstitution of the freeze-dried composition of the invention with suitable aqueous solution, allows to determine the microbubble yield after said reconstitution, in comparison to the microvesicles concentration measured before the freeze-drying process.

**[0125]** In the present invention, the calibrated microvesicles yield after the reconstitution of the freeze-dried composition of the invention is at least 50%, preferably at least 55%, more preferably at least 60% even more preferably at least 65%, up to e.g. 85%, preferably 90%, more preferably 95%, even more preferably 100%.

**[0126]** The expression "calibrated microvesicles yield" refers to the ratio between the microvesicles concentration measured before freeze-drying and the microvesicles concentration measured after reconstitution of the freeze-dried product with physiologically acceptable solution (in short "after freeze-drying") (see **Equation 2**):

**Eq.2:** CMV yield after freeze-drying (%) = (CMV concentration after freeze-drying /mL) / (CMV concentration before freeze-drying /mL)

Use

**[0127]** The microvesicles prepared according to the method of the invention may be used in a variety of diagnostic and/or therapeutic techniques, including in particular Ultrasound and Magnetic Resonance.

**[0128]** Diagnostic methods include any method where the use of the gas-filled microvesicles allows enhancing the visualisation of a portion or of a part of an animal (including humans) body, including imaging for preclinical and clinical research purposes. A variety of imaging techniques may be employed in ultrasound applications, for example including fundamental and harmonic B-mode imaging, pulse or phase inversion imaging and fundamental and harmonic Doppler imaging; if desired three-dimensional imaging techniques may be used.

**[0129]** Microvesicles according to the invention may typically be administered in a concentration of from about 0.01 to about 1.0  $\mu\text{L}$  of gas per kg of patient, depending e.g. on their respective composition, the tissue or organ to be imaged and/or the chosen imaging technique. This general concentration range may of course vary depending on specific imaging applications, e.g. when signals can be observed at very low doses such as in colour Doppler or power pulse inversion.

**[0130]** In an embodiment said method of diagnosing comprises:

1. (i) administering to a patient a suspension of gas-filled microvesicles obtained by reconstitution of a freeze-dried product obtained according to the process of the invention; and
2. (ii) detecting an ultrasound signal from a region of interest in said patient.

**[0131]** According to an embodiment, said suspension of gas-filled microvesicles comprises an amphiphilic material and a freeze-drying protecting component.

**[0132]** Reconstitution of the freeze-dried product is preferably made by dispersing it into a physiologically acceptable aqueous carrier, e.g. saline, in the presence of a physiologically acceptable gas, e.g.  $\text{C}_4\text{F}_{10}$ , under gentle agitation.

**[0133]** Possible other diagnostic imaging applications include scintigraphy, light imaging, and X-ray imaging, including X-ray phase contrast imaging.

**[0134]** Another aspect of the invention relates to the use in a method of therapeutic treatment of a suspension of microvesicles reconstituted from freeze-dried product according to the

invention.

**[0135]** Therapeutic techniques include any method of treatment (as above defined) of a patient which comprises the combined use of ultrasounds and gas-filled microvesicles either as such (e.g. in ultrasound mediated thrombolysis, high intensity focused ultrasound ablation, blood-brain barrier permeabilization, immunomodulation, neuromodulation, radiosensitization) or in combination with a therapeutic agent (i.e. ultrasound mediated delivery, e.g. for the delivery of a drug or bioactive compound to a selected site or tissue, such as in tumor treatment, gene therapy, infectious diseases therapy, metabolic diseases therapy, chronic diseases therapy, degenerative diseases therapy, inflammatory diseases therapy, immunologic or autoimmune diseases therapy or in the use as vaccine), whereby the presence of the gas-filled microvesicles may provide a therapeutic effect itself or is capable of enhancing the therapeutic effects of the applied ultrasounds, e.g. by exerting or being responsible to exert a biological effect in vitro and/or in vivo, either by itself or upon specific activation by various physical methods (including e.g. ultrasound mediated delivery).

**[0136]** Microvesicles according to the invention can typically be administered for therapeutic purposes in a concentration of from about 0.01 to about 5.0  $\mu\text{L}$  of gas per kg of patient, depending e.g. from their respective composition, the type of subject under treatment, the tissue or organ to be treated and/or the therapeutic method applied.

