(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2018 (15.02.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/029015 A1

(51) International Patent Classification:
A61K 49/22 (2006.01)

(21) International Application Number:
PCT/EP2017/069190

(22) International Filing Date:
28 July 2017 (28.07.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16382392.5 08 August 2016 (08.08.2016) EP

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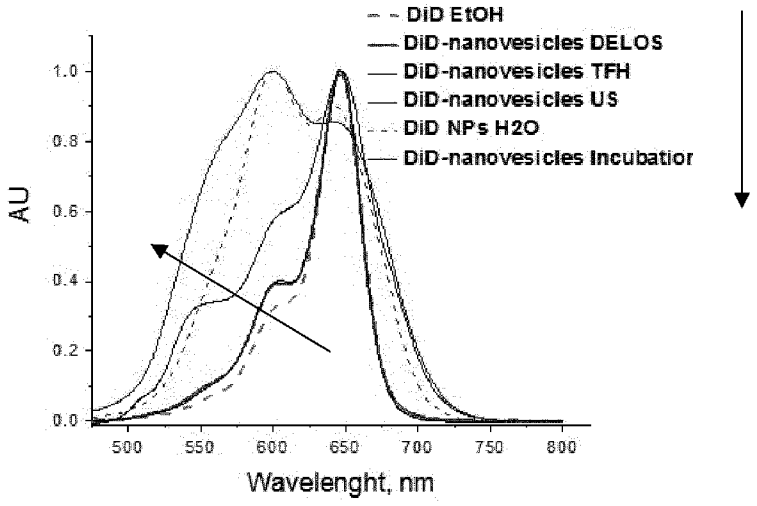
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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(54) Title: FLUORESCENT NANOVESICLES, METHOD FOR OBTAINING THEM AND USES THEREOF

FIG 11



(57) Abstract: The invention provides a fluorescent nanovesicles with stability in an aqueous medium, and particularly a new fluorescent nanovesicle maintaining the fluorescence's property for a period of time in an aqueous medium. The invention is also directed to their uses as a fluorescent probe and to a method for preparing the stable fluorescent nanovesicles in an easy and feasibly way.

WO 2018/029015 A1

[Continued on next page]

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

FLUORESCENT NANOVESICLES,

METHOD FOR OBTAINING THEM AND USES THEREOF

Field of the invention

The present invention relates to fluorescent nanovesicles. In particular, the present invention relates to new fluorescent nanovesicles capable of maintaining stable over time the fluorescence's property in an aqueous medium.

The present invention also relates to a method for preparing the stable fluorescent nanovesicles, as well as to their uses as fluorescent probes in an aqueous media.

Background of the invention

Liposomes made with phospholipids are among the most-studied self-assembled nano-objects. They are vesicles composed of one or more concentric lipid bilayers, which separate a small-enclosed liquid compartment, the lumen, from its surroundings.

Particularly, nanoscopic vesicles, composed of sterols and quaternary ammonium surfactants, have been prepared by a compressed fluid-based methodology. The internalization of hydrosoluble proteins within the small enclosed compartment of these nanoscopic vesicles, that is, within their lumen, protects the activity of the protein up to their deliverance, see "*Multifunctional Nanovesicle-bioactive conjugates prepared by a one-step scalable method using CO_2-expanded solvents*" of Nano letters, ACS Publications, July 5, 2013.

EP0185680 discloses methods and compositions for the entrapment of water-soluble compounds, partially water-soluble compounds, or water-insoluble compounds in liposomes composed of salt forms of organic acid derivatives of sterols that are capable of forming bilayers. The sterol liposomes according to this patent can be prepared with or without the use of organic solvents, and different ways for compound entrapment are also described.

Despite versatility of the known vesicles, the stability of these structures is kinetically limited because their lipid building blocks are highly insoluble, and therefore, the collapsed planar lamella is the equilibrium state of aggregation.

On the other side, molecular fluorescent dyes have been also studied for fluorescence microscopy. It is known that small organic molecules can be highly fluorescent. However, they suffer drawbacks such as a low photostability, preventing long time imaging, low biocompatibility and high toxicity [see, Liu, P., Liu, P., Zhao, K. & Li, L. "Photostability

enhancement of azoic dyes adsorbed and intercalated into Mg–Al-layered double hydroxide". Opt. Laser Technol. 74, 23–28 (2015)], and in some cases they have a poor solubility in aqueous medium, which limits their use for *in vitro* and *in vivo* applications.

Moreover, inorganic nanostructures with size-dependent optical properties, such as quantum dots (QDs), offer an interesting alternative thanks to their high brightness and photostability. Nevertheless, they require a surface functionalization for reducing the toxicity, improving biocompatibility and attaining site-specificity.

Still another possible alternative are the fluorescent organic nanoparticles (FONs) which are photostable and biocompatible and have a good brightness, due to the high concentration of dyes molecules in a small volume. However, they do not allow the use of water-soluble dyes in aqueous media.

Although, nanovesicles loaded with cationic probes such as rhodamine 6G perchlorate (R6G) have been studied in a vesicle bilayer composed by cholesterol-cetyltrimethylammonium bromide (CTAB) by Ghosh et al. "*How Does the Surface Charge of Ionic Surfactant and Cholesterol Forming Vesicles Control Rotational and Translational Motion of Rhodamine 6G Perchlorate (R6G ClO4)*" from Langmuir (2015), 31(8), 2310-2320, such studies reveal that the hydrophobicity and electrostatic repulsion induce the migration of R6G from the vesicle bilayer to the aqueous phase.

Therefore, there is still the need to provide a new structure for use as a fluorescent probe, where fluorescent dyes can be introduced, irrespective of their solubility, and that maintains good fluorescence properties, while having a good chemical and kinetical stability in an aqueous medium, overcoming among others the problems of aggregation and migration of the dye to the aqueous phase.

Summary of the invention

The present invention was made in view of the prior art described above, and the first object of the present invention is to provide a fluorescent nanovesicle with improved properties, as well as their use for particular applications, which derive from the improved fluorescent structures mentioned above.

To solve the problem, the present invention provides in a first aspect, a fluorescent nanovesicle composed of an amphiphilic bilayer membrane with an inner compartment enclosing an aqueous medium, the amphiphilic bilayer membrane comprising sterol molecules or derivatives thereof assembled with quaternary ammonium surfactant molecules which define hydrophobic and hydrophilic regions in the bilayer membrane, which is

characterized in that the amphiphilic bilayer membrane further comprises at least a non-water soluble organic dye, wherein the non-water soluble dye is bonded to the bilayer membrane by a linkage selected from a covalent bond and/or a hydrophobic bond, thereby the fluorescence of the nanovesicle is stable over time in an aqueous medium.

Surprisingly, the fluorescent nanovesicle designed by the present inventors provides a new scaffold for nanostructuring dyes in water media, in which the chemical and colloidal stability (physical stability) of the dye at the scaffold is maintained over time. Surprisingly, the absorbance and emission spectra of the fluorescent nanovesicle are also maintained for at least two months.

Advantageously, a fluorescent nanovesicle is provided whose fluorescence is stable for at least two months in aqueous media and having chemical and physical stability in aqueous media for ever a longer time.

Therefore, according to the first aspect of the present invention, the term “stable” in the sentence “stable fluorescent nanovesicle” includes two stability effects, that is, on one side, it refers to the fluorescence stability of the dye in aqueous media upon light irradiation and, on the other side, it refers to the chemical and physical stability of the nanovesicle containing the dye in aqueous media, thereby providing a stable fluorescent nanovesicle in an aqueous media suitable for use as a fluorescent probe in aqueous media.

Advantageously, the present inventors found that a fluorescent nanovesicle as claimed in claim 1 does not undergo chemical degradation, neither aggregation, the fluorescence being stable at least over two months.

Moreover, the nanovesicle according to the first aspect is designed to be able to interact with a non-water soluble organic dye, making the new fluorescent scaffold more versatile and suitable for using as a fluorescent probe.

In a preferable embodiment, the non-water soluble organic dye can be an amphiphilic organic dye, an apolar organic dye or a fluorescent dye of a sterol derivative.

The non-water soluble organic dye preferably comprises a polar or an apolar head and at least one alkyl tail having 10 to 24 carbons in length. It is still more preferable that the non-water soluble organic dye comprises a polar or an apolar head and at least two alkyl tails, each one having from 10 to 24 carbons in length. It is desirable that the alkyl chain length (tail) of the dye be similar or close to the alkyl chain length (tail) of the quaternary ammonium surfactant that forms the nanovesicle. In another preferable embodiment, the non-water soluble organic dye interaction with hydrophobic and/or hydrophilic regions of the bilayer

membrane includes a covalent binding, or a hydrophobic binding.

That is, the fluorescent nanovesicles according to the present invention comprise at least a non-water soluble organic dye, the nanovesicle being formed by a bilayer membrane including a sterol molecule or derivatives thereof assembled with a quaternary ammonium surfactant molecule in which the at least the non-water soluble organic dye is linked with said bilayer membrane.

In a preferable embodiment, the nanovesicle can have an average size of from 25 to 500 nm as measured by dynamic light scattering and nanoparticle tracking analysis, still more preferable from 50 nm to 300 nm. In a still preferable embodiment, the nanovesicle can be substantially spherical in shape.

Geometrically, the quaternary ammonium surfactant molecule has a conical shape, whereas the sterol molecule has an inverted conical shape, and the assembly of the conical molecule and the inverted conical molecule forms a synthon structure, which has a quasi-cylindrical shape.

A pair of synthons can form a closed and concentric bilayer membrane having (in a cross section view) a hydrophobic region in the central zone of the bilayer membrane and two hydrophilic regions each one at each side of said central zone. Each one of said two hydrophilic regions defines, by one side, the inner layer, which separates the inner and small enclosed aqueous liquid compartment, the lumen, and, by the other side, the outer layer of the bilayer membrane, which is in contact with the outer of the nanovesicle, usually an aqueous medium. The surfactant molecules can be arranged into two-layered leaflets with the head groups in contact with the aqueous medium outside the nanovesicles, and the tails in the interior of the bilayer, whereas sterol molecules can arrange themselves, including the hydroxyl groups, in the hydrophobic region generated by the surfactant tails to avoid any contact with the aqueous medium of the inner compartment or of that of the outside the nanovesicles.

Therefore, the nanovesicles according to this invention are amphiphilic nanovesicles.

Specifically, the non-water soluble organic dye interaction with hydrophobic and/or hydrophilic regions of the bilayer membrane can be a covalent binding to the sterol molecule in the hydrophobic region, or a hydrophobic binding at the hydrophilic and/or hydrophobic regions of the bilayer membrane.

