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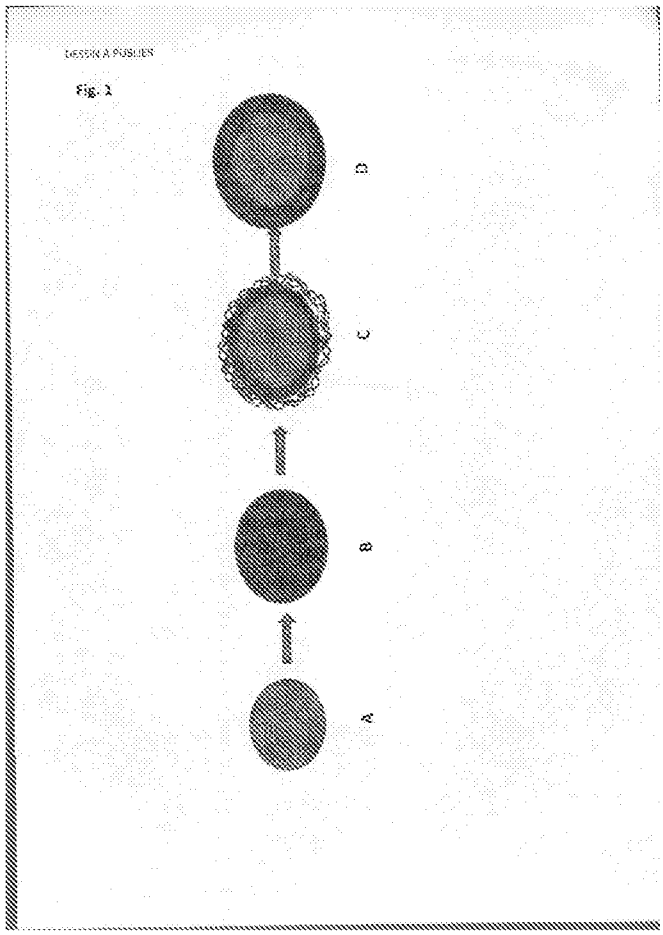
(54) **Nanocapsules and method for manufacturing thereof.**

(57) The invention is directed to a method for manufacturing supported lipid bilayer on a porous silica nanoparticle with a ^-potential comprised between -10 mV and +10 mV, said method comprising the steps of (a) providing a negatively charged supported lipid bilayer on a porous silica nanoparticle, wherein said negatively charged supported lipid bilayer has a (^-potential inferior to -15 mV and wherein said negatively charged supported lipid bilayer comprised at least one phospholipid and; (b) adding a formulation of lipids, said lipids being 1,2-dioleoyl-3-trimethylammonium-propane alias DOTAP, cholesterol and at least one lipid different from DOTAP and cholesterol. Said method is remarkable in that it further comprises the step of (c) performing an ultra-sonication for promoting DOTAP incorporation. Said method can be supplemented by the step of addition of alginate and the step of cross-linking said alginate. The invention is also directed to nanocapsule and composition comprising said nanocapsule.



Nanocapsules and method for manufacturing thereof

Description

Technical field

[0001] The invention is directed to the field of synthesis of porous silica nanomaterials adapted to be used as nanovector for the encapsulation and the delivery of materials.

Background art

[0002] Porous silica nanomaterials allow different biomedical applications such as drug delivery, therapeutic imaging, and diagnosis. In this context, porous and mesoporous silica nanoparticles (MSNPs) have been hugely studied as a vector for drug delivery applications.

[0003] Nanocapsules suitable for delivering an active moiety are known in the art as being protected by an outer layer. Said outer layer can be for instance a supported lipid bilayer (SLB). This outer layer is important for the release properties of the nanocapsule.

[0004] Liposomes are artificial membranes composed mostly of phospholipids Cholesterol is often used in the preparation of liposome, in order to reduce the fluidity of the hydrocarbon chains and to make the liposomes less permeable. The liposomes alone cannot be used as nanocapsule, since they are very rapidly taken up by the immune system of an organism, which limits the duration for which the liposomes circulate and can release the entrapped active moiety inside the blood stream (see study entitled "Factors affecting protein release from microcapsule prepared by liposome in alginate", by Dai C. *et al.*, *Colloids and Surfaces B: Biointerfaces*, **2005**, *42*, 253-258).

[0005] The study entitled "pH-responsive liposome-templated polyelectrolyte nanocapsules" (Cuomo F. *et al.*, *Soft Matter*, **2012**, *8*, 4415-4420) relates to hollow nanocapsule made of polyectrolyetolytes. They are produced by implementing a layer-by-layer (LBL) approach, using liposomes as template. Negatively charged alginate and positively charged chitosan, two biocompatible polymers are alternatively adsorbed onto liposomes,

producing nanocapsules with an average diameter of about 280 nm. When the outer layer is made of chitosan, the nanocapsules are stable only at acidic pH, while when the outer layer is made of alginate, the nanocapsules remain stable at a pH range comprised between 4.6 and 8.

[0006] The study entitled "Loading and protection of hydrophilic molecules into liposome-templated polyelectrolyte nanocapsules" (Cuomo F. *et al.*, *Langmuir*, **2014**, *30*, 7993-7999) relates to structure with submicrometer dimension that can entrap dextran. The system shows an ability to retain the molecules both at slightly acidic or neutral pH and consists also of LBL approach, using liposomes as template and negatively charged alginate and positively charged chitosan.

[0007] Ideally, a controlled release active moiety system must have high encapsulation efficiency, must provide maximal stability and a low initial burst. It is however often difficult to provide an homogeneous outer layer of alginate, especially when supported lipid bilayer bearing a negative charge is surrounding the inner core of the nanocapsule, namely the porous silica nanoparticle. When trying to combine negatively charged alginate on negatively charged supported lipid bilayer, agglomeration issues occur and then, those nanocapsules often present leakage issues, leading to uncontrolled released of the active moiety, which renders them unsuitable for therapeutic, pharmaceuticals and/or other similar applications.

Summary of invention

Technical Problem

[0008] The invention has for technical problem to alleviate at least one of the drawbacks present in the prior art.

[0009] More particularly, the invention has for technical problem to provide a biocompatible nanocapsule with an outer layer that is sufficiently resistant to prevent the leakage of the active moiety and to be sufficiently biodegradable at the same time to favour the slow and control release of the active moiety, especially when negatively charged alginate is to be

designed as the outer layer of a nanocapsule surrounded by negatively charged lipid bilayer.

Technical solution

[0010] In general, the particular embodiments of each object of the invention are also applicable to other objects of the invention. To the extent possible, each object of the invention is combinable with other objects.

[0011] The first object of the invention is directed to a method for manufacturing supported lipid bilayer on a porous silica nanoparticle with a ζ-potential comprised between -10 mV and +10 mV, said method comprising the steps of (a) providing a negatively charged supported lipid bilayer on a porous silica nanoparticle, wherein said negatively charged supported lipid bilayer has a ζ-potential inferior to -15 mV and wherein said negatively charged supported lipid bilayer comprised at least one phospholipid and; (b) adding a formulation of lipids, said lipids being 1,2-dioleoyl-3-trimethylammonium-propane alias DOTAP, cholesterol and at least one lipid different from DOTAP and cholesterol. Said method is remarkable in that it further comprises the step of (c) performing an ultra-sonication.

[0012] According to a preferred embodiment, said step of (c) performing an ultra-sonication is achieved for promoting DOTAP incorporation.

[0013] According to a preferred embodiment, said ultra-sonication is carried out at a temperature comprised between 40°C and 60°C.

[0014] According to a preferred embodiment, the concentration of DOTAP in the formulation of lipids is comprised between 50% and 150% of the concentration of said at least one phospholipid, preferentially in a concentration amounting to 100% of the concentration of said at least one phospholipid.