**[0137]** In an embodiment said method of ultrasound therapeutic treatment comprises:

1. (i) administering to a patient a suspension of gas-filled microvesicles obtained by reconstitution of a freeze-dried product obtained according to the process of the invention;
2. (ii) identifying a region of interest in said patient to be submitted to a therapeutic treatment, said region of interest comprising said suspension of gas-filled microvesicles; and
3. (iii) applying an ultrasound beam for therapeutically treating said region of interest;

whereby said ultrasound therapeutic treatment is enhanced by the presence of said suspension of gas-filled microvesicles in said region of interest.

**[0138]** The following examples will help to further illustrate the invention.

### ***Examples***

#### **Example 1**

#### **Preparation of gas-filled microvesicles**

[0139] Gas-microvesicles were synthesized using a commercially available microfluidic flow-focusing device (CU4553.007 N30 design, Micronit Microfluidics, NL), mounted in a commercially available chip holder (Micronit microfluidics, Fluidic Connect PRO Chip Holder with 4515 Inserts). The microvesicles formation channel had a width of 19  $\mu\text{m}$ . The chip and its holder were positioned in an optically transparent temperature controlled water bath that was mounted on an inverted microscope equipped with a 20 times magnification objective (Olympus, LMPLAN 20x) and a CCD camera (Lumenera, LM156M). The temperature of the thermostatic bath was set at 50  $^{\circ}\text{C}$ .

[0140] The amphiphilic materials in the liquid flow were: DSPC:DPPE-PEG5000 in a respective molar ratio of 9:1.

[0141] The materials were added with the above molar ratios at a concentration of 20 mg/mL to a 2:1 (volume ratio) chloroform/methanol mixture under stirring at 60  $^{\circ}\text{C}$  until complete dissolution the amphiphilic material. The solvent was then evaporated under reduced pressure and the obtained film was dried overnight under reduced pressure. The dried material was then redispersed (at concentrations of 15 mg/mL) in saline (0.9% NaCl) at 60  $^{\circ}\text{C}$  under stirring for 30 minutes. The dispersion was then sonicated by using a tip sonicator (Branson Sonifier 250) to homogeneously disperse the material. The preparations were then filtered using a polycarbonate filter (0.45  $\mu\text{m}$  pore size), cooled down to room temperature and degassed.

[0142] Gas-filled microvesicles comprising  $\text{CO}_2/\text{C}_4\text{F}_{10}$  in a volume ratio of 85/15 were prepared with a gas-mixing device similar to the one schematically illustrated in **Figure 3**. Briefly, two gas containers were filled with  $\text{CO}_2$  and  $\text{C}_4\text{F}_{10}$ , respectively. The gas flow of each gas was regulated by respective mass flow controllers: (i) EL-Flow: F200CV-002-RAD-11-K, for the  $\text{CO}_2$  and (ii) Low- $\Delta\text{P}$ -Flow: F-200DV-RAD-11-Z for  $\text{C}_4\text{F}_{10}$  (both gas controllers from Bronkhorst, Ruurlo, The Netherlands). The mass flow controllers were controlled by a customized software program implemented in Matlab (Mathworks), which was installed on a personal computer, in order to set and keep the desired mixing ratio. A pressure sensor (PSE530-M5-L; SMC Corp., Tokyo, Japan) measured the actual pressure in the gas mixture in the outlet channel leading to the microfluidic chip; a gas pressure of 2 bars was used for the formation of the microvesicles. The liquid co-flow rate was controlled by using a separate mass flow controller (Mini Cori Flow: M13V14I-MAD-11-K-S; Bronkhorst, Ruurlo, The Netherlands). A liquid co-flow rate of around 150  $\mu\text{L}/\text{min}$  was used to operate the flow-focusing device in the jetting regime and produce microvesicles with a diameter (mode) of around 4  $\mu\text{m}$ .