In an embodiment, the fluorescent dye of a sterol derivative is covalently bonded to the sterol molecule in the hydrophobic region of the bilayer membrane.

In an embodiment, the amphiphilic organic dye is linked at the hydrophilic and hydrophobic regions of the bilayer membrane by a hydrophobic linkage, in which the hydrophobic part of the amphiphilic dye is in contact with the hydrophobic region of the membrane, while the polar part of the amphiphilic dye is in contact with the hydrophilic region of the membrane.

In an embodiment, the apolar organic dye is linked at the hydrophobic region of the bilayer membrane by a hydrophobic linkage.

The stable fluorescent nanovesicle according to the first aspect of this invention may comprise at least two organic dyes that interact simultaneously in the same nanovesicle, each one having a different optical spectrum, the first dye being selected to have an emission spectrum that partly overlaps the absorption spectrum of the second dye. The dyes can be separated from each other by a distance of typically lower than 10 nm, as needed to observe Forster resonant energy transfer (FRET). The FRET effect is disclosed, for example, by Terenziani et al., "*Dipolar versus Octupolar Triphenylamine-Based Fluorescent Organic Nanoparticles as Brilliant One- and Two-Photon Emitters for (Bio)imaging*" from Small 2011, 7, No. 22, 3219–3229.

Advantageously, the fluorescent nanovesicle comprises two organic dyes configured to generate the FRET effect. Thus, the fluorescent nanovesicles can be used as fluorescence resonance energy transfer (FRET)-based scaffolds having different emission spectra that can be excited by a single wavelength excitation source as needed for multicolour detection applications.

The fluorescent nanovesicles can be excited by both one-photon and multi-photon excitation. Advantageously, the fluorescent nanovesicles can be designed for in vivo and in vitro imaging application in human and animal tissues, for excitation with different laser sources, and reaching different depths. Additionally or alternatively, the stable fluorescent nanovesicle can have a bioactive compound and/or a targeting agent.

The fluorescent nanovesicles can be functionalized with large numbers and varieties of functional molecules, allowing their use as biosensors.

Thus, the fluorescent nanovesicles can be functionalized using a plurality of ligands. Suitable ligands are known in the art. In some embodiments, the ligands can be attached to the outer layer of the fluorescent nanovesicles, for example, by coordination bonds. Ligands can also be associated with the outer layer of the fluorescent nanovesicles via non-covalent bond. The ligands can comprise coordinating or bonding functional groups, such as thiol, amine, phosphine, CO, N_2, alkene, chloride, hydride, alkyl, and derivatives thereof, and

combinations thereof.

It is well understood by a skilled person in the art that the various optional features disclosed herein can be combined with each other whenever technically possible without departing the scope of protection of the present invention.

In a further aspect, this invention provides a stable colloidal dispersion comprising a plurality of fluorescent nanovesicles according to the first aspect of the present invention. The plurality of fluorescent nanovesicles can be presented in a lyophilized state.

Surprisingly, the stability of the fluorescent nanovesicles of this invention upon dilution is excellent. The term “stable” can also refer to the stability of the average size of the fluorescent nanovesicles over time and/or upon dilution.

Advantageously, the colloidal dispersion comprising a plurality of fluorescent nanovesicles maintains its stability in an aqueous medium even upon dilution. Particularly, the stability upon dilution of the colloidal dispersion comprising a plurality of nanovesicles according to the first aspect of this invention is maintained even when compared with different systems such as micelles loaded with the same organic dye, at a dilution concentrations below the critical micellar concentration (CMC) required to form the micelles.

Therefore, the colloidal dispersion of this invention can be advantageously used in applications where the concentration of the fluorescent nanovesicles is diluted in use like when they are used under physiological conditions such as in body fluids.

Therefore, the fluorescent nanovesicles disclosed herein have potential applications in a number of fields such as imaging (e.g., biological imaging, fluorescence imaging, biomedical imaging), sensing (e.g., chemical sensing, biological sensing), theranostic and medical applications (e.g., imaging, therapy, diagnostics, photothermal therapy, combinations thereof).

In other words, devices comprising the fluorescent nanovesicles according to the first aspect of the present invention have been also disclosed herein. Examples of such devices include, but are not limited to optical devices (e.g., light emitting diodes), optoelectronic devices, bioanalytical devices, chemical sensors, biosensors, and combinations thereof.

As stated above, the nanovesicle can comprise a sterol molecule and a quaternary ammonium surfactant molecule. The nanovesicle can, for example, be formed by self-assembly of the sterol and the quaternary ammonium surfactant molecule. As used herein, a lipid is not a surfactant, such that the nanovesicles are not liposomes.

In some embodiments, the sterol molecule or a derivative thereof, and the quaternary ammonium surfactant molecule can be present in the nanovesicle in a molar ratio of from 10:1 to 1:5. It is preferable a molar ratio within the range from 2:1 to 1:2, still more preferable a molar ratio of 1:1.

In a preferable embodiment, the sterol is cholesterol.

In a preferable embodiment, the quaternary ammonium surfactant is selected from the group consisting of cetyl trimethylammonium bromide (CTAB), tetradecyldimethylbenzylammonium chloride (MKC), cetrimide and benzalkonium chloride (BKC) or mixtures thereof. The scope of the present invention contemplates the presence of additional surfactants together with the quaternary ammonium surfactants, the additional surfactants not necessarily require to be of the quaternary ammonium type.

In a preferable embodiment, the bilayer membrane of the nanovesicle comprises a cholesterol and a quaternary ammonium surfactant. In some examples, the bilayer membrane comprises cholesterol and cetyl trimethylammonium bromide (CTAB).

An additional object of the present invention is to provide a practical method capable of preparing the fluorescent nanovesicles according to the first object of the present invention, the method being suitable to be scalable at industrial scale.

To solve this problem, the present invention provides a method as claimed in the attached method claims for preparing the fluorescent nanovesicles of the first aspect of the present invention.

In a preferable embodiment, the nanovesicles can be prepared by using CO_2 expanded solvents by the method named “Depressurization of an Expanded Liquid Organic Solution-suspension” (DELOS-SUSP).

The method for preparing nanovesicles by using the technology “Depressurization of an Expanded Liquid Organic Solution-suspension” comprises the general steps a) to c) as included below: a) preparation of an aqueous solution of the quaternary ammonium surfactant, b) dissolution of the sterol or derivatives thereof in an organic solvent and then expanding the solution by using a compressed fluid (CF), and c) vesicle synthesis by depressurization of the resulting solution from step b) on the solution resulting from step a).

In order to prepare fluorescent nanovesicles comprising non-water soluble organic dyes by this technology, the method further comprises:

in step b) adding a non-water soluble organic dye to the sterol or derivatives thereof and the

organic solvent in order to prepare an organic solution, and dissolving the non-water soluble organic dye and the sterol or derivatives thereof in the organic solvent and then expanding the organic solution by using a compressed fluid; and, in step c), synthetizing the fluorescent nanovesicles by depressurization of the expanded solution on the quaternary ammonium surfactant solution, previously prepared in step a).

In an alternative method for preparing the fluorescent nanovesicles comprising non-water soluble organic dyes by this technology, the method further comprises:

a) water, preferably sterile water is reserved;

b) dissolution of the sterol or derivatives thereof, the quaternary ammonium surfactant, and the non-water soluble organic dye in the organic solvent and, then, expanding the solution by using a compressed fluid (CF); and

c) synthetizing the fluorescent nanovesicles by depressurization of the expanded solution on the water of step a).

The meaning of the non-water soluble organic dye, the quaternary ammonium surfactant, the sterol or derivatives thereof, and the molar ratio of the quaternary ammonium surfactant molecule to sterol molecule or derivatives thereof are the same as described herein.

Non-liposomal nanovesicular structures can be formed, for example, using equimolar amounts of sterols, such as cholesterol (chol), and quaternary ammonium surfactants, such as CTAB. None of the individual components of a nanovesicle self-assemble to form vesicular structures, since in water quaternary ammonium surfactants form micelles and the insoluble sterol species form crystals, though in combination the components form amphiphilic bimolecular building blocks that assemble into closed bilayers. These nanovesicles do not tend to aggregate, and they keep their structure for periods as long as several years.

The obtained nanovesicular structures exhibit a high nanovesicle to nanovesicle homogeneity regarding size, lamellarity, and membrane supramolecular organization, which are all properties that can impact the use of such nanovesicles in medical or diagnosis imaging. Contrary to micellar structures formed exclusively with quaternary ammonium surfactants, the morphology of these nanovesicules is substantially unaffected upon increasing the temperature or by dilution, making them attractive candidates for use *in-vivo*. The nanovesicles are stable aqueous colloidal structures and they have antibacterial and anti-biofilm properties.

In a preferable embodiment, the organic solvent is selected from a monohydric alcohol a

polyhydric alcohol, a ketone, ethylenediamine, acetonitrile, ethyl acetate and mixtures thereof; and the CF is selected from CO_2, ethane, propane, a hydrochlorofluorocarbon, and a hydrofluorocarbon.

Surprisingly, the interaction of the organic dye with the bilayer membrane does not disturb the stability of the nanovesicle, nor migration of the organic dye to the aqueous phase is detected.

There is not a specific threshold non-water soluble organic dye concentration. However, it is postulated that the threshold non-water soluble organic dye concentration is function of the maximum interaction, which is possible of the organic dye with the nanovesicle that at the same time does not affect the stability of the nanovesicles themselves. It is well understood by a skilled person in the art that this threshold concentration can vary depending on the specific organic dye and on the specific type of interaction. However, for all tested organic dyes fluorescent properties are stable for over two months, indicating that the organic dye was stable in this media, being the organic dye covalently bonded, or hydrophobically bonded to the bilayer membrane of the nanovesicle.

Advantageously, the method for forming the fluorescent nanovesicles according to the present invention provides an adequate covalent binding, and a hydrophobic anchorage to the bilayer membrane of the nanovesicle. Advantageously, the method for forming the fluorescent nanovesicles according to the present invention ensures chemical and physical stability in aqueous media of the nanovesicle having the non-water soluble organic dye bonded thereto.

Another aspect of the present invention is the use of the fluorescent nanovesicle according to the first aspect of the invention as a fluorescent probe in an aqueous medium.

In a preferable embodiment, the fluorescent probe can be used for bioimaging and/or biodetection in an *in vitro* or *in vivo* aqueous media.

Definitions

According to the scope of the present invention, the term “aqueous medium” or “aqueous media” is intended to encompass/to mean a liquid system in which the main component is water, including a cell media (*in vitro*), and any physiological condition such as a body fluid (*in vivo*). The main component is water means that at least 50% is water.