[0015] According to a preferred embodiment, said at least one phospholipid is 1,2 dioleoyl-sn-glycero-3-phospho-L-serine alias DOPS.

[0016] According to a preferred embodiment, said step of ultra-sonication is performed between 10 minutes and 30 minutes, preferentially during 20 minutes.

[0017] According to a preferred embodiment, said at least one lipid different from DOTAP and cholesterol in step (b) is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine alias DPPC.

[0018] The second object of the present invention is directed to a method for manufacturing a nanocapsule based on a supported lipid bilayer on a charged porous silica nanoparticle with a ζ-potential comprised between -10 mV and +10 mV, said method comprising the method in accordance with the first object of the invention and the steps of addition of an aqueous solution of sodium alginate; and of cross-linking of said sodium alginate.

[0019] According to a preferred embodiment, the concentration of sodium alginate in said aqueous solution is comprised between 1% (wt%) and 5% (wt%), preferentially is equal to 2% (wt%).

[0020] According to a preferred embodiment, said addition is performed in a buffer solution, said buffer solution being based on 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid alias HEPES or on phosphate-buffered saline alias PBS.

[0021] According to a preferred embodiment, said step of cross-linking of said sodium alginate is performed by mixing an aqueous solution of divalent cation, preferentially calcium-based cation or barium-based cation, at a concentration comprised between 40 mM and 60 mM, preferentially at a concentration of 50 mM.

[0022] According to a preferred embodiment, said porous silica nanoparticle is a mesoporous silica nanoparticle, preferentially a positively charged mesoporous silica nanoparticle.

[0023] The third object of the present invention is directed to a nanocapsule suitable for encapsulating at least one active moiety, said nanocapsule comprising an inner core, an inner layer and an outer layer, said inner core being formed by a porous silica nanoparticle, said inner layer being formed by a lipid bilayer, wherein said lipid bilayer comprised at least one phospholipid, characterized in that said outer layer comprises cross-linked sodium alginate.

[0024] According to a preferred embodiment, said outer layer of cross-linked sodium alginate has a thickness comprised between 3 nm and 100 nm, preferentially between 20 nm and 30 nm, more preferentially 25 nm.

[0025] According to a preferred embodiment, said porous silica nanoparticle is a mesoporous silica nanoparticle, preferentially a positively charged mesoporous silica nanoparticle.

[0026] The fourth object of the invention is directed to a composition comprising the nanocapsule in accordance with the third object of the invention and at least one active moiety, said at least one active moiety being in the inner core of said nanocapsule.

[0027] According to a preferred embodiment, said at least one active moiety is one contrasting agent preferentially selected from the group comprising calcein, rhodamine, methylene blue and indocyanine green, more preferentially indocyanine green.

[0028] According to a preferred embodiment, said composition comprising the nanocapsule in accordance with the third object of the invention, and at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties, preferentially a molecule presenting anticancerous properties, said at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties being in the inner core of said nanocapsule, is suitable for oral administration.

[0029] According to a preferred embodiment, said composition comprising the nanocapsule in accordance with the third object of the invention, and at least one cosmetic agent, preferentially an antioxidant, and/or at least one dermatological agent, preferentially a wound-healing agent, said at least one cosmetic and/or dermatological agent being in the inner core of said nanocapsule, is suitable for topical administration.

[0030] According to a preferred embodiment, said composition comprising the nanocapsule in accordance with the third object of the invention, and at least one active moiety presenting therapeutic and/or pharmaceutical properties, is suitable for loco-regional administration such as intraocular and/or intratumoral administration.

[0031] The fifth object of the invention is directed to a method for manufacturing a negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle, said method comprising the steps of (a) preparing a first formulation of lipids, said lipids from said first formulation being 1,2 dioleoyl-sn-glycero-3-phospho-L-serine alias DOPS, cholesterol and at least one lipid different from DOPS and cholesterol, said formulation of lipids being dissolved in a solvent; (b) evaporation said solvent; (c) adding an aqueous formulation of positively charged mesoporous silica nanoparticles; (d) performing a first ultra-sonication; (e) performing a centrifugation. Said method is remarkable in that it further comprises the steps of (f) addition of a second formulation of lipids, said lipids from said second formulation being 1,2-dioleoyl-3-trimethylammonium-propane alias DOTAP, cholesterol and at least one lipid different from DOTAP and cholesterol, said second formulation of lipids being dissolved in a solvent; and (g) performing a second ultra-sonication; and wherein said negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle has a ζ potential comprised between -10 mV and + 10 mV.

[0032] According to a preferred embodiment, said step of performing a second ultra-sonication is achieved for promoting DOTAP incorporation.

[0033] According to a preferred embodiment, the number of equivalent of said DOTAP relative to one equivalent of DOPS is comprised between 0.5 and 1.5, preferentially is equal to 1.

[0034] According to a preferred embodiment, the number of equivalents of cholesterol relative to one equivalent of DOPS is comprised between 2.30 and 2.70, preferentially is equal to 2.50.

[0035] According to a preferred embodiment, said at least one lipid different from DOPS and cholesterol in step (a) is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine alias DPPC.

[0036] According to a preferred embodiment, said at least one lipid different from DOTAP and cholesterol in step (f) is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine alias DPPC.

[0037] According to a preferred embodiment, the number of equivalent of said DPPC relative to one equivalent of DOPS is comprised between 3.55 and 3.95, preferentially is equal to 3.75.

[0038] According to a preferred embodiment, said solvent is a mixture of chloroform and methanol.

[0039] The sixth object of the invention is directed to a method for manufacturing a nanocapsule based on a negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle with a ζ potential comprised between – 10 mV and + 10 mV, said method comprising the method in accordance with the fifth object of the invention followed by the steps of addition of an aqueous solution of sodium alginate; and the step of cross-linking of said sodium alginate.

[0040] According to a preferred embodiment, said step of addition of alginate is performed by adsorption of an aqueous solution of sodium alginate at a concentration comprised between 1% (v/v) and 5% (v/v), preferentially at a concentration of 2% (v/v).

[0041] According to a preferred embodiment, said step of cross-linking of said sodium alginate is performed by mixing an aqueous solution of calcium, at a concentration comprised between 40 mM and 60 mM, preferentially at a concentration of 50 mM.

[0042] The seventh object of the invention is directed to a nanocapsule suitable for encapsulating at least one active moiety, said nanocapsule comprising an inner core, an inner layer and an outer layer, said inner core being formed by a mesoporous silica nanoparticle, said inner layer being formed by a lipid bilayer. Said nanocapsule is remarkable in that said outer layer comprises cross-linked sodium alginate.

[0043] According to a preferred embodiment, said outer layer of cross-linked sodium alginate has a thickness comprised between 3 nm and 100 nm, preferentially between 20 nm and 30 nm, more preferentially 25 nm.

[0044] The eighth object of the invention is directed to a composition comprising the nanocapsule in accordance with the seventh object of the invention and at least one active moiety, said at least one active moiety being in the inner core of said nanocapsule.

[0045] According to a preferred embodiment, said at least one active moiety is a contrasting agent preferentially selected from the group comprising calcein, rhodamine, methylene blue and indocyanine green, more preferentially indocyanine green.

[0046] According to a preferred embodiment, the composition is suitable for oral administration, and comprises at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties, preferentially a molecule presenting anticancerous properties, said at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties being in the inner core of said nanocapsule.

[0047] According to a preferred embodiment, the composition is suitable for topical administration, and comprises at least one cosmetic agent, preferentially an antioxidant, and/or at least one dermatological agent, preferentially a wound-healing agent, said at least one cosmetic agent being in the inner core of said nanocapsule.