## **Example 2**

### **Preparation of the freeze-dried composition**

[0143] Firstly, 3 mL of the calibrated microvesicles suspension obtained through the microfluidic flow-focusing method, as described in Example 1, were transferred in a Pyrex tube (Pyrex disposable tube 12 × 75 mm) without further addition of C<sub>4</sub>F<sub>10</sub> in the tube. Then, the calibrated microvesicles suspension was centrifuged (Sigma 3-16 Centrifuge) for 6 minutes at 64 g, and the infranatant was withdrawn by means of a syringe equipped with a needle. The remaining 200 µL of washed microvesicles were redispersed with 3 mL of a solution comprising the freeze-drying protecting component as detailed in the following examples.

[0144] The calibrated microvesicles suspended in the solution of the freeze-drying protecting component were then aliquoted in DIN8R glass vials (1.5 mL suspension/vial) and transferred in the freeze dryer.

[0145] The vials were cooled at temperatures between -30 °C and -60 °C (as detailed in the subsequent examples) and freeze-dried for approximately 1 hour under vacuum. At the end of the procedure, a freeze-dried composition was obtained as a white homogenous dry solid. The head space was then filled with pure C<sub>4</sub>F<sub>10</sub>.

### **Example 3**

#### **Effect of the freeze-drying protecting components on calibrated microvesicles characteristics**

[0146] Table 2 lists the freeze-drying protecting components which were investigated.

**Table 2** Selected freeze-drying protecting components, classified by chemical class

<b>Molecule</b>	<b>Class</b>	<b>Molecular Weight (g/mol)</b>
PEG4000	Polymer	4'000
PEG8000	Polymer	8'000
Xylitol	Polyol	152.2
Sorbitol	Polyol	182.2
Mannitol	Polyol	182.2
Glucose	Monosaccharide	180.2
Maltose	Disaccharide	342.3
Sucrose	Disaccharide	342.3
Dextran 6000	Polysaccharide	6'000

[0147] The above materials were used, at various concentrations, in the preparation of the freeze-dried composition illustrated in Example 2 at a freezing temperature of -60 °C. **Table 3** illustrates the composition and concentration of the investigated freeze-dried protecting



components (1A, 1B, 2A, 2B, S3-S13).

**Table 3** Composition and concentration of the evaluated solutions of freeze-drying protecting components

Prep No.	Freeze-drying protecting components	Total concentration of the freeze-drying protecting components (mg/mL)
1A	PEG4000	100
1B	PEG4000	200
2A	PEG8000	100
2B	PEG8000	200
S3A	Sorbitol	100
S3B	Sorbitol	200
S4	Xylitol	100
S5	Mannitol	100
S6	Sucrose	100
S7A	Maltose	100
S7B	Maltose	200
S8	Raffinose	100
S9	Dextran 6000	100

### **Characterization**

**[0148]** After the freeze-drying process, each freeze-dried composition was reconstituted with 1.5 mL of aqueous solution in the presence of a biocompatible gas, in order to obtain a calibrated and stable microvesicles suspension. The freeze-dried composition was thus reconstituted with a volume of aqueous solution similar to the volume of suspension which underwent the freeze-drying process. Accordingly, the obtained concentrations of the freeze-drying protecting components in the reconstituted suspension were 93,75 mg/mL and 188 mg/mL, respectively starting from a solution at a concentration of 100 mg/mL and 200 mg/mL.

**[0149]** After reconstitution, the reconstituted calibrated microvesicles suspensions were let 5 min on the bench before being characterized using a Coulter Counter Multisizer 3 fitted with a 30  $\mu$ m aperture tube, to measure the size, the geometric standard deviation (GSD), the concentration of microvesicles and the yield after the freeze-drying process.

### **Results**

[0150] The main results of the characterization of the reconstituted calibrated microvesicles suspensions are reported in **Table 4**.

[0151] The GSD value and the concentration of microvesicles were measured before and after the freeze-drying process to evaluate the efficacy of the freeze-drying protecting components in preserving the initial characteristics of the calibrated microvesicles.

**Table 4** GSD, CMV yield values and concentration of calibrated vesicles suspensions obtained after reconstitution of lyophilized compositions comprising a single freeze-drying protecting component (FD= freeze-drying).