In the present invention, the term "nanovesicles" refers to colloidal vesicles, which are between 25 nm and 5 μm in size and are formed by one bilayer of amphiphilic molecules that

contain an aqueous phase in the small-enclosed liquid compartment. In particular, the nanovesicle comprises a bilayer membrane having hydrophobic and hydrophilic regions, in which the bilayer membrane comprises a sterol molecule and/or derivatives thereof assembled with a quaternary ammonium surfactant molecule.

In the invention, the term “quaternary ammonium surfactant” refers to those cationic surfactants with at least one positive charge in the molecule and also includes the combination of one or more cationic surfactants. Particularly, quaternary ammonium surfactants are quaternary ammonium salts in which at least one nitrogen substituent is a long chain. Compounds such as CTAB, cetrimide and BKC or their mixture are included. The cationic surfactants can be obtained from commercially available sources, with pharmaceutical and cosmetic qualities.

The term "derivatives" of sterol, in the present invention, includes derivatives of cholesterol and refers to molecules of the steroids family, generally obtained from the cholesterol precursor molecule and having lipophilic character.

The term “organic dye” refers to an organic molecule, which is capable to absorb light in the visible region, and possibly emits light in the same region upon light irradiation. By definition, a dye comprises in its molecule a chromophore part. The chromophore part is the molecule part which is responsible of the absorption of light within the visible spectrum, thereby reflecting or transmitting light at a determined wavelength which determines the object’s color.

The term “fluorescence stability” in the present invention refers to the stability over time of the emission spectra of fluorescent nanoparticles in an aqueous medium.

The term “stable fluorescent nanovesicle” in the present invention refers to the above fluorescence stability and to the chemical and physical stability of the nanovesicles in an aqueous medium.

The term “organic solvent” which has been used in the method for forming the fluorescent nanovesicles according to this invention is a solvent selected from the group formed by monohydric alcohols, such as: ethanol, methanol, 1-propanol, 2-propanolol, 1-butanol, 1-hexanol, 1-octanol and trifluoroethanol; polyhydric alcohols, such as: propylene glycol, PEG 400 and 1,3-propanediol; ketones, such as acetone, methyl ethyl ketone and methyl isobutyl ketone; ethylenediamine, acetonitrile, ethyl acetate and mixtures thereof. In any case, whatever is the nature of the organic solvent, the lipid component has to be soluble in it and further, said solvent has to necessarily be miscible in CF and water. Moreover, the selected

organic solvent must have relatively low toxicity.

The term "CF" which has been used in the method for forming the fluorescent nanovesicles according to this invention is a component selected between CO_2, ethane, propane, hydrochlorofluorocarbons (eg., CFC-22), and hydrofluorocarbons (eg., HFC-134A). Preferably, the CF in step b) is CO_2, considered an ecological solvent, because it is non-toxic, non-flammable, non-corrosive, is not harmful for the environment and moreover, is very abundant in nature.

Brief Description of the Drawings

Figure 1 depicts size distribution of plain nanovesicles measured by Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA).

Figure 2 shows CryoTEM images of DiD-Nanovesicules (1.3 mol*10^{-3}) a) DELOS-SUSP, b) Ultrasonication, c) Thin Film Hydration, d) Incubation.

Figure 3 depicts graphs of time evolution of DiD-nanovesicles (1.3 mol*10^{-3}) evaluated by absorption, emission (top) and size distribution over 2 months (bottom in combination with Table A).

Figure 4 depicts graphs of absorption (top) and size evolution (bottom) of DiD-NPs over two weeks.

Figure 5 depicts graphs of stability upon dilution of DiD-nanovesicles (1.3 mol*10^{-3}) (continuous lines) compared with DiD-CTAB micelles (disrupted lines).

Figure 6 shows size distribution by NTA and CryoTEM images of DID-nanovesicles at increasing concentrations (from a→e); and the integration efficiency (EE) of DELOS-SUSP of DID-nanovesicles are included in Table B.

Figure 7 depicts a graph of absorption of DiD-nanovesicles at different compositions.

Figure 8 depicts a graph of absorption change upon irradiation of DID-nanovesicles (1.3 mol*10^{-3}) at different irradiation times.

Figure 9 show bar charts of size (top), Z-potential (bottom) and CryoTEM images of NBD6Chol-nanovesicles at different compositions after one day of preparation and after 1 month.

Figure 10 depicts a graph of emission profiles along with the fluorescence and photodecomposition quantum yields of Chol6NBD-nanovesicles.

Figure 11 is a graph comparative summary of DiD, in which emission of DiD-nanovesicles and DiD-NPs (top) and absorption spectra of DiD-nanovesicles prepared by different routes (bottom). The direction of the arrow in the legends identifies each curve in the graph also according to the direction of the arrow drawn in the graph.

Figure 12 depicts the FRET occurring between DiI (Donor) and DiD (acceptor), both dyes being loaded in the bilayer membrane of a nanovesicle (QS). Exciting the DiI, it is possible to collect the emission from DiD (top), and depicts the FRET (cascade FRET) occurring between DiI, DiD and DiR (bottom), which reveals that exciting the DiI it is possible to collect emission from DiD and DiR.

Figure 13 depicts the one-(solid line) and two-photon (line+symbols) absorption spectra of DID and DID nanovesicles at two different loadings, $3*10^{-3}$ (triangles) and $6.6*10^{-3}$ (squares). This result is a proof of concept that the DiD and the DiD nanovesicles can emit under one-photon and multi-photon excitation.

Detailed Description of the Invention

Hereinafter, the best mode for carrying out the present invention is described in detail.

Here the authors of the present invention disclose a preferable embodiment of a fluorescent nanovesicle. In particular, three simple strategies will be described below to allow for the interaction of the organic dyes having different physicochemical and optical properties with the bilayer membrane of these nanovesicles, resulting in highly stable bright nanovesicles that show a great colloidal stability over time and a good photostability. The nanovesicles in this embodiment are formed by the self-assembly in water of CTAB and cholesterol in a 1:1 ratio (Figure 1) that create a supramolecular synthon with the proper shape and dimensions to self-assembly forming a spherical double-layer membrane with diameters of around 70 nm. Advantageously nanovesicles show a great colloidal stability during several years, as well as upon dilution and rising temperature, which is conferred by their strongly positively charged membrane. They can be prepared by the one-step method using compressed fluids (CFs), named Depressurization of Expanded Liquid Organic Solution-Suspension (DELOS-SUSP), which showed several advantages over conventional routes for the preparation of functionalized vesicles. Thanks to these properties nanovesicles resulted to be a versatile scaffold for the engineering of organic dyes-linked vesicles using dyes with different physico-chemical properties, in terms of solubility.

Different interaction strategies are possible depending on the nature and characteristics of the dyes:

- Lipophilic dyes with long alkyl chains can be “anchored” to the bilayer membrane by means of hydrophobic interactions which take place between the chains of the dyes and the hydrophobic compartment of the double-layer membrane. The dye used as a proof-of-concept for this embodiment was 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indodicarbocyanine perchlorate (DiD) a carbocyanine dye with two 18-carbons chains.

- By the other side, cholesterol of nanovesicle can be partially substituted by a cholesterol probe (dye), as demonstrated herein by using NBD-6-Cholesterol, NBD6Chol leading to bilayer membrane nanovesicle with a covalently linked dye. In some examples, the concentration of the NBD-6-Cholesterol to the cholesterol molecule can be lower than 2%. Advantageously, a concentration of the cholesterol derivative lower than 2% does not disturb the morphology and sizes of the resulting fluorescent nanovesicles. In a preferable embodiment, the molar ratio of the cholesterol molecules, including NBD-6-Cholesterol, with respect to the quaternary ammonium surfactant molecules can range from 10:1 to 1:5, preferably in the range of 2:1 to 1:2, still more preferably 1:1.

DiD and NBD-6-Chol are not soluble in water. Hence, the innovation here reported is the use of the nanovesicles as robust and versatile scaffolds enabling nanostructuring in water a variety of dyes with different physico-chemical characteristics without losing their optical properties providing in some case an enhancement of their photostabilities.

Nanovesicles linked with DiD (DiD-nanovesicles)

The interaction of the DiD with the nanovesicle was performed using the DELOS-SUSP methodology (Cano-Sarabia M et al. *Langmuir* 2008, 24, 2433–2437; Cabrera I et al. *Nano Lett.* 2013, 13, 3766–3774) and the membrane functionalization of resulting labeled nanovesicles, DiD-nanovesicles, compared with other methods generally used for loading vesicles, such as incubation (IC), ultrasonication (US) and thin film hydration (TFH). See below Example section for details on preparations.

The normalized absorbances in the visible range of DiD-nanovesicles prepared by different routes are shown in Figure 11 for comparison along with the spectra of DiD in Ethanol (EtOH) and nanoparticles of pure DiD (DiD-NPs), prepared by DELOS method.

Cyanines dyes are well known for their tendency to self-aggregate at high concentrations, even in organic solvents. The band present at 550 nm, ascribed to the presence H-aggregates of DiD, is prominent in samples obtained by incubation and ultrasonication. The spectra of DiD-nanovesicles prepared by DELOS-SUSP and TFH are very similar to the spectrum of DiD in EtOH. This confirms that by DELOS-SUSP and TFH methods, isolated

dye molecules are evenly distributed inside the membrane of nanovesicles. In any case, as shown in Figure 2, CryoTEM images of DiD-nanoparticles (1.3 mol*10-3) prepared by different techniques. DiD-nanovesicles prepared by DELOS-SUSP are more homogenous in terms of lamellarity than DiD-nanovesicles by TFH. UV-vis spectra show that aggregates of DiD are forming depending on the preparation route followed. The image c) shows that by TFH it is impossible to obtain, without further purification steps, unilamellar nanovesicles with homogeneous size distribution.

DiD-nanovesicles were found to be colloidally stable during months and no noticeable changes were found in the size distribution neither in absorbance/emission spectra over two months (Figure 3 and Table A).

Chemical stability of DiD-nanovesicles was studied by monitoring absorption and emission spectra of the samples over time. After an initial reorganization of the dye, the emission and absorbance are maintained over time. This proves the chemical and colloidal stability of the dye when inserted in nanovesicle's membrane, in fact it doesn't undergo chemical degradation neither aggregation over two months. See Figure 3.