[0048] According to a preferred embodiment, the composition is suitable for loco-regional administration, and comprises at least one therapeutic and/or pharmaceutical agent, said at least one therapeutic and/or pharmaceutical agent being in the inner core of said nanocapsule.

[0049] The ninth object of the invention is directed to a method for manufacturing a negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle, said method comprising the following steps (a) preparing a first formulation of lipids, said lipids from said first formulation being 1,2 dioleoyl-sn-glycero-3-phospho-L-serine alias DOPS, cholesterol and at least one lipid different from DOPS and cholesterol, said first formulation of lipids being dissolved in a solvent; (b) preparing a second formulation of lipids, said lipids from said second formulation being 1,2-dioleoyl-3-trimethylammonium-propane alias DOTAP, cholesterol and at least one lipid different from DOTAP and cholesterol, said second formulation of lipids being dissolved in a solvent; and (c) mixing said first formulation with said second formulation at a

temperature comprised between 40°C and 60°C; wherein said negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle has a ζ potential comprised between -10 mV and -10 mV.

[0050] According to a preferred embodiment, the number of equivalent of said DOTAP relative to one equivalent of DOPS is comprised between 0.5 and 1.5, preferentially is equal to 1.

[0051] According to a preferred embodiment, the number of equivalents of cholesterol relative to one equivalent of DOPS is comprised between 2.30 and 2.70, preferentially is equal to 2.50.

[0052] According to a preferred embodiment, said at least one lipid different from DOPS and cholesterol in step (a) is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine alias DPPC.

[0053] According to a preferred embodiment, said at least one lipid different from DOTAP and cholesterol in step (b) is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine alias DPPC.

[0054] According to a preferred embodiment, the number of equivalent of said DPPC relative to one equivalent of DOPS is comprised between 3.55 and 3.95, preferentially is equal to 3.75.

[0055] According to a preferred embodiment, said solvent is a mixture of chloroform and methanol.

[0056] The tenth object of the invention is directed to a method for manufacturing a nanocapsule based on a negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle with a ζ potential comprised between – 10 mV and + 10 mV, said method comprising the method in accordance with the ninth object of the invention followed by the steps of addition of an aqueous solution of sodium alginate; and the step of cross-linking of said sodium alginate.

[0057] According to a preferred embodiment, said step of addition of alginate is performed by adsorption of an aqueous solution of sodium alginate at a concentration comprised between 1% (v/v) and 5% (v/v), preferentially at a concentration of 2% (v/v).

[0058] According to a preferred embodiment, said step of cross-linking of said sodium alginate is performed by mixing an aqueous solution of calcium,

at a concentration comprised between 40 mM and 60 mM, preferentially at a concentration of 50 mM.

[0059] The eleventh object of the invention is directed to a nanocapsule suitable for encapsulating at least one active moiety, said nanocapsule comprising an inner core, an inner layer and an outer layer, said inner core being formed by a mesoporous silica nanoparticle, said inner layer being formed by a lipid bilayer. Said nanocapsule is remarkable in that said outer layer comprises cross-linked sodium alginate.

[0060] According to a preferred embodiment, said outer layer of cross-linked sodium alginate has a thickness comprised between 3 nm and 100 nm, preferentially between 20 nm and 30 nm, more preferentially 25 nm.

[0061] The twelfth object of the invention is directed to a composition comprising the nanocapsule in accordance with the eleventh object of the invention and at least one active moiety, said at least one active moiety being in the inner core of said nanocapsule.

[0062] According to a preferred embodiment, said at least one active moiety is a contrasting agent preferentially selected from the group comprising calcein, rhodamine, methylene blue and indocyanine green, more preferentially indocyanine green.

[0063] According to a preferred embodiment, the composition is suitable for oral administration, and comprises at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties, preferentially a molecule presenting anticancerous properties, said at least one active moiety presenting therapeutic, pharmaceutical, nutraceutical and/or cosmeceutical properties being in the inner core of said nanocapsule.

[0064] According to a preferred embodiment, the composition is suitable for topical administration, and comprises at least one cosmetic agent, preferentially an antioxidant, and/or at least one dermatological agent, preferentially a wound-healing agent, said at least one cosmetic agent being in the inner core of said nanocapsule.

[0065] According to a preferred embodiment, the composition is suitable for loco-regional administration, and comprises at least one therapeutic

and/or pharmaceutical agent, said at least one therapeutic and/or pharmaceutical agent being in the inner core of said nanocapsule.

Advantages of the invention

[0066] The invention is particularly interesting in that the reinforcement of the SLB allows for a better sealing of such nanocapsule and will also render them less or no permeable. The leaking issue will be suppressed or diminished. It will favour the stability and the slow-release of the active moiety, especially in presence of surfactants, inside of the nanocapsule.

[0067] The improvement of the stability is an advantage for incorporating those nanocapsules within creams, emulsions, creams based on emulsions, and hydrogel.

[0068] The method of the present invention will also render the nanocapsule more biocompatible, since the outer layer of the nanocapsule will be resistive at acidic pH. The drug release can be in this case better controlled.

[0069] As the slow-release of the active moiety is controlled by the outer layer of cross-linked alginate, various types of implementation of the nanocapsules according to the present invention can be derivatized. In particular, the slow and controlled release of hormonal product(s) and/or growth factor(s) can be envisioned with the nanocapsules in accordance with the present invention.

[0070] Various compositions that can be suitable for oral, topical, loco-regional administration could thus be developed.

Brief description of the drawings

[0071] Figure 1: General scheme showing the synthesis of nanocapsule in accordance with the present invention.

[0072] Figure 2: Size analysis of silica nanoparticles by Nano Tracking Analysis (NTA).

[0073] Figure 3: ζ-potential graph for MSNPs, MSNPs$^+$, and SLB on MSNPs performed on a Malvern instruments. Data are mean ± SE and represent three independent experiments.

[0074] Figure 4: Cryo-TEM image of a sample of nanocapsules with a cross-linked alginate as outer layer.

[0075] Figure 5: Cryo-TEM image of two nanocapsules with a cross-linked alginate as outer layer.

Description of an embodiment

[0076] The method of the invention consists in adding a cationic lipid onto a nanocapsule surrounded by a negatively charged lipid bilayer in order to be able to adsorb negatively charged polymer (such as sodium alginate) on nanocapsule surface. The first part of the method is performed by adding 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) in the form of liposome and by performing an ultra-sonication step. The step of ultra-sonication is essential since it will increase the temperature of the system and will allow the incorporation of DOTAP into the negatively charged supported lipid bilayer.

[0077] On figure 1, the nanocapsule **A** is a nanocapsule with an outer layer of negatively charged supported lipid bilayer.

[0078] In addition to the cholesterol, the SLB comprises a combination of various lipids and/or anionic and/or neutral phospholipids from the following list:

DPPG: 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)

DOPG: 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)

DOPS: 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine

DPPI: 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-myo-inositol)

DOPI: 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol)

DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine

DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

DPPE: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine

DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine

[0079] The nanocapsule B schematically shows that there was an incorporation of a layer of DOTAP. Any other cationic fatty acid chain might also be used.

[0080] The incorporation of DOTAP provides a nanocapsule with a supported lipid bilayer showing a ζ-potential comprised between -10 mV and +10 mV, so sensibly more positive than the nanocapsule with a SLB without such incorporation of DOTAP.

[0081] Advantageously, the ultra-sonication step can be carried out at a temperature comprised between 40°C and 60°C, in order to promote the activation of the system and thus the incorporation of DOTAP into the outer layer.

[0082] Advantageously, the ultra-sonication step can be carried out during 10 minutes to 30 minutes. It is preferentially performed for a period of 20 minutes.