Prep No.	Total concentration of the freeze-drying protecting component (mg/mL)	GSD	CMV Yield after FD (2-6 $\mu$ m) (%)	Concentration (CMV/mL)
1A	100	1.20	39	$1.6 \times 10^8$
1B	200	1.22	63	$2.3 \times 10^8$
2A	100	1.21	26	$0.9 \times 10^8$
2B	200	1.20	64	$2.0 \times 10^8$
S3A	100	1.33	3	$0.1 \times 10^8$
S3B	200	1.25	5	$0.2 \times 10^8$
S4	100	1.34	8	$0.3 \times 10^8$
S5	100	1.34	14	$0.5 \times 10^8$
S6	100	1.28	13	$0.4 \times 10^8$
S7A	100	1.28	22	$1.0 \times 10^8$
S7B	200	1.27	21	$0.9 \times 10^8$
S8	100	1.22	23	$0.9 \times 10^8$
S9	100	1.26	21	$0.8 \times 10^8$

[0152] Considering the GSD value and the microvesicles yield after freeze-drying, results clearly showed that the addition of polyethylene glycols to the calibrated microvesicles suspension, before the freeze-drying process, is much more effective than the addition of polyols or saccharides in term of preservation of the microvesicles characteristics.

[0153] In particular, freeze-drying of calibrated microvesicles in polyethylene glycols (PEG4000 and PEG8000) solutions enabled to improve the GSD values and/or the microvesicles yield.

[0154] Furthermore, the addition of polyethylene glycols solutions at a concentration of 200 mg/mL to the CMV suspension gave better results than adding polyethylene glycols at a concentration of 100 mg/mL. In particular, the CMV suspensions containing PEG4000 at a

concentration of about 200 mg/mL (1B) were characterized by a GSD of 1.22 and a remarkable CMV yield of 63%. Still more advantageously, using a solution of PEG8000 at 200 mg/mL (2B) allowed to reach a GSD of 1.20 and CMV yield of 64%.

#### **Example 4**

#### **Characterization of the calibrated microvesicles characteristics after freeze-drying at different freezing temperatures.**

[0155] The preparation of the freeze-dried composition was further investigated by evaluating different freezing temperatures at which the freshly-prepared calibrated microvesicles are cooled at the beginning of the lyophilization procedure.

[0156] The preparation process was performed as previously described in Example 2, except that the vials were cooled at different freezing temperatures, as reported in **Table 5**.

[0157] At the end of the freeze-drying process, each lyophilized composition was reconstituted with 1.5 mL of aqueous solution in the presence of a biocompatible gas, in order to obtain a calibrated and stable microvesicles suspension. The freeze-dried composition was thus reconstituted with a volume of aqueous solution similar to the volume of suspension which underwent the freeze-drying process. Accordingly, the obtained concentrations of the freeze-drying protecting components in the reconstituted suspension were 93,75 mg/mL and 188 mg/mL, respectively starting from a solution of freeze-drying protecting components at a concentration of 100 mg/mL and 200 mg/mL.

[0158] After reconstitution, the reconstituted calibrated microvesicles suspensions were let 5 min on the bench before being characterized using a Coulter Counter Multisizer 3 fitted with a 30 µm aperture tube, to measure the size, the geometric standard deviation (GSD), the concentration of microvesicles and the yield after the freeze-drying process.

[0159] **Table 5** reports the GSD values and the CMV yield of the microvesicles suspensions, obtained after reconstitution of lyophilized compositions comprising different freeze-drying protecting components. Formulations 2A, 2B and S3A were selected and different freezing temperatures were tested.

#### **Results**

[0160] Results confirmed that the addition of PEG8000 to the calibrated microvesicles suspension allowed to improve the freeze-drying performance at any investigated freezing temperature, when compared with the use of sorbitol. For instance, the use of PEG8000 at

100 mg/mL was much more advantageous in terms of GSD and CMV yield than the use of sorbitol at 100 mg/mL, which was not able to efficiently preserve the initial CMV conditions. In fact, the CMV suspension containing sorbitol at about 100 mg/mL (S3A) was characterized by low CMV yield, with a maximum value of 6%, and by high GSD values, comprised between 1.27 and 1.38.