Table A

--	After 1 month		After 2 months	
Mean	104.8	+/- 2.7	101.1	+/- 3.6
Mode	76.8	+/- 2.3	75.2	+/- 3.3
SD	42.5	+/- 6.3	50.9	+/- 2.5
D10	57.5	+/- 2.1	53.9	+/- 1.6
D50	83.5	+/- 7.7	76.4	+/- 3.2
D90	149.5	+/- 10.6	137.9	+/- 6.9

Moreover, the stability of DiD-nanovesicles was compared to that of DiD-NPs, as shown in Figure 4. DiD-NPs prepared by DELOS method were found to be unstable over time. The continuous decrease in absorption (top) is probably due to the aggregation occurring between the particles (bottom). DiD was found to precipitate after two months from the preparation of the nanoparticles.

The advantages of the vesicular system DiD-nanovesicles over other formulations of DiD were analyzed by comparing DiD-nanovesicles with a sample of DiD-CTAB in which the CTAB micelles solubilize the DiD, which was obtained by DELOS-SUSP method but without cholesterol. The stability upon dilution (Figure 5) of DiD-nanovesicles (1.3 mol*10^{-3}) was studied by absorption (top) and emission (bottom) divided by absorption at excitation wavelength. The vesicular system was compared with a formulation of DiD-CTAB micelles. Absorption and emission at concentration above and below the Critical Micellar

Concentration (CMC) of CTAB were compared. When diluting below CMC, normalized absorption of DiD-nanovesicles doesn't change while in DiD-CTAB some bands attributed to aggregation of DiD molecules start to appear. Moreover, DiD-nanovesicles do not lose brightness upon dilution while DiD-CTAB is almost not fluorescent at concentration lower than the CMC.

Advantageously, the stability upon dilution was excellent for DiD-nanovesicles, which maintains its brightness, while DiD-CTAB formulations do not fluoresce at concentrations below the critical micellar concentration (C<CMC) of CTAB. The effect of local concentration of DiD on the spectroscopic properties of DiD-nanovesicles was also studied.

Moreover, the fluorescence spectra (normalized by absorbance at excitation wavelength) of DiD-nanovesicles and DiD-NPs are compared, showing that the luminescence of the last formulation is quenched (Figure 11).

Integration efficiency (EE) of DELOS-SUSP was calculated by measuring the real concentration of DiD in nanovesicles and dividing by the nominal concentration (i.e. the theoretical concentration that the sample would have if all the DiD placed into the reactor would go inside nanovesicle's membrane).

Five different samples with increasing fluorophore concentration were prepared by DELOS-SUSP, which all showed a high integration efficiency (Figure 6).

Table B

DiD mol $*10^{-3}$	Integration Efficiency of DELOS-SUSP
0.57	0.8
1.3	0.9
3	0.84
4.2	0.79
6.6	0.92

Size distribution of said samples (Figure 6, top) shows that the increased concentration does not appreciably affect the size distribution and stability is confirmed over two months for all the samples. CryoTEM images (Figure 6, bottom) show good homogeneity. The black arrow in Fig 6e signs the presence of some other supramolecular structure in the suspension. Size distribution of the samples can be measured by Nanoparticle Tracking Analysis (NTA), equipped with a blue laser (488 nm), where DiD molecules do not absorb. CryoTEM images show that there is almost no effect of the dye on the morphology neither on lamellarity of the vesicles. Only in the sample with the highest DiD concentration some structures not attributable to nanovesicles appears, probably due to different supramolecular organization of DiD molecules.

UV-vis normalized absorption spectra (Figure 7) show that as more dye is loaded in the bilayer membrane of nanovesicles more aggregates are formed. Normalized absorption spectra of DiD-nanovesicles at different compositions are compared to that of DiD in EtOH. As long as DiD concentration increase, a deformation of the band-shape with increase absorbance on the blue-side is observed, possibly due to the presence of some dimers and aggregates on the nanovesicles membrane.

Likely these aggregates are mainly present on the bilayer membrane of the nanovesicles itself, considering that CryoTEM images do not evidence the presence of other supramolecular structures, except in a few cases such the one previously mentioned. Fluorescence quantum yield (QY) of DiD-nanovesicles, see Table 1 below, decreases at high DiD concentrations. QY of DiD and Nanovesicles-DiD was measured exciting at 631 nm. Cresyl Violet in Methanol was used as standard. Photodecomposition QY was also measured.

The decrease of QY is ascribed to the proximity effect of the dye molecules and/or polarity effect of the solvent considering that it is likely that part of the dye stays in contact with water. Table 1 also shows a different composition of the nanovesicle, particularly PEG-Chol/Chol/CTAB, and Chol/MKC at different molar ratios, as well as different Cyanines dyes and more than one dye at the same nanovesicle to obtain fluorescent nanovesicles showing the FRET-effect.

Table 1

Membrane anchoring				
Nanovesicles composition and molar ratio	Probe (dye)	Probe concentration $*10^{-3}$ (mol/mol_{tot})	QY Φ(%)	Observations
Chol/CTAB 1/1	DiD	0.57	23	Uptake in epithelial cells and feasible for STORM imaging
/	/	1.3	19	
/	/	3	11	
/	/	4.2	10	
/	/	6.6	7	
/	DiI	0.57	18	STORM imaging
/	/	3	13	
/	/	6.6	10	
PEG-Chol/Chol/CTAB 1/25/26	/	0.57	20	STORM imaging
PEG-Chol/Chol/CTAB 1/6/7	/	0.57	20	STORM imaging

(continuation)

Chol/CTAB 1/1	DiR	0.57	3	
/	/	6.6	1 ca.	
/	DiI+DiD (FRET sample)	0.57 (DiI) - 0.57 (DiD)	(FRET ratio≈35-40%)	
/	/	6.6 (DiI) – 6.6 (DiD)	(FRET ratio≈55-60%)	
/	DiI+DiD+DiR (cascade FRET)	0.57 (DiI) - 0.57 (DiD)-0.57 (DiR)		
/	/	6.6 (DiI) – 6.6 (DiD) - 6.6 (DiR)		
Chol/MKC 1/2	DiR	0.57		Injected in mousses (biodistribution study)

- DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate)
- DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide)

Photostabilities of DiD in EtOH and of DiD-nanovesicles were analyzed by monitoring the evolution over time of absorbance of the samples under continuous laser irradiation. The data are shown in Table below and are also represented in Figure 8.

Additional column in Table 1 for rows of DiD

Probe	Photodecomposition QY*10^{-5}
DiD EtOH	<0,008
DiD mol *10^{-3} (mol/mol_{tot})	Photodecomposition QY*10^{-5}
0.57	1.3±0.4
1.3	1.4±0.2
3	1.9±0.3
4.2	1.9±0.4
6.6	1.8±0.4

The quantum yields of photoreaction were calculated following the method proposed by Belfield *et al.* "*Photophysical and photochemical properties of 5,7-di-methoxycoumarin under one- and two-photon excitation*". *J. Phys. Org. Chem.* **16,** 69–78 (2003).

No relevant effect of DiD concentration was noticed on photostability of DiD-nanovesicles.

The resonance energy transfer between DiI and DiD (top) and cascade resonance energy transfer (FRET effect) between DiI, DiD and DiR is shown in Figure 12, top and bottom. Said Figures show that by selectively exciting the DiI (donor) it is possible to collect emissions of the acceptors, DiD (top) and DiD-DiR (bottom). Thus, the fluorescent nanovesicles can be used as fluorescence resonance energy transfer (FRET)-based scaffolds with different

emission spectra, that can be excited by a single wavelength excitation source resulting in a tunable emission wavelengths.

Moreover, the fluorescent nanovesicles can fluoresce both upon one-photon and multi-photon excitation and therefore, they can be designed to imaging at different deeps in a human or animal tissue (Figure 13).

Nanovesicles linked with NBD-6-Cholesterol (NBD6Chol-nanovesicles)

Cholesterol was partially substituted by NBD-6-Cholesterol for the preparation by DELOS-SUSP method of nanovesicles with this dye covalently attached to their membranes. Five samples were prepared with different NBD-6-Chol/Chol ratios by DELOS-SUSP shown in the Table 2 below.

Table 2

Covalent binding			
Nanovesicles composition	Probe (dye)	Probe concentration $*10^{-3}$ (mol/mol_{tot})	QY Φ (%)
Chol/CTAB 1/1	NBD-6-Cholesterol	0.46	8
/	/	1.3	4
/	/	2.2	4
/	/	5.2	2
/	/	6.4	3

The ratio between NBD-6-Chol and Chol was held lower than 2% in order to have minor effects on the morphology and sizes of the resulting NBD6Chol-nanovesicles. Size and Z-potential (measured by DLS) of the samples over 1 month (Figure 9) showed a constant and homogenous size distribution revealing they are stable with high positive Z-potentials (≈ 80mV). Likewise CryoTEM micrographs (Figure 9) showed homogeneous structures confirming that the partial substitution of cholesterol does not affect the morphology of the vesicles.

Therefore, no relevant variations have been noticed on distribution of size and Z-potential and CryoTEM images show homogeneous and unilamellar vesicles at all the concentration assayed. The increased concentration of NBD-6-Chol, NBD6Chol-nanovesicles reduces the QYs of the samples as can be seen in Table 2 above and Figure 10, probably due to aggregations of the dyes on nanovesicle's membrane or the proximity of the NBD groups on nanovesicle's surface. This assumption is further supported by the broadening and the red-shift of the emission spectra (Figure 10).

Although the scope of the present invention is not restricted to a selected non-water soluble organic dye, it is preferable an amphiphilic organic dye or a fluorescent dye of a sterol derivative.

Examples

Hereinafter, the present invention is described in more detail and specifically with reference to the Examples and Figures, which however are not intended to limit the present invention.

Materials

5-Cholesten-3β-ol (Chol, purity 95%) was purchased from Panreac (Barcelona, Spain). Hexadecyltrimethylammonium bromide (CTAB, BioUltra for molecular biology ≥99.0%) was purchased from Sigma-Aldrich. Sodium hydroxide (NaOH, ≥98,0%) was obtained from Panreac. 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indodicarbocyanine perchlorate (DiD oil) was purchased from Life Technologies (Carlsbad, USA). NBD-6-Cholesterol was supplied by Avanti Polar Lipids (Alabaster, AL, USA). Milli-Q water was used for all the samples preparation (Millipore Ibérica, Madrid, Spain). Ethanol (Teknocroma Sant Cugat del Vallès, Spain) was purchased with high. Carbon dioxide (99,9% purity) was purchased by Carburos Metálicos S.A. (Barcelona, Spain). All the chemicals were used without further purification.