[0083] DOTAP is incorporated under the form of a liposome. The concentration of DOTAP in the liposome that is added is comprised between 50% and 150% of the concentration of at least one phospholipid (for example, DOPS) which is part the supported lipid bilayer. Advantageously, the concentration amounts to 100% of the concentration of said phospholipid.

[0084] Once DOTAP has been incorporated to the supported lipid bilayer protecting the nanoparticles, the electrostatic properties, demonstrated by the ζ-potential comprised between -10 mV and +10 mV, which control the adsorption of further components (ζ-potential is important to control the thickness of alginate layer and avoid the aggregation).

[0085] One example is the adsorption of alginate (see nanocapsule C of figure 1), or alginate derivatives, which are further cross-linked (see nanocapsule D of figure 1) in order to reinforce the structure of the outer layer of the nanocapsule.

[0086] Thus, the addition of an aqueous solution of sodium alginate is achieved, followed by a cross-linking step. The concentration of sodium alginate in the aqueous solution is relatively low, amounting to 1%-5% (by weight). Preferentially, the concentration that is used is about 2% (by weight). In

order to facility the adsorption, the nanocapsule can be placed in a solution of a buffering agent, for instance 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). In order to perform the cross-linking step, and to make a gel of sodium alginate, divalent cations are employed. Examples of compounds are $CaCl_2$ or $BaCl_2$. They can be preferentially used at a concentration comprised between 40 mM and 60 mM, preferentially at a concentration of 50 mM.

[0087] Therefore, nanocapsule suitable for encapsulating at least one active moiety can be made. Such nanocapsule comprises an inner core formed by a porous silica nanoparticle, preferentially a mesoporous silica nanoparticle, more preferentially a positively charged mesoporous silica nanoparticle. The inner core is surrounded by an inner layer and an outer layer. The inner layer is formed by a supported lipid bilayer (SLB), preferentially a supported lipid bilayer comprising at least one phospholipid. The outer layer comprises cross-linked sodium alginate.

[0088] Moreover, the inner core can be formed by mica, clays, montmorillonite, alumina or TiO_2 particles. This allows for different manner of entrapping the active moiety.

[0089] The thickness of said outer layer made of cross-linked sodium alginate is comprised between 3 nm and 100 nm. It is preferentially comprised between 20 nm and 30 nm, more preferentially it is equal to 25 nm.

[0090] Once the nanocapsule has been incubated with at least one active moiety, composition of all kinds can be obtained. The active moiety can be one contrasting agent, for instance calcein, rhodamine, methylene blue and/or indocyanine green. The active moiety can be one compound presenting therapeutic and/or pharmaceutical properties, for instance a molecule presenting anticancerous properties. The active moiety can also be one cosmetic agent, for instance an antioxidant. The incubation of the active moiety into the nanocapsule is performed before the encapsulation with the supported lipid bilayer and thus before the incorporation of DOTAP and the adsorption of alginate. Incubation of the active moiety is generally performed in milliQ water but can also be performed in citrate buffer.

[0091] In the implementation of a project related to the synthesis of mesoporous silica nanomaterials which are completely surrounded by supported lipid bilayer, the particular synthesis of negatively charged supported lipid bilayer (SLB) on a positively charged mesoporous silica nanoparticle MSNPs^{+}) have been achieved. In the implementation of the process, the ζ-potential measured in milliQ water at a pH of 5.8 was inferior to -50 mV. The following paragraphs describe the synthesis of such nanocapsules with a highly negative ζ-potential.

[0092] Anionic supported lipid bilayers (SLB) on functionalized mesoporous silica nanoparticles (MSNPs) are synthesized in a one pot process using ultra-sonication. The ultra-sonication process allows indeed the incorporation of the MSNPs+ within the SLB.

[0093] The lipid formulations of supported lipid bilayer covering mesoporous silica nanoparticles were composed of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and cholesterol.

[0094] When preparing the formulation, the lipids are dissolved in a solvent, preferably a mixture of chloroform/methanol at a molar ratio of 9/1.

[0095] To form the thin lipid film, 1 ml of said formulation is then evaporated to remove said solvent.

[0096] The lipid film constituted by DPPC/DOPS/cholesterol was thus ultra-sonicated in the presence of 4 ml of an aqueous solution of functionalized MSNPs with a nanoparticle size of 50.9±3.6 nm.

[0097] The concentration of said aqueous solution of functionalized MSNPs is fixed at 5 mg/mL.

[0098] Ultra-sonication of the lipid film in water induces very high shearing forces generating the formation of Small Unilamellar Vesicles (SUV) from MultiLamellar Vesicles (MLV).

[0099] At the end of the process, the stable colloidal suspension was then centrifuged to remove the excess of liposomes and the particles were suspended in water.

[00100] After formulation, lipids were extracted and then quantified using the Liquid Chromatography-Mass Spectrometry (LC-MS) method. Table I summarizes the different molar ratios for each lipid before and after the synthesis of the MSNPs incorporated within the SLB.

Table I: Initial molar ratios of each lipid before synthesis and final ratios after synthesis and purification of the MSNPs with SLB. The data are mean±SE, performed by LC-MS in three independent experiments.

		DPPC	DOPS	cholesterol
Initial ratio	SLB	52	14	34
Ratio after synthesis	SLB	41.5±2.9	8.1±2.3	50.3±8.2

[00101] Variations can be seen between the lipids molar ratios before ultra-sonication and the final ratios found by LC-MS after the synthesis. The final molar ratio of cholesterol in the SLB increases from 34% to 50%.

[00102] This high amount of cholesterol (36%-50%) in the final SLB is necessary to give colloidal stability to the SLB. This is provided by the number of equivalents (2.50) of cholesterol relative to one equivalent of DOPS as initial molar ratio.

[00103] The ζ-potential properties of SLB on MSNPs were investigated in different media (milliQ water, HEPES buffer and human serum) and were compared with the similar physicochemical properties of MSNPs and MSNPs+ (see figures 2 and 3). These measurements have been made without performing any filtration or size exclusion prior to analysis. The size has been measured using NTA whereas ζ-potential has been measured by using Malvern Nano Zetasizer®. In milliQ water, at a pH value of 5.8, MSNPs+ have a charge of +26.47 mV whereas SLB on MSNPS have a charge of -52 mV.

[00104] Further details relating to the synthesis and use of said negatively charged supported lipid bilayer on a positively charged mesoporous silica

nanoparticle are to be found in the International patent application numbered **PCT/EP2016/067564**.

[00105] In order to increase the ζ-potential, the method of the present invention has been applied on these highly negatively charged nanocapsules.

[00106] The method is exemplified herein: in a first alternative, directly after the centrifugation step (used to remove the excess of liposomes), a formulation comprising DOTAP liposomes is added. In a second alternative, a first formulation of lipids in a solvent can be prepared, on which a second formulation comprising DOTAP liposomes is added. The first formulation may for example comprise DOPS, cholesterol and at least one lipid different from DOPS and cholesterol, for instance DPPC.

[00107] The formulation of DOTAP liposomes comprises DOTAP, cholesterol and a lipid different from DOTAP and cholesterol, and is prepared in a solvent, preferentially in a mixture of chloroform/methanol 9/1. The overall concentration of the lipids in the solvent is comprised between 2 and 12 mg/l. The lipid different from DOTAP and cholesterol may be DPPC. The ratio DPPC/DOTAP/cholesterol may thus be 75/20/50. An evaporation of the solvent is performed in order to obtain a lipid film. After addition of about 5 mL of milliQ water, the suspension is then ultrasonicated. The excess of lipid aggregates was removed after centrifugation at 8000 rpm during 10 minutes by Eppendorff® 64R centrifuge. The DOTAP liposomes obtained were stored at 4 °C under argon. A ζ-potential value has been determined to be equal to +27.4 ± 4 mV for this second formulation based on DOATP liposomes.