[0161] Moreover, these results confirmed that the CMV suspension containing PEG8000 at a concentration of about 200 mg/mL (2B) was characterized by a lower GSD value and a remarkable higher CMV yield when compared with the CMV suspension containing PEG8000 at about 100 mg/mL (2A). For instance, in the former case the CMV yield was found to be comprised between 56 and 68%, while in the latter case the higher CMV yield was found to be 45% at -40°C. Furthermore, also the GSD values were lower for the formulation 2B, with values lower than 1.20, confirming the higher performance of the solution of PEG8000 at 200mg/mL in preserving the initial characteristics of the CMV.

**Table 5** GSD value, CMV yield and concentration measured after freeze-drying (FD) at different freezing temperatures.

Prep No.	FD protecting components (mg/mL)	Freezing T° (°C)	GSD	CMV Yield after FD (2-6 µm) (%)	Concentration (CMV/mL)
2A	PEG8000 (100 mg/mL)	-60 °C	1.21	26	$0.9 \times 10^8$
		-50 °C	1.21	39	$1.3 \times 10^8$
		-40 °C	1.22	45	$2.1 \times 10^8$
		-30 °C	1.21	34	$1.5 \times 10^8$
2B	PEG8000 (200 mg/mL)	-60 °C	1.20	64	$2.0 \times 10^8$
		-50 °C	1.20	68	$2.0 \times 10^8$
		-40 °C	1.19	61	$2.3 \times 10^8$
		-30 °C	1.20	56	$2.5 \times 10^8$
S3A	Sorbitol (100 mg/mL)	-60 °C	1.33	3	$0.1 \times 10^8$
		-50 °C	1.29	3	$0.1 \times 10^8$
		-40 °C	1.38	6	$0.3 \times 10^8$
		-30 °C	1.27	4	$0.2 \times 10^8$

### **Example 5**

#### **Effect of the concentration of the freeze-drying protecting component on calibrated microvesicles characteristics**

[0162] The concentration of the freeze-drying protecting component was also investigated in order to evaluate the freeze-drying efficiency, in terms of preservation of GSD value and microvesicles yield after freeze-drying.

[0163] For this study, PEG4000 and PEG8000 were selected as freeze-drying protecting components, since they evolved as the most promising among those here investigated. Different solutions in a range of concentrations between 50 mg/mL and 250 mg/mL were prepared for each investigated formulation.

[0164] The calibrated microvesicles suspended in the different solutions of freeze-drying protecting components were then aliquoted in DIN8R glass vials (1.5 mL suspension/vial) and transferred in the freeze dryer.

[0165] Two different freezing temperatures were investigated. The vials were cooled at -60°C and -40°C, and freeze-dried for approximately 1 hour under vacuum. At the end of the procedure, a freeze-dried composition was obtained as a white homogenous dry solid. The head space was then filled with pure C<sub>4</sub>F<sub>10</sub>.

## Results

[0166]

**Table 6** GSD and CMV yield values of the CMV suspensions containing different concentrations percentages of PEG4000, after freeze-drying.

PEG4000 <i>mg/mL</i>	Freezing at -60°C		Freezing at -40°C	
	<i>GSD</i>	<i>CMV yield (%)</i>	<i>GSD</i>	<i>CMV yield (%)</i>
50	1.230	10%	1.224	19%
100	1.224	35%	1.204	38%
150	1.227	59%	1.193	46%
200	1.246	65%	1.191	57%
250	1.197	71%	1.198	52%

**Table 7** GSD and CMV yield values of the CMV suspensions containing different concentrations percentages of PEG8000, freeze-drying.

PEG8000 <i>mg/mL</i>	Freezing at -60 °C		Freezing at -40 °C	
	<i>GSD</i>	<i>CMV yield (%)</i>	<i>GSD</i>	<i>CMV yield (%)</i>
50	1.259	4%	1.243	9%
100	1.188	29%	1.213	24%
150	1.182	56%	1.202	49%
200	1.188	63%	1.193	60%

PEG8000 <i>mg/mL</i>	Freezing at - 60 °C		Freezing at -40 °C	
	<i>GSD</i>	<i>CMV yield (%)</i>	<i>GSD</i>	<i>CMV yield (%)</i>
250	1.195	60%	1.192	58%

[0167] Results showed that there is a linear relationship between the final microvesicles yield after freeze-drying and the total concentration of the freeze-drying protecting component. As displayed in **Figure 4**, an increasing improvement of the freeze-drying efficiency was obtained using concentrations of freeze-drying protecting component between 50 mg/mL and 200 mg/mL for both PEG4000 and PEG8000.