Preparation of fluorescent nanovesicles by DELOS-SUSP

The formation of the fluorescent nanovesicles is based on the one-step DELOS-SUSP method. DELOS-SUSP (*depressurization of an expanded liquid organic solution-suspension*), a compressed fluids (CFs) based method, was used for the preparation of nanovesicles (Cano-Sarabia M et al. Langmuir 2008, 24, 2433–2437; Cabrera I et al. Nano Lett. 2013, 13, 3766–3774).

Briefly, 111 mgr of Cholesterol was first dissolved in 4.2 mL of EtOH at working temperature T_w (T_w=308 K) along with the hydrophobic dye (DiD or NBD-6-Chol). The solution was then added to a high pressure vessel (V=11.8 mL) at atmospheric pressure and T_w. After 20 minutes of equilibration the vessel was pressurized with CO_2 at the working pressure P_w (P_w=10MPa) in order to have an expanded liquid ethanol solution with a molar fraction of CO_2 of X_{CO2}=0.63. The reactor was kept at the working condition for one hour, in order to homogenize the system. The organic solution was then depressurized over 35mL of water, where 100 mgr of CTAB had been previously dissolved. N_2 at 10MPa was added to the vessel during the depressurization in order to maintain constant Pw inside it. The vessel is equipped with a gas filter, in order to prevent any not solute compound to come out from the reactor during depressurization. With this one-step method, nanovesicles comprising a

bilayer membrane with homogeneous size can be prepared. Furthermore it allows the straightforward loading of nanovesicles with hydrophobic compounds, such as DiD and NBD-6-Cholesterol, yielding the labeled nanovesicles: DiD-nanovesicles and NBD6Chol-nanovesicles.

Five different samples of DiD-nanovesicles were prepared with the following molar ratios DiD/(Chol+CTAB): 0.57-1.3-3-4.2-6.6 mol*10^{-3}.

5 different samples of NBD6Chol-nanovesicles were prepared with the following molar ratios NBD-6-Chol/(Chol+CTAB): 0.46, 1.3, 2.2, 5.2, 6.4 mol*10^{-3}. In this case cholesterol was partially substituted by the NBD-6-Cholesterol with the cited ratios.

DiD Nanoparticles (DiD-NPs) were prepared by DELOS method. Briefly, DiD was dissolved in EtOH (without cholesterol) and placed inside the high pressure vessel. The same steps of DELOS-SUSP for nanovesicles preparation were followed, but depressurization was performed over MilliQ water (without CTAB).

CTAB micelles loaded with DiD (CTAB-DiD) were prepared by the same route, but depressurization was performed over water with CTAB.

Preparation of DiD-nanovesicles by other methods

The ability of DELOS-SUSP for the straightforward integration of hydrophobic dyes, such as DiD, was compared with other routes for cholesterol-rich vesicles preparations, such as Ultrasonication (US) and Thin Film Hydration (TFH). The loading capability was as well compared with Incubation of preformed plain nanovesicles with DiD.

Thin Film Hydration (TFH):

Proper quantities of membrane components, with same concentrations of DiD-nanovesicles prepared by DELOS-SUSP, were initially dissolved in chloroform. The solvent was then rotevaporated to remove the solvent and form a thin film. The film was further dried by placing it under vacuum for 4 hours. Once dried, the film was hydrated at room temperature over night using water+10%vol of EtOH. With a further step, the obtained nanovesicles were downsized by ultrasonication for 10 minutes.

Ultrasonication (US):

Proper quantities of Cholesterol and CTAB as shown for DELOS-SUSP and TFH were mixed in a vial. MilliQ water and a solution of DiD in Ethanol (in volume ratio 9:1) were then added to the solids immediately before placing the US tip inside the vial. The sample was sonicated for 8 minutes. No further steps were required.

Incubation (US):

Few uL of a concentrated solution of DiD in EtOH were added to preformed nanovesicles and incubated for 24 hours. During this incubation time the UV-vis absorption spectra of the sample was monitored. The sample was unstable over time.

Purification

All samples were purified by diafiltration by using KrosFlo Research II*i* TFF System (SpectrumLabs, USA) equipped with mPES MicroKros filter column (100 kDa MWCO) in order to remove ethanol and excess of CTAB.

The real concentration of DiD and Chol-6-NBD in nanovesicles was determined by adding a known volume of ethanol to the samples (volume ratio ethanol/nanovesicle sample in at least 10:1). The EtOH causes the rupture of the vesicles (verified by Nanoparticle Tracking Analysis NTA) and the dissolution of the dyes. The maximum absorption was then measured and divided by the calculated extinction coefficient of the dye in ethanol

$$(\varepsilon_{\downarrow}DiD^{\uparrow}(MAX\ Abs) = [\![\ 246500\ M]\!]^{\uparrow}(-1)\ [\![cm]\!]^{\uparrow}(-1);\ \varepsilon_{\downarrow}(NBD-6-Chol)^{\uparrow}(MAX\ Abs) = 22500\ M^{\uparrow}(-1)\ [\![cm]\!]^{\uparrow}(-1))\ .$$

No effect of cholesterol and CTAB on shape and intensity of absorption bands of DiD and NBD-6-Chol in ethanol was detected.

Characterization of dye-labelled nanovesicles

Size and morphology

Dynamic Light Scattering (DLS):

Size, Polidispersity Index (PdI) and Z-potential of plain nanovesicles, and NBD6Chol-nanovesicles were studied using Dynamic Light Scattering (Malvern Zetasizer Nano ZS, Malvern Instruments, UK) with non-invasive backscatter optics, equipped with He-Ne laser at 633 nm. All the values reported were the average of 3 consecutive measurements of the same samples at 25°C.

Nanoparticle tracking analysis (NTA):

Mean size and size distribution of DiD-nanovesicles were analyzed by NTA using a Nanosight NS300 equipped with a laser at 488 nm. It was not possible to study DID-nanovesicles by DLS due to the relevant absorbance of this sample at 633 nm. The sample is placed into a chamber and a laser beam is passed through it. Light scattered by the particles can be visualized by a 20X magnification microscope equipped with a sCMOS

camera. The camera captures a video of the particles moving under Brownian motion. The diffusivity of the particles is then calculated by the software and the size calculated by using Stokes-Einstein equation. Samples were diluted 10000 times to fit the concentration range suggested by manufacturer. The values reported are average of results from five videos of each sample.

Cryo-TEM images:

Cryogenic transmission electronic microscopy images (Cryo-TEM) were acquired with a JEOL JEM microscope (JEOL, Tokyo, Japan) operating at 120kV. The sample was placed in a copper grid coated with a perforated polymer film and then plunged into liquid ethane to freeze it. Then it was placed into the microscope for the analysis.

Spectroscopic Measurements

UV-vis absorption spectra were recorded using a Lambda 650 (Perkin Elmer) spectrophotometer. Steady-state fluorescence and excitation spectra were collected with a Fluoromax-3 (Horiba Jobin Yvon) spectrofluorimeter for dilute solutions (maximum absorbance <0.1) in spectroscopic grade ethanol, methanol and MilliQ water. Fluorescence quantum yields were determined using comparative method with fluorescein in 0.1 M NaOH (QY, Φ: 92%) and cresyl violet in methanol (QY, Φ: 56%) as reference. Fluorescence decay curves were measured using time correlated-single photon counting method.

Sample photostability under continuous-wave excitation was determined by measuring the photochemical decomposition quantum yield. The photochemical decomposition quantum yield Φ_{ph} is defined as the ratio of number of bleached molecules N_{mol} per number of absorbed photons N_{phot}:

$$\Phi_{ph} = N_{mol} / N_{phot}$$

Quantum yields of photochemical decomposition were measured in ethanol (for DID) and water (NDB and all nanovesicle samples) using absorption and fluorescence methods, where the number of bleached molecules is determined by a kinetic decrease in absorbance or fluorescence intensity under the assumption that fluorescence and absorbance of the photoproducts are negligible in the spectral region of dye fluorescence and main absorption band. Fluorescence method was used to estimate the photostability of dilute samples (with maximum absorbance < 0.1), while photochemical decomposition quantum yields of concentrated solutions (with maximum absorbance ≈ 1) were determined by absorption method. The output of a Kr^+ laser (at 476.2 nm and 482.5 nm) and a diode laser (660 nm) was used for excitation of NBD-6-Cholesterol and DID samples respectively. The entire

volume of a sample was illuminated simultaneously in order to exclude the influence of diffusion on photochemical process.

It is a matter of course that the features mentioned above and those explained below can be used in other combinations in addition to those described, on in isolation, without departing from the scope of the invention.

- . -

CLAIMS

1. A fluorescent nanovesicle composed of an amphiphilic bilayer membrane with an inner compartment enclosing an aqueous medium, the amphiphilic bilayer membrane comprising sterol molecules or derivatives thereof assembled with quaternary ammonium surfactant molecules which define hydrophobic and hydrophilic regions in the bilayer membrane, **characterized in that** the amphiphilic bilayer membrane further comprises at least a non-water soluble organic dye, wherein the non-water soluble organic dye is bonded to the bilayer membrane by a linkage selected from covalent and/or hydrophobic, thereby the fluorescence of the nanovesicle is stable over time in an aqueous medium.

2. The fluorescent nanovesicle according to claim 1, wherein the non-water soluble organic dye is a fluorescent dye of a sterol derivative bonded to the bilayer membrane by a covalent bond at the hydrophobic region of the bilayer membrane to the sterol molecule forming the bilayer membrane.

3. The fluorescent nanovesicle according to claim 1, wherein the non-water soluble organic dye is an amphiphilic organic dye which is bonded to the bilayer membrane by a hydrophobic linkage at the hydrophobic and hydrophilic regions of the bilayer membrane.

4. The fluorescent nanovesicle according to claim 1, wherein the non-water soluble organic dye is an apolar organic dye which is bonded to the bilayer membrane by a hydrophobic linkage at the hydrophobic region of the bilayer membrane.

5. The fluorescent nanovesicle according to claim 1, wherein the non-water soluble organic dye comprises a polar or an apolar head and at least one alkyl tails, each tail having from 10 to 24 carbons in length.

6. The fluorescent nanovesicle according to any one of previous claims, wherein the amphiphilic bilayer membrane comprises at least two non-water soluble organic dye, each one having a different optical spectrum, the first dye being selected to have an emission spectrum that partially overlaps the absorption spectrum of the second dye.

7. The fluorescent nanovesicle according to any one of previous claims, wherein the fluorescent nanovesicle further comprises a bioactive compound and/or a targeting agent.

8. The fluorescent nanovesicle according to any one of previous claims, wherein the quaternary ammonium surfactant is selected from CTAB, MKC, cetrimide and BKC or mixtures thereof, the sterol molecule is cholesterol, and the molar ratio of the quaternary ammonium surfactant to sterol is within the range from 10:1 to 1:5.