[00108] After addition of said formulation of DOTAP liposome on said highly negatively charged nanocapsules, an ultra-sonication step is carried out, preferentially at a temperature comprised between 40°C and 60°C. This thermal activation (ultra-sonication and/or heating) is performed between 10 and 30 minutes, usually during 20 minutes.

[00109] ζ-potential measured by using Malvern Nano Zetasizer® in milliQ water, at a pH value of 5.8, is of -4.33 ± 5 mV. That is the indication that the highly initial negative charge has been largely reduced, since now, the nanocapsule is almost bearing no charges at all.

[00110] The next step, concerning the adsorption and cross-linking, is thus carried out. An aqueous solution of sodium alginate is added, preferentially in a buffer based on HEPES). The concentration of sodium alginate is comprised between 1% and 5% by weight of the nanocapsule and is preferentially equal to 2%.

[00111] After the adsorption of sodium alginate, a calcium solution (based on $CaCl_2$) was mixed with the nanocapsule suspension during 1 hour, resulting in a formation of a protective gel of alginate. A ζ-potential value has been determined to be equal to -15.1 ± 3 mV after the formation of the gel.

[00112] Table II summarizes the different characteristics of the nanocapsule obtained according to the method of the present invention:

Table II: Size and ζ-potential of DOTAP liposomes, and the nanocapsules A, B, C and D as schematically represented on figure 1. The data are mean ± SE, performed in HEPES buffer.

References on figure 1	Composition of the samples	Size (nm)[1]	ζ-potential (mV)[2]
N/A[3]	**liposomes DOTAP (DPPC/DOTAP/CHOL)**	70 ± 15	+27.4 ± 4
A	**nanocapsule with DPPC/DOPS/CHOL**	120 ± 16	- 40 ± 10
B	**nanocapsule with at least DOPS + liposomes DOTAP**	172 ± 11	-4.33 ± 5
C	**nanocapsule with at least DOPS + liposomes DOTAP + Alginate 2%**	168 ± 16	-18.53 ± 6
D	**nanocapsule with at least DOPS + liposomes DOTAP + cross-linked Alginate 2%**	114 ± 15	-15.1 ± 3

[1]Size obtained by Dynamic Light Scattering (DLS)

[2]ζ-potential obtained by DLS

[3] N/A = non-applicable

These results indicate a weak aggregation of the nanocapsule obtained through the method of the present invention. The cross-linking of alginate with a calcium or barium cations also reduces the aggregation of the nanocapsules, since the average diameter is reduced. It is highlighted that the ζ-potential was highly negative at the beginning of the experiment and has become almost neutral once the DOTAP liposomes have been incorporated to the supported lipid bilayer.

[00113] Analysis of the nanocapsule has been made by cryo-TEM analysis. The average size is equal to 69 ± 22 nm (as there is not solvatation layer apparent in the Cryo-TEM analysis, the average diameter size is smaller than the size measure by DLS, which is 114 ± 15 nm).

[00114] Figure 4 shows an image Cryo-TEM. It demonstrates that no aggregation is present among the nanocapsule presenting an alginate gel as outer layer.

[00115] Figure 5 shows a detailed Cryo-TEM image, where two nanocapsules are present. The protective outer layer of alginate gel is clearly visible.

[00116] The leakage of the nanocapsule of the present invention was then studied. The fluorescent dye calcein was used in order to measure the properties of the nanocapsule to release or to hold the active moiety in its inner core which is a (meso)porous silica nanoparticle.

[00117] Table III shows the percentage of calcein leakage in the negatively charged supported lipid bilayer on a positively charged mesoporous silica nanoparticle (nanocapsule of the type **A** on figure 1), with a ζ-potential inferior or equal to -40 mV.

Table III: Leakage of calcein from highly negatively charged nanocapsules

	% calcein leakage		
Time in hours	**HEPES**	**Cell medium**	**HEPES + 1% Triton X-100**
2h	29	43	83
24h	38	50	96
48h	40	55	96
96h	43	67	96

[00118] Table IV shows the percentage of calcein leakage in the nanocapsule presenting an outer layer made of cross-linked alginate in accordance with the method of the present invention (nanocapsule of type D on figure 1).

Table IV: Leakage of calcein from nanocapsule in accordance with the present invention.

	% calcein leakage				
Time in hours	**HEPES**	**Cell medium**	**pH 2**	**HEPES + 1% Triton X-100**	**HEPES + 1% MYRJ™ S40**
2h	25	26	9	37	37
24h	28	27	10	41	32
48h	28	28	10	41	31
96h	27	26	10	41	30

[00119] By comparison of tables III and IV, one can observe that the leakage of calcein is relatively more important in the case where the nanocapsule are not protected with an outer layer made of cross-linked alginate. Thus, the loss of active moiety is prevented when an outer layer of cross-linked alginate is present.

[00120] The implementations of such nanocapsules with the protective outer layer of cross-linked sodium alginate are various. Compositions comprising contrasting agents, therapeutic molecules, pharmaceutical agents, cosmetic agents, dermatological agents (wound-healing agents), anticancerous agents, nutraceutical agents, cosmeceutical agents, hormonal products, growth factors and/or compounds adapted for loco-regional therapies (bone implants and/or cardiac implants), for intraocular administration and/or for intratumoral agent, can be thus easily derivatized.

Experimental section

[00121] **Incubation of MSNP+ with excess of calcein in milliQ water**

[00122] The incubation is performed with an amount of 1 mg of calcein for an amount of 2 mg of (meso)porous silica nanoparticles. The incubation is performed in milliQwater.

[00123] **DOTAP liposomes**

[00124] Phospholipids were dissolved in chloroform/methanol 9:1 at a range of concentrations of 2 to 12 mg/mL. For DOTAP liposomes, the phospholipids ratio was DPPC/DOTAP/Cholesterol 75/20/50. Then, these lipids were evaporated to produce a lipid film. Five mL of milliQ water were mixed with the lipid film. The suspension was ultrasonicated with Ultrasonicator Sonics Vibra Cell® during 15 minutes at room temperature under Argon flow at 29% of amplitude pulsed mode 10s/10s. The excess of lipid aggregates was removed after centrifugation at 8 000 rpm during 10 minutes by Eppendorff® 64R centrifuge. The DOTAP liposomes obtained were stored at 4 °C under Argon.

[00125] **Aniocells**

[00126] Phospholipids were dissolved in chloroform/methanol 9:1 at a range of concentrations of 2 to 12 mg/mL. For Aniocells, the phospholipids ratio was DPPC/DOPS/Cholesterol 75/20/50. Then, these lipids were evaporated to produce a lipid film. Five mL of calcein $MSNP^+$ at 2 mg/mL were mixed with the lipid film. The suspension was ultrasonicated with Ultrasonicator Sonics Vibra Cell® during 15 minutes at room temperature under Argon flow at 29% of amplitude pulsed mode 10s/10s.

[00127] **Ultra-sonication of Aniocells and DOTAP liposomes**

The ultra-sonication step was carried out during 4 minutes at a pulse 10/10. The excess of liposomes (DOPS-DOTAP) was removed after centrifugation at 45 000g, 20 minutes by Eppendorff® centrifuge. The aniocells-DOTAP liposomes was dispersed in HEPES buffer.

[00128] **Adsorption of the alginate layer**

[00129] The suspension of aniocells and DOTAP liposomes was mixed in presence of 2% alginate solution during 1 hour. The excess of alginate was removed after centrifugation at 45 000g, 20 minutes by Eppendorff® centrifuge. The aniocells-DOTAP liposomes-aginate was dispersed in HEPES buffer.