[0168] The above results show that an increase of the concentration of the PEG solution has in general a positive effect on the GSD values and/or CMV yields.

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[0169]

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## REFERENCES CITED IN THE DESCRIPTION

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## Patentkrav

1. Frysetørret sammensætning, som omfatter (i) et amfifilt materiale, der omfatter et phospholipid og (ii) en polyethylenglycol (PEG) med en molekylvægt på mellem 2000 og 10000 g/mol, som ved rekonstitution med en farmaceutisk acceptabel opløsning i nærvær af en biokompatibel gas tilvejebringer en suspension af kalibrerede gasfyldte mikrovesikler, hvor den rekonstituerede suspension af mikrovesikler har en geometrisk standardafvigelse (GSD) på mindst 1,22 eller derunder, og hvor polyethylenglycol (PEG) har en koncentration på mellem 12 og 25 vægt/volumen-%.
2. Frysetørret sammensætning ifølge krav 1, hvor polyethylenglycol (PEG) har en molekylvægt på mellem 4000 og 8000 g/mol.
3. Frysetørret sammensætning ifølge krav 2, hvor polyethylenglycol (PEG) er valgt blandt en PEG med en molekylvægt på 4000 g/mol ( $\pm 10\%$ ) eller en PEG med en molekylvægt på 8000 g/mol ( $\pm 10\%$ ).
4. Frysetørret sammensætning ifølge et hvilket som helst af ovennævnte krav 1 til 3, hvor den rekonstituerede suspension af kalibrerede mikrovesikler er kendetegnet ved en koncentration på mindst  $2,0 \times 10^8$  mikrovesikler/ml.
5. Frysetørret sammensætning ifølge et hvilket som helst af ovennævnte krav 1 til 4, hvor polyethylenglycol (PEG) har en koncentration på mellem 14 og 24 vægt/volumen-%.
6. Suspension af gasfyldte mikrovesikler opnået ved rekonstituering af en frysetørret sammensætning ifølge et hvilket som helst af ovennævnte krav med en farmaceutisk acceptabel opløsning i nærvær af en biokompatibel gas.



7. Suspension af gasfyldte mikrovesikler ifølge krav 6, hvor den frysetørringsbeskyttende bestanddel er til stede ved en koncentration på mellem 12 % og 25 %.

- 5 8. Fremgangsmåde til fremstilling af en frysetørret sammensætning til fremstilling af en rekonstitueret suspension af kalibrerede gasfyldte mikrovesikler med en geometrisk standardafvigelse (GSD) på 1,22 eller derunder, som omfatter trinene:
- 10 a. fremstilling af en suspension af kalibrerede gasfyldte mikrovesikler, der omfatter en polyglycol som frysetørringsbeskyttende bestanddel, hvor de kalibrerede mikrovesikler har en størrelsesfordeling, der er kendetegnet ved en geometrisk standardafvigelse (GSD) på 1,22 eller derunder; og
- 15 b. frysetørring af suspensionen af kalibrerede mikrovesikler.

9. Fremgangsmåde ifølge krav 8, hvor fremstillingsmetoden i trin a. er mikrofluidisk flow-fokuseringsteknikken.

- 20 10. Fremgangsmåde ifølge krav 9, hvor den frysetørringsbeskyttende bestanddel har en samlet koncentration på mellem 100 mg/ml og 300 mg/ml, fortrinsvis mellem 120 mg/ml og 250 mg/ml, mere fortrinsvis hvor den samlede koncentration er 200 mg/ml.