9. A plurality of fluorescent nanovesicles according to any one of previous claims presented in a lyophilized state.

10. A colloidal dispersion comprising a plurality of fluorescent nanovesicles according to any one of previous claims.

11. Method for preparing a fluorescent nanovesicle according to any one of claims 1 to 8 comprising the steps of:

a) preparing an aqueous solution of a quaternary ammonium surfactant,

b) dissolving a cholesterol or derivatives thereof in an organic solvent and then expanding the solution by using a compressed fluid (CF), and

c) vesicle synthesis by depressurization of the expanded solution on the quaternary ammonium surfactant solution previously prepared in step a),

characterized in that the method further comprises:

in step b) adding a non-water soluble organic dye to the sterol or derivatives thereof and the organic solvent in order to prepare an organic solution, and dissolving the non-water soluble organic dye and the sterol or derivatives thereof in the organic solvent and then expanding the organic solution by using a compressed fluid, and, in step c) synthesizing the fluorescent nanovesicles by depressurization of the expanded solution on the quaternary ammonium surfactant solution, previously prepared in step a).

12. Method according to claim 11, wherein organic solvent is selected from a monohydric alcohol, a polyhydric alcohol, a ketone, ethylenediamine, acetonitrile, ethyl acetate and mixtures thereof; and the compressed fluid is selected from CO_2, ethane, propane, a hydrochlorofluorocarbon, and a hydrofluorocarbon.

13. Method according to claim 11, wherein the quaternary ammonium surfactant is selected from CTAB, MKC, cetrimide and BKC or mixtures thereof, the sterol molecule is cholesterol, and the molar ratio of the quaternary ammonium surfactant to sterol is within the range from 10:1 to 1:5.

14. Use of the fluorescent nanovesicle according to any one of claims 1 to 10 as a fluorescent probe in an aqueous medium.

15. Use of the fluorescent nanovesicle according to claim 14 for bioimaging and/or biodetection in an *in vitro* or *in vivo* method and for theragnostic applications.

FIG 1

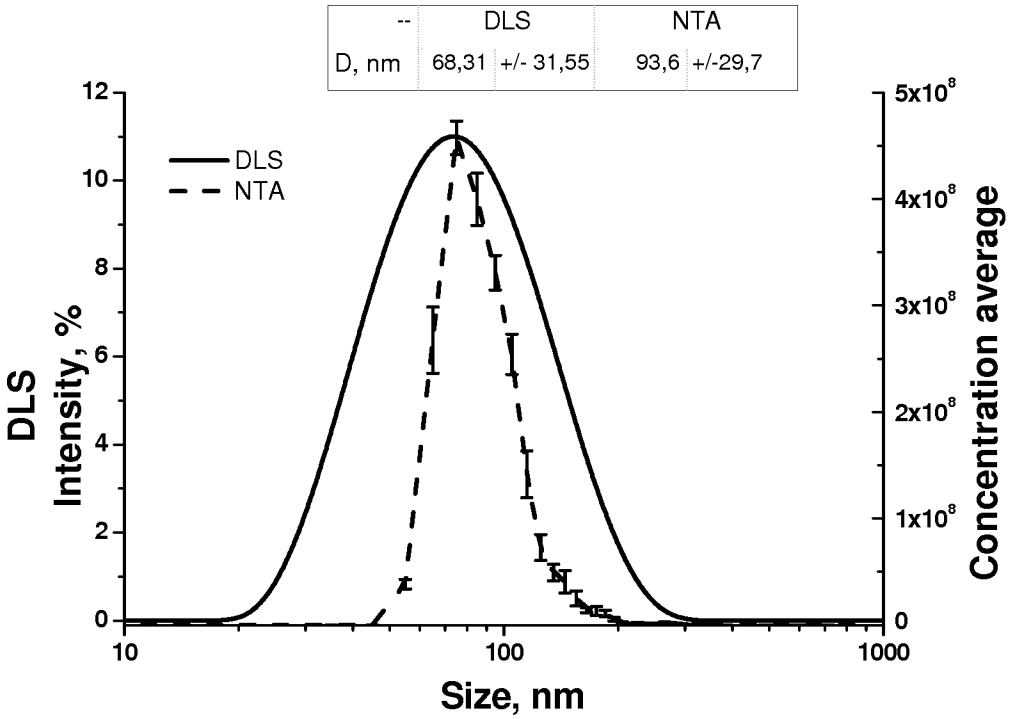

--
DLS
NTA
D, nm
68,31
+/- 31,55
93,6
+/-29,7
DLS
NTA
DLS Intensity, %
Concentration average
Size, nm
12
10
8
6
4
2
0
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4x10^8
3x10^8
2x10^8
1x10^8
0
10
100
1000

FIG 2

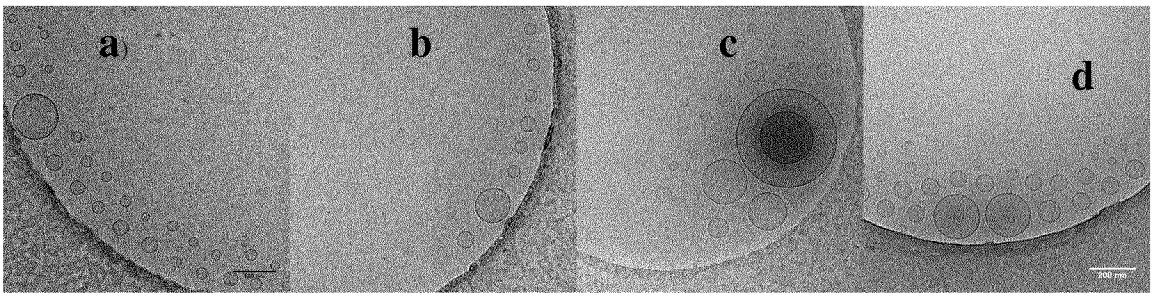
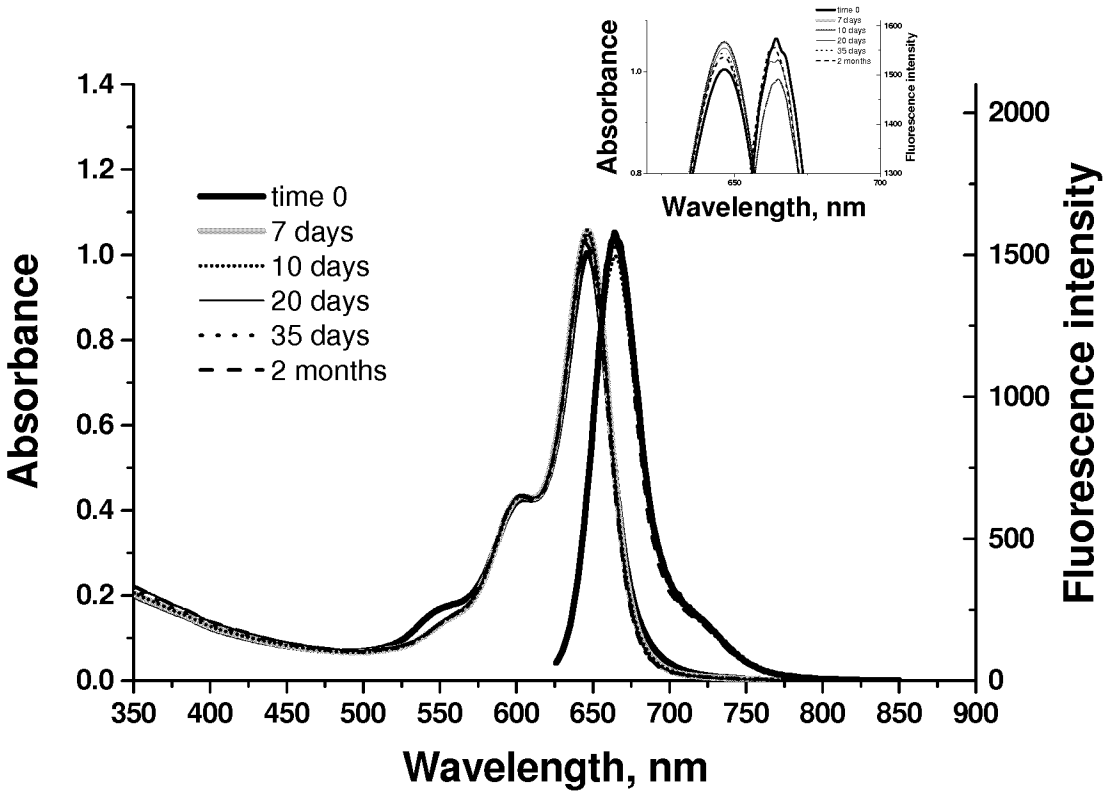

a)
b
c
d

FIG 3


Absorbance
Fluorescence intensity
Wavelength, nm
time 0
7 days
10 days
20 days
35 days
2 months
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2000
1500
1000
500
0
350 400 450 500 550 600 650 700 750 800 850 900
Absorbance
Fluorescence intensity
Wavelength, nm
1.0
0.8
1600
1500
1400
1300
650
700