[00130] **Cross-linking of the alginate layer**

[00131] 50 mM of calcium solution was mixed with the alginate suspension during 1 hour. The excess of calcium was removed after centrifugation at 45 000g, 20 minutes by Eppendorff® centrifuge. The suspension was then dispersed in HEPES buffer.

[00132] **LC-MS experiments**

[00133] The LC-MS Thermo Scientific Dionex BIO LC system is coupled with the mass of LTQ Orbitrap Elite. The system consisted of a GS50 gradient pump, AS50 Auto Sampler with oven column of thermal compartment. The separation was performed at 40 °C on a GRACE visionHT C18 HL column (150×2.1 mm i.d., 3 µm) from Dionex Bio LC with the scan mass of 300 and 1 000. The flow rate was 0.25 mL/min for the mobile phases (mobile phase C, 5 mM ammonium acetate in water (pH 4.0) and mobile phase D, 5 mM ammonium acetate in methanol). The binary linear gradient began from a mixture of 20 % C and 80 % D and ended at 100 % D.

[00134] **NTA analysis**

[00135] Nanoparticles Tracking Analysis (NTA) used a light scattering method which relates the rate of Brownian motion to particle size. This method allows direct and real time visualizing and analyzing of the NPs in liquids. During NTA measurement, NPs are illuminated by a focused laser beam and analyzed by the light scattered by each individual particle in the microscope onto the image sensor of a charge-coupled device (CCD) camera. The camera visualizes and records the frames of the particles in solution. The NTA software identifies and individually tracks the particles

moving under Brownian motion. This measurement uses the temperature and the viscosity of the liquid to calculate particle size through the Stokes-Einstein equation. The Nanosight® analyses the particles with a size range from 30 to 1 µm. The samples were diluted at 0.01 mg/mL for analysis.

[00136] **DLS analysis**

[00137] Malvern Nano Zetasizer® measures the size and ζ-potential of nanoparticles by using dynamic light scattering size (DLS). The analysis was performed with 0.1 mg/mL for each sample.

[00138] **CryoTransmission Electron Microscopy (CRYO-TEM)**

[00139] The purpose of the CRYO-TEM analysis is to determine the presence of the lipid bilayer surrounding silica NPs. The samples were frozen with liquid nitrogen in carbon grids by FEI tool™ for sample preparation. Analyses were performed using FEI Titan Krios™ CRYO-TEM operated at 200 kV.

Revendications

1. Méthode de fabrication d'une bicouche lipidique supportée sur une nanoparticule de silice poreuse avec un potentiel ζ compris entre -10 mV et +10 mV, ladite méthode comprenant les étapes suivantes :
    a) provision d'une bicouche lipidique supportée chargée négativement sur une nanoparticule de silice poreuse, dans laquelle ladite bicouche lipidique supportée chargée négativement a un potentiel ζ inférieur à -15 mV et dans laquelle ladite bicouche lipidique supportée chargée négativement comprend au moins un phospholipide et ;
    b) addition d'une formulation de lipides, lesdits lipides étant le 1,2-dioléoyle-3-triméthylammonium-propane, dit DOTAP, le cholestérol et au moins un lipide différent du DOTAP et du cholestérol ;

    caractérisée en ce que ladite méthode comprend en outre l'étape de
    c) réalisation d'une ultrasonication.

2. Méthode selon la revendication 1, caractérisée en ce que ladite étape (c) est réalisée à une température comprise entre 40 °C et 60 °C.

3. Méthode selon l'une quelconque des revendications 1 à 2, caractérisée en ce que la concentration de DOTAP dans la formulation de l'étape (b) est comprise entre 50% et 150% de la concentration dudit au moins un phospholipide, préférentiellement en une concentration correspondant à 100% de la concentration dudit au moins un phospholipide.

4. Méthode selon l'une quelconque des revendications 1 à 3, caractérisée en ce que ladite étape d'ultrasonication est réalisée entre 10 minutes et 30 minutes, préférentiellement pendant 20 minutes.

5. Méthode selon l'une quelconque des revendications 1 à 4, caractérisée en ce que ledit au moins un lipide différent de DOTAP et de cholestérol est le 1,2-dipalmitoyle-sn-glycéro-3-phosphocholine, dit DPPC.

6. Méthode de fabrication d'une nanocapsule à base d'une bicouche lipidique

supportée sur une nanoparticule de silice poreuse chargée d'un potentiel ζ compris entre -10 mV et +10 mV, ladite méthode comprenant la méthode selon l'une quelconque des revendications 1 à 5 et les étapes suivantes :

a) addition d'une solution aqueuse d'alginate de sodium ; et

b) réticulation dudit alginate de sodium.

7. Méthode selon la revendication 6, caractérisée en ce que la concentration en alginate de sodium dans ladite solution aqueuse est comprise entre 1% (en poids) et 5% (en poids), préférentiellement est égale à 2% (en poids).

8. Méthode selon l'une quelconque des revendications 6 à 7, caractérisée en ce que ladite addition est effectuée dans une solution tampon, ladite solution tampon étant à base d'acide 4-(2-hydroxyéthyle)-1-pipérazine-éthane sulfonique, dit HEPES, ou de tampon phosphate salin, dit PBS.

9. Méthode selon l'une quelconque des revendications 6 à 8, caractérisée en ce que ladite étape de réticulation dudit alginate de sodium est réalisée en mélangeant une solution aqueuse de cation divalent, préférentiellement cation à base de calcium ou cation à base de baryum, à une concentration comprise entre 40 mM et 60 mM, préférentiellement à une concentration de 50 mM.

10. Méthode selon l'une quelconque des revendications 1 à 9, caractérisée en ce que ladite nanoparticule de silice poreuse est une nanoparticule de silice mésoporeuse, préférentiellement une nanoparticule de silice mésoporeuse chargée positivement.

11. Nanocapsule appropriée pour encapsuler au moins un fragment actif, ladite nanocapsule comprenant un noyau interne, une couche interne et une couche externe, ledit noyau interne étant formé par une nanoparticule de silice poreuse, ladite couche interne étant formée par une bicouche lipidique, dans laquelle ladite bicouche lipidique comprenant au moins un phospholipide, caractérisée en ce que ladite couche externe comprend de l'alginate de sodium réticulé.

12. Nanocapsule selon la revendication 11, caractérisée en ce que ladite couche externe d'alginate de sodium réticulé présente une épaisseur comprise entre 3 nm et 100 nm, de préférence entre 20 nm et 30 nm, plus préférentiellement 25 nm.

13. Composition comprenant la nanocapsule selon l'une quelconque des revendications 11 à 12 et au moins un fragment actif, ledit au moins un fragment actif étant dans le noyau interne de ladite nanocapsule.

14. Composition selon la revendication 13, caractérisée en ce que ledit au moins un fragment actif est un agent de contraste choisi préférentiellement parmi le groupe comprenant la calcéine, la rhodamine, le bleu de méthylène et le vert d'indocyanine, plus préférentiellement le vert d'indocyanine.