25

11. Frysetørret sammensætning til fremstilling af en suspension af kalibrerede gasfyldte mikrovesikler med en geometrisk standardafvigelse (GSD) på 1,22 eller derunder, hvilken frysetørrede sammensætning kan opnås ved hjælp af en
- 30 fremgangsmåde, der omfatter følgende trin:
- a. fremstilling af en første suspension af gasfyldte kalibrerede mikrovesikler ved hjælp af en mikrofluidisk flow-fokuseringsproces, idet suspensionen yderligere omfatter en polyethylenglycol (PEG) med en molekylvægt på mellem 2000 og
- 35 10000 g/mol, og hvor de kalibrerede mikrovesikler har en størrelsesfordeling, der er kendetegnet ved en geometrisk standardafvigelse (GSD) på 1,22 eller derunder; og
- b. frysetørring af suspensionen.

12. Fremgangsmåde til fremstilling af et injicerbart  
kontrastmiddel, der omfatter en suspension af gasfyldte  
mikrovesikler, hvor fremgangsmåden omfatter rekonstituering af  
5 en frysetørret sammensætning ifølge et hvilket som helst af  
ovennævnte krav 1-5 med en farmaceutisk acceptabel opløsning i  
nærvær af en biokompatibel gas.

13. Fremgangsmåde ifølge krav 12, hvor suspensionen af  
10 kalibrerede mikrovesikler er kendetegnet ved en GSD på 1,22  
eller derunder.