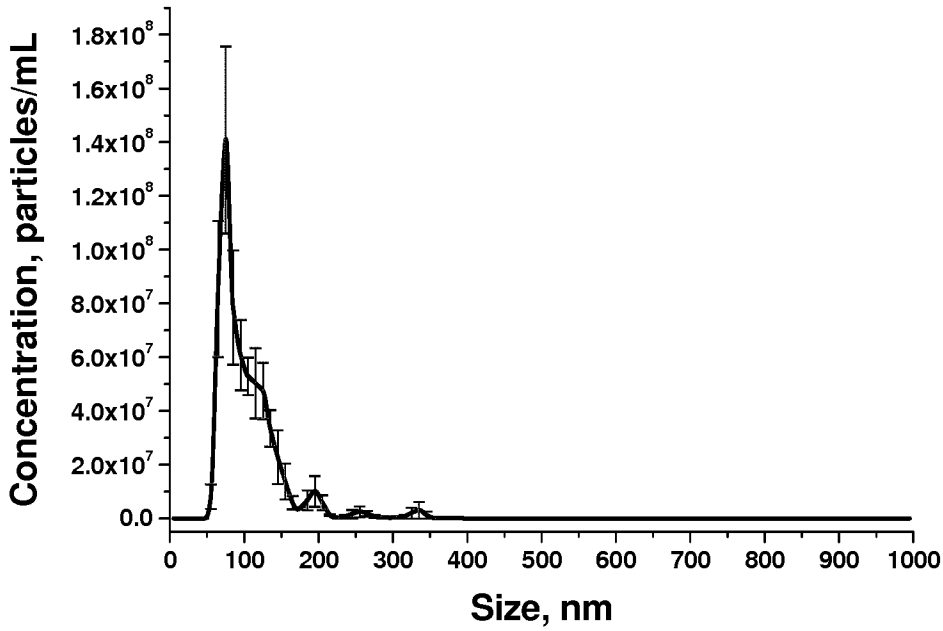
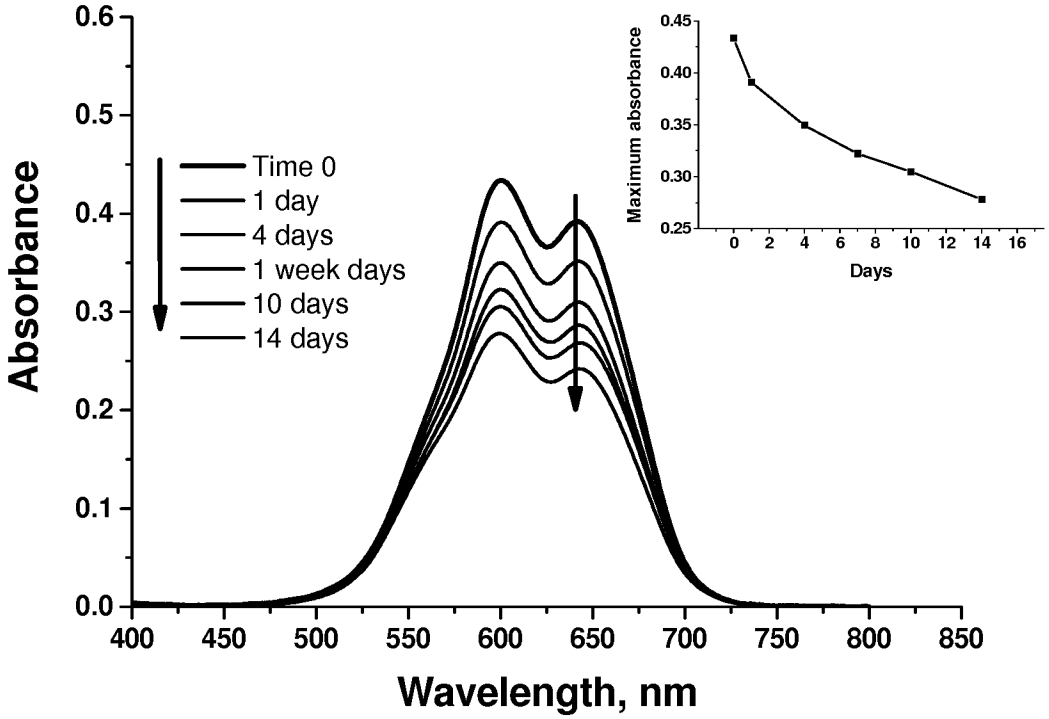

Concentration, particles/mL
Size, nm
1.8x10^8
1.6x10^8
1.4x10^8
1.2x10^8
1.0x10^8
8.0x10^7
6.0x10^7
4.0x10^7
2.0x10^7
0.0
0 100 200 300 400 500 600 700 800 900 1000

FIG 4


Absorbance
Wavelength, nm
Time 0
1 day
4 days
1 week days
10 days
14 days
Maximum absorbance
Days

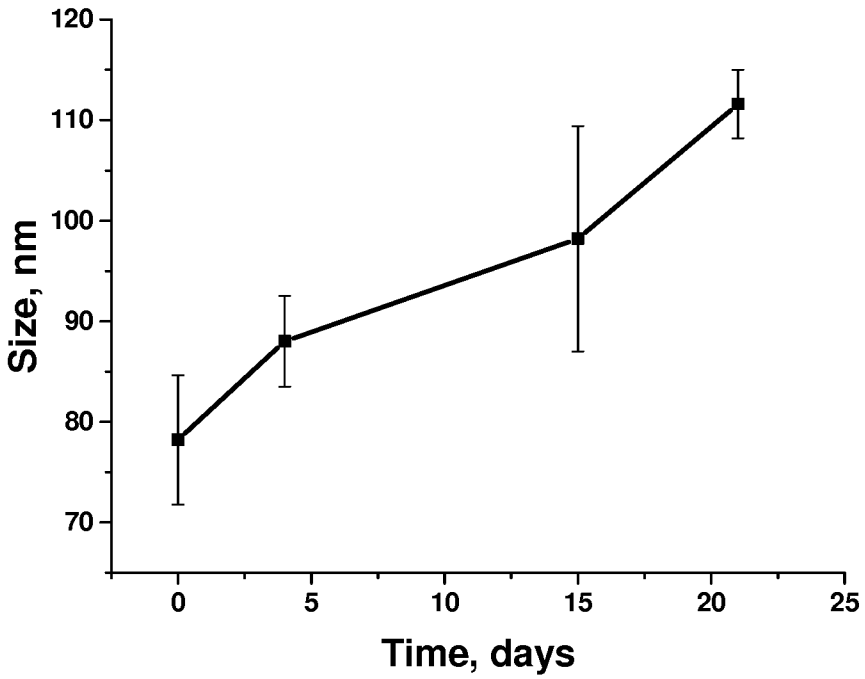
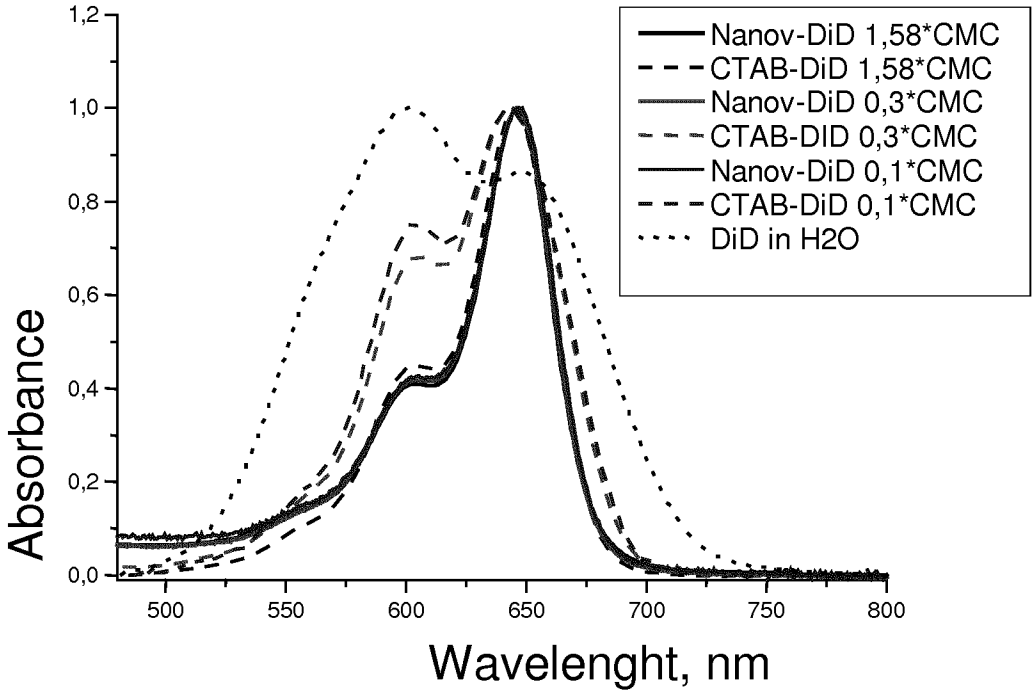

Size, nm
Time, days

FIG 5


Absorbance
Wavelenght, nm
Nanov-DiD 1,58*CMC
CTAB-DiD 1,58*CMC
Nanov-DiD 0,3*CMC
CTAB-DID 0,3*CMC
Nanov-DiD 0,1*CMC
CTAB-DiD 0,1*CMC
DiD in H2O

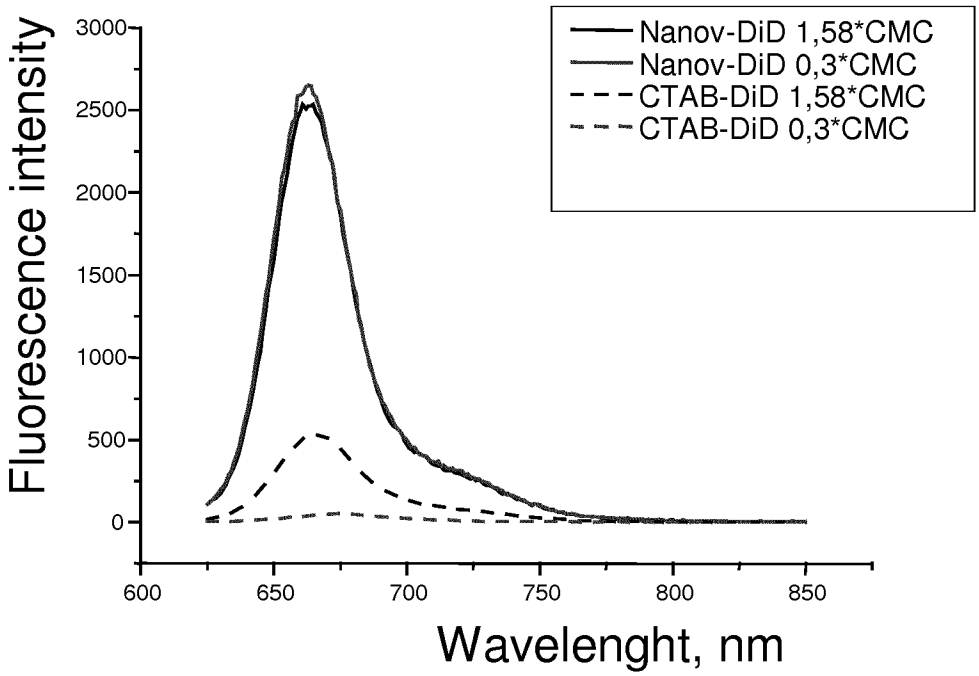
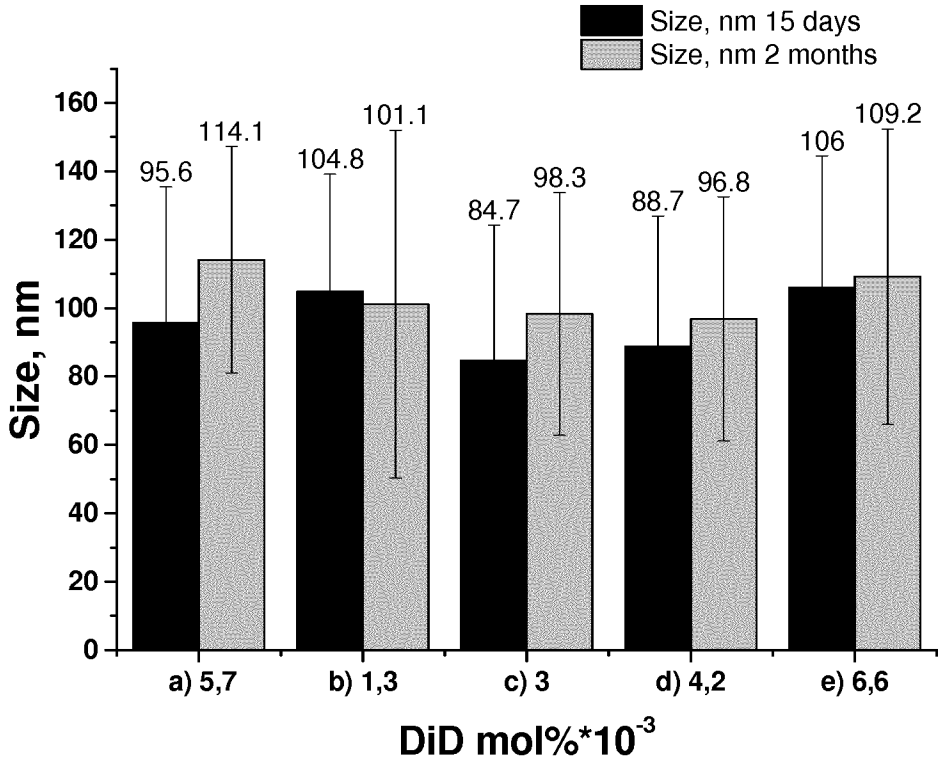