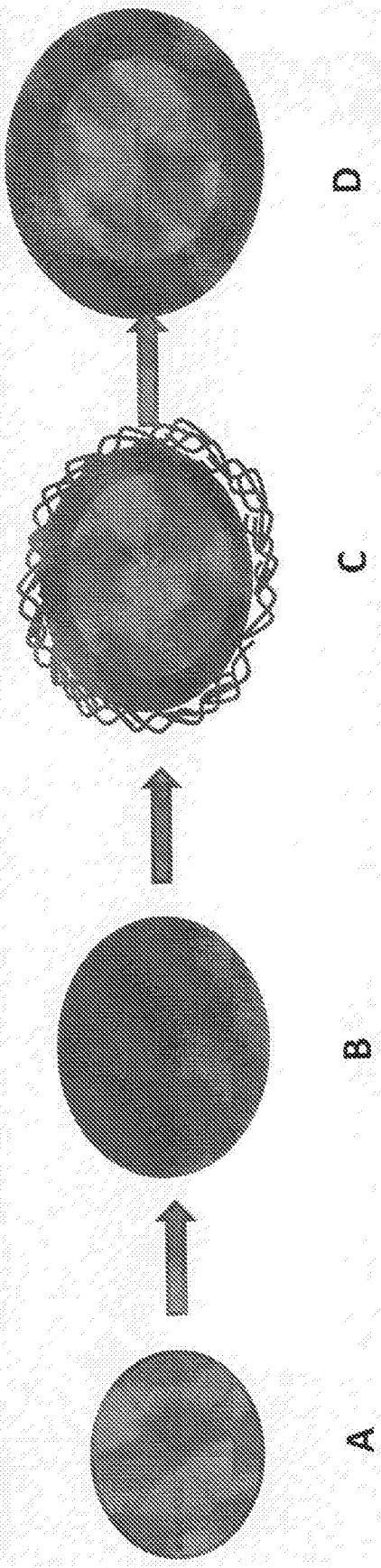
15. Composition qui convient à une administration orale, ladite composition comprenant la nanocapsule selon l'une quelconque des revendications 11-12, et au moins un fragment actif présentant des propriétés thérapeutiques, pharmaceutiques, neutraceutiques et/ou cosméceutiques, préférentiellement une molécule présentant des propriétés anticancéreuses, ledit au moins un fragment actif présentant des propriétés thérapeutiques, pharmaceutiques, neutraceutiques et/ou cosméceutiques étant dans le noyau interne de ladite nanocapsule.

16. Composition qui convient à une administration topique, ladite composition comprenant la nanocapsule selon l'une quelconque des revendications 11-12, et au moins un agent cosmétique, préférentiellement un antioxydant et/ou un agent dermatologique, préférentiellement un agent cicatrisant, ledit au moins un agent cosmétique et/ou ledit au moins un agent dermatologique étant dans le noyau interne de ladite nanocapsule.

17. Composition qui convient à une administration loco-régionale telle qu'une administration intraoculaire et/ou intratumorale, ladite composition

comprenant la nanocapsule selon l'une quelconque des revendications 11-12, et au moins un fragment actif présentant des propriétés thérapeutiques et/ou pharmaceutiques. LU100023

Fig. 1



Samples	NTA Size in milliQ water (nm)	NTA Size in HEPES Buffer (nm)	NTA Size in Human Serum (nm)
MSNPs	68 ± 36	127 ± 71	124 ± 66
MSNPs+	99 ± 42	187 ± 83	NA
SLB@MSNPs	127 ± 43	145 ± 69	139 ± 72