# DRAWINGS

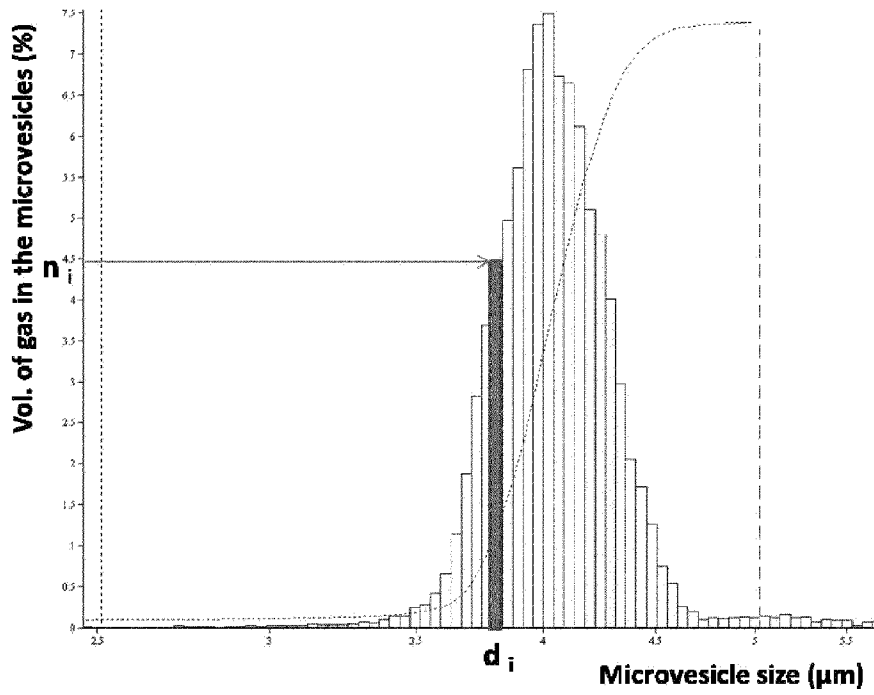


Figure 1

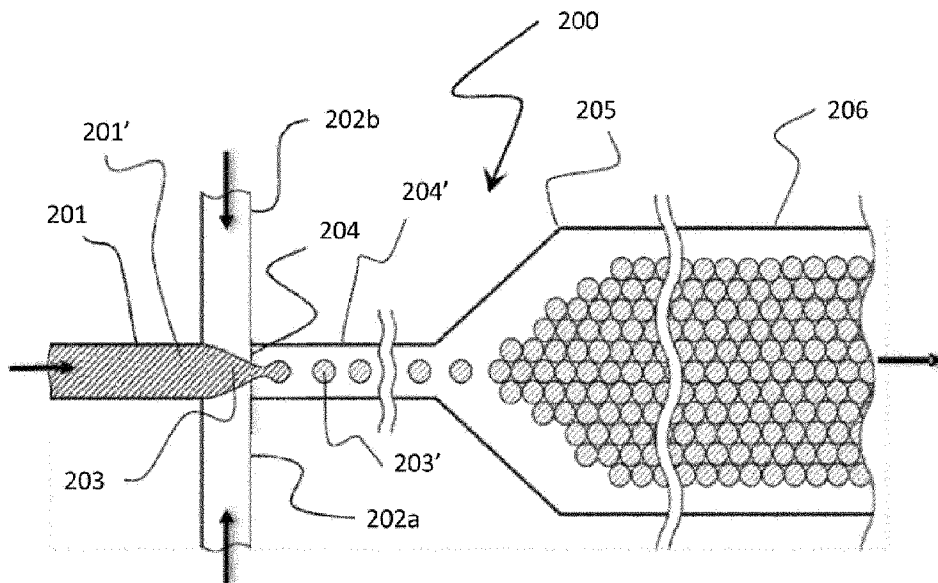


Figure 2

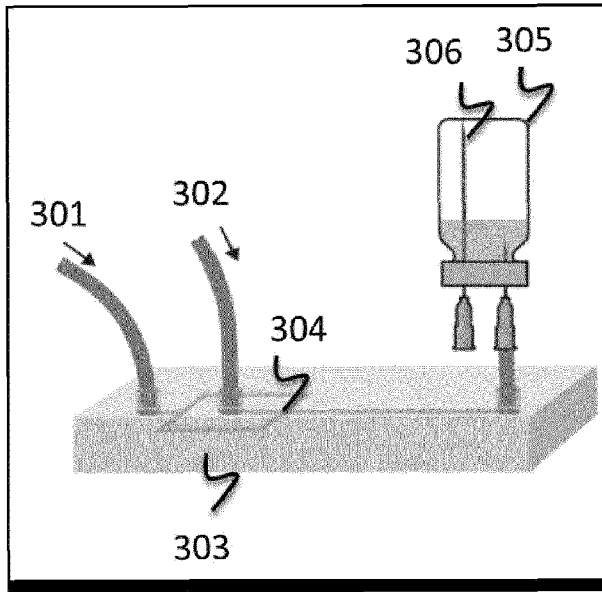


Figure 3

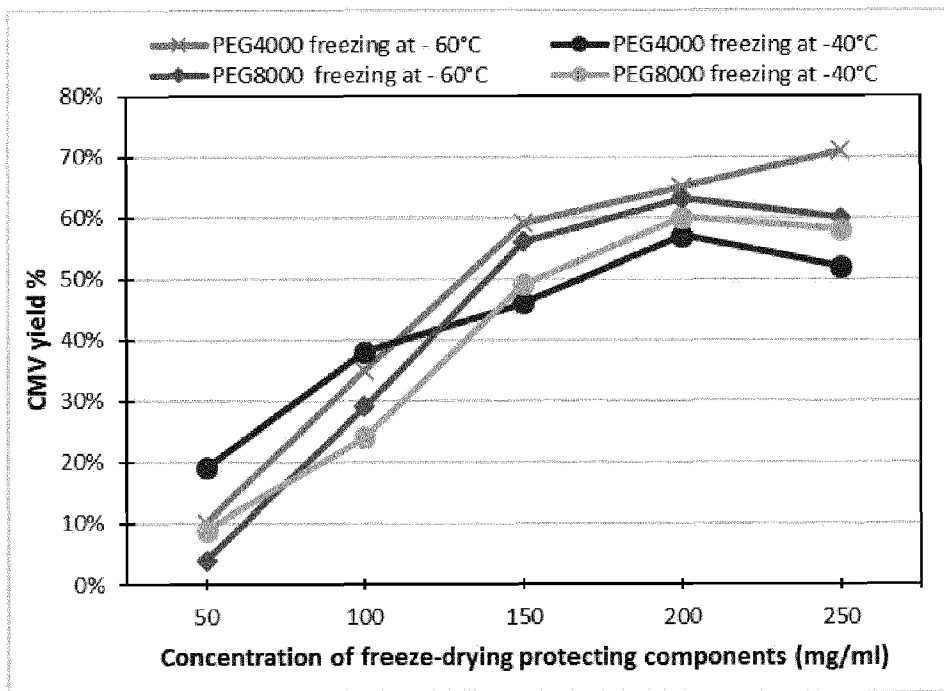


Figure 4