Fluorescence intensity
Wavelenght, nm
Nanov-DiD 1,58*CMC
Nanov-DiD 0,3*CMC
CTAB-DiD 1,58*CMC
CTAB-DiD 0,3*CMC

FIG 6


Size, nm 15 days
Size, nm 2 months
Size, nm
160
140
120
100
80
60
40
20
0
95.6
114.1
104.8
101.1
84.7
98.3
88.7
96.8
106
109.2
a) 5,7
b) 1,3
c) 3
d) 4,2
e) 6,6
DiD mol%*10-3

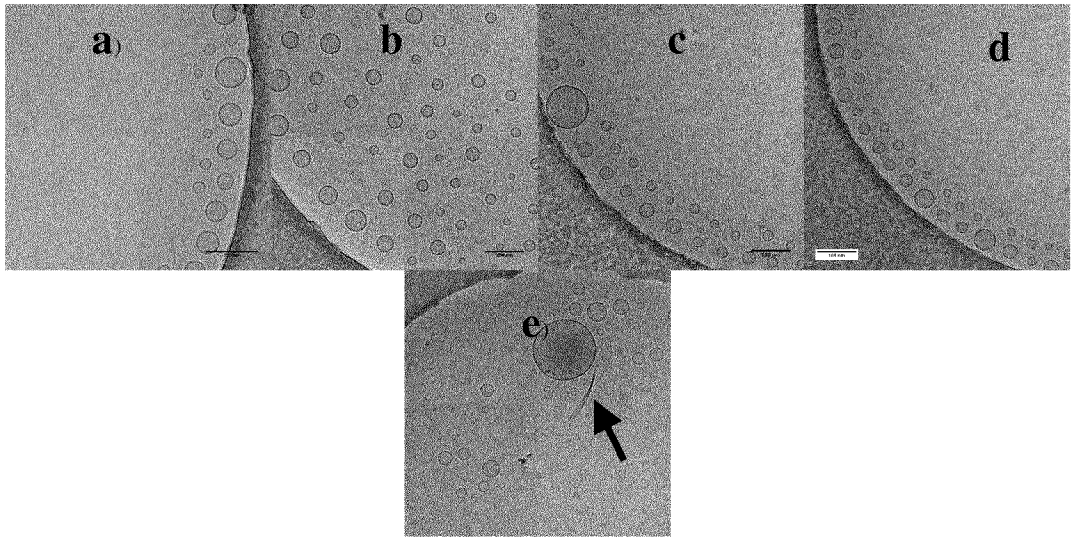

a)
b)
c)
d)
e)

FIG 7

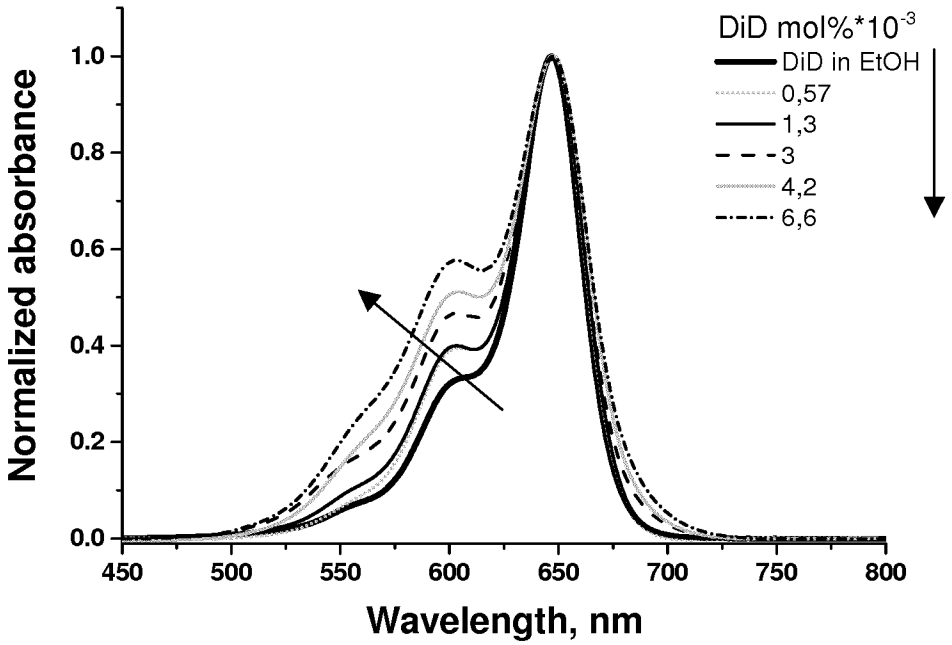

DiD mol%*10-3
DiD in EtOH
0,57
1,3
3
4,2
6,6
Normalized absorbance
1.0
0.8
0.6
0.4
0.2
0.0
450
500
550
600
650
700
750
800
Wavelength, nm

FIG 8

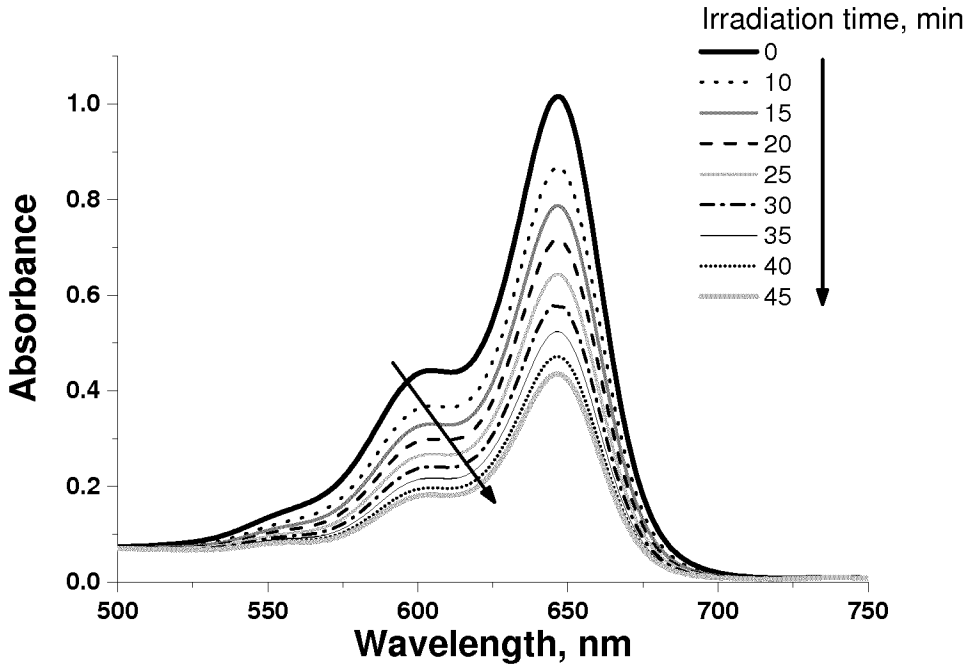
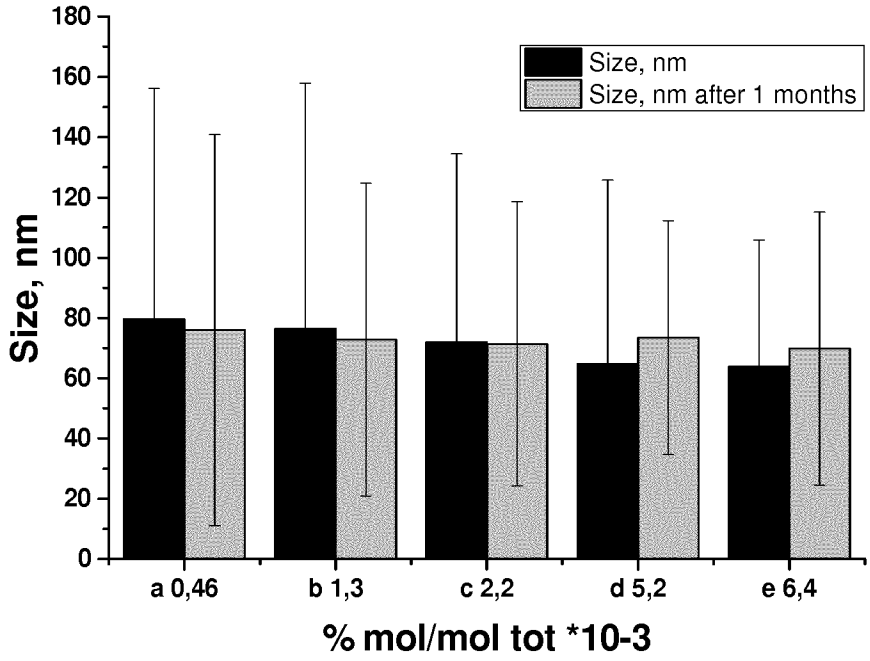