Fig. 2

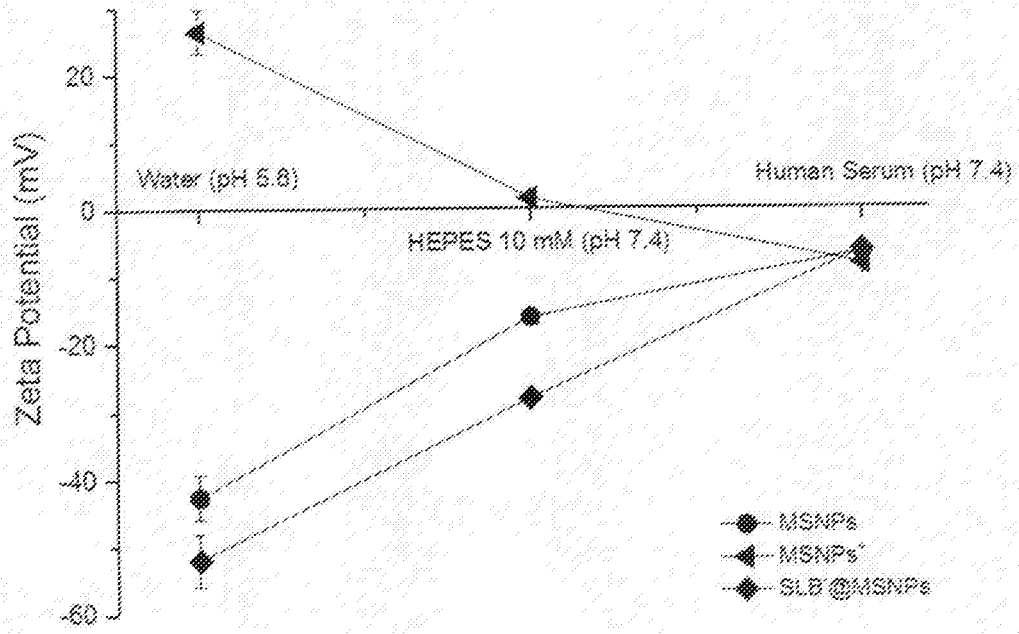


Fig. 3

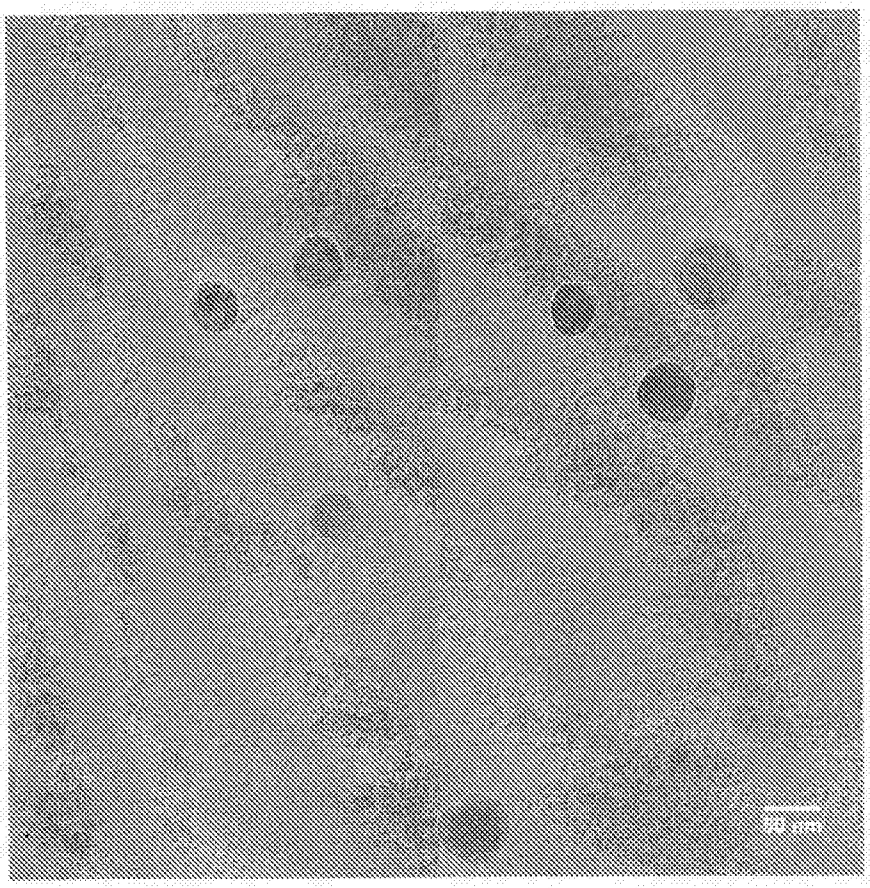


Fig. 4

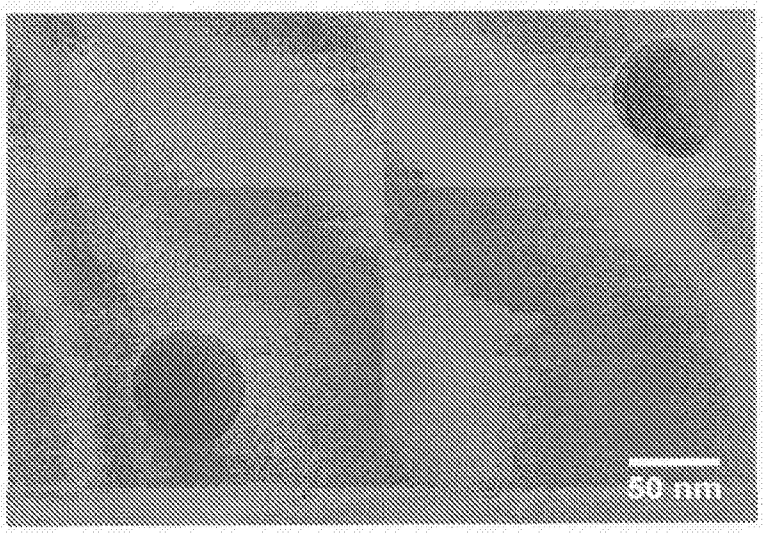


Fig. 5

Abstract

The invention is directed to a method for manufacturing supported lipid bilayer on a porous silica nanoparticle with a ζ-potential comprised between -10 mV and +10 mV, said method comprising the steps of (a) providing a negatively charged supported lipid bilayer on a porous silica nanoparticle, wherein said negatively charged supported lipid bilayer has a ζ-potential inferior to -15 mV and wherein said negatively charged supported lipid bilayer comprised at least one phospholipid and; (b) adding a formulation of lipids, said lipids being 1,2-dioleoyl-3-trimethylammonium-propane alias DOTAP, cholesterol and at least one lipid different from DOTAP and cholesterol. Said method is remarkable in that it further comprises the step of (c) performing an ultra-sonication for promoting DOTAP incorporation. Said method can be supplemented by the step of addition of alginate and the step of cross-linking said alginate. The invention is also directed to nanocapsule and composition comprising said nanocapsule.

(Fig. 1)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie

National Application Number

LO 1572
LU 100023

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	US 8 992 984 B1 (BRINKER C JEFFREY [US] ET AL) 31 March 2015 (2015-03-31) * column 6, line 56 - column 7, line 35; figure 1D; examples 4,6 *	1-17	INV. A61K9/127 A61K9/51
A	WO 2014/138278 A1 (UNIV CALIFORNIA [US]) 12 September 2014 (2014-09-12) * page 12, lines 1-12 * * example II *	1-17	
A	WO 2010/078569 A2 (STC UNM [US]; LIU JUEWEN [US]; BRINKER C JEFFREY [US]; ASHLEY CARLEE [] 8 July 2010 (2010-07-08) * paragraph [0071]; examples *	1-17	
A	US 2015/272885 A1 (ASHLEY CARLEE ERIN [US] ET AL) 1 October 2015 (2015-10-01) * paragraph [0367]; figure 17 *	1-17	
E	WO 2017/013250 A1 (LUXEMBOURG INST OF SCIENCE AND TECH [LU]) 26 January 2017 (2017-01-26) * the whole document *	1-17	

TECHNICAL FIELDS SEARCHED (IPC)

A61K

The present search report has been drawn up for all claims

Date of completion of the search	Examiner
29 June 2017	Giménez Miralles, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C59)

1

ANNEX TO THE SEARCH REPORT ON LUXEMBOURG PATENT APPLICATION NO.

LO 1572
LU 100023

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

29-06-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2015164798 A1	18-06-2015
		US 2015320681 A1	12-11-2015
WO 2014138278 A1	12-09-2014	EP 2964201 A1	13-01-2016
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		WO 2014138278 A1	12-09-2014
WO 2010078569 A2	08-07-2010	US 2011268791 A1	03-11-2011
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		CA 2852064 A1	18-04-2013
		CN 104023711 A	03-09-2014
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		SG 11201401499X A	26-09-2014
		US 2015272885 A1	01-10-2015
		WO 2013056132 A2	18-04-2013
WO 2017013250 A1	26-01-2017	LU 92784 B1	31-01-2017
		WO 2017013250 A1	26-01-2017

EPO FORM P0457

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
LO1572	20.01.2017		LU100023

International Patent Classification (IPC)
INV. A61K9/127 A61K9/51

Applicant
Luxembourg Institute of Science and Technology (LIST)

This report contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☐ Box No. VIII Certain observations on the application

	Examiner
Form LU237A (Cover Sheet) (January 2007)	Giménez Miralles, J

Application No.
LU100023

WRITTEN OPINION

Box No. I Basis of the opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-17
	No:	Claims	
Inventive step	Yes:	Claims	1-17
	No:	Claims	
Industrial applicability	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

1. The relevant prior art documents are referred to as D1 to D5 as in the order of appearance in the Search Report (SR). Unless otherwise indicated, reference is made to the passages of said documents cited in the SR.

2. Citations and explanations supporting the statement with regard to novelty (N), inventive step (IS) and industrial applicability (IA):

(N) The subject-matter of independent claims 1, 6, 11, 13, 15, 16 and 17 is considered to be novel.

The prior art D1, which can be considered as the closest prior art, discloses a method of manufacturing supported lipid bilayer (SLB) on porous silica nanoparticles ("protocells"), wherein the surface charge of the "protocells" can be made positive or negative depending on the lastly added liposome; e.g. by mixing positively charged DOTAP liposomes with negatively charged SLB based on DOPS (figure 1D) the surface charge of the resulting SLB can be controlled, thus preventing premature cargo release (e.g. calcein).

However, D1 does not disclose providing a SLB on porous silica nanoparticles, adding a lipid formulation *comprising DOTAP, cholesterol and a third lipid,* and performing *ultra-sonication to promote lipid exchange* incorporating DOTAP in the SLB. For these reasons, claim 1 is novel.

Further, claims 6, 11, 13, 15, 16 and 17 are also novel, since the prior art does not disclose a nanocapsule comprising supported lipid bilayer (SLB) on porous silica nanoparticles further coated with a cross-linked alginate layer.

(IS) The subject-matter of independent claims 1, 6, 11, 13, 15, 16 and 17 is considered to involve an inventive step for the following reasons.

As explained above, the closest prior art D1 does not disclose providing a SLB on porous silica nanoparticles, adding a lipid formulation *comprising DOTAP, cholesterol and a third lipid,* and performing *ultra-sonication to promote lipid exchange* incorporating DOTAP in the SLB.

The problem solved in claim 1 is therefore the provision of an efficient method of manufacturing SLB on porous silica nanoparticles allowing to control the lipid composition of the SLB and hence the zeta-potential of the lipid bilayer.

While D2 teaches forming the phospholipid bilayer of SLB on porous silica nanoparticles by contacting a suspension of silica nanoparticles with a solution of phospholipids and supplying with energy e.g. via *sonication* to facilitate coating of the silica nanoparticles with the phospholipid bilayer, D2 does not suggest exchanging the lipid composition of a SLB on porous silica nanoparticles by contacting with a second lipid composition comprising DOTAP, let alone under conditions of *ultra-sonication to promote lipid exchange.*

For this reason, the subject-matter of claim 1 is not obvious.

Further, the prior art does not indicate or suggest coating SLB on porous silica nanoparticles with a cross-linked alginate layer to form nanocapsules. Therefore, claims 6, 11, 13, 15, 16 and 17 are also non-obvious.

(IA) The subject-matter of claims 1-17 is considered to be industrially applicable. The possibility of industrial application is beyond any doubt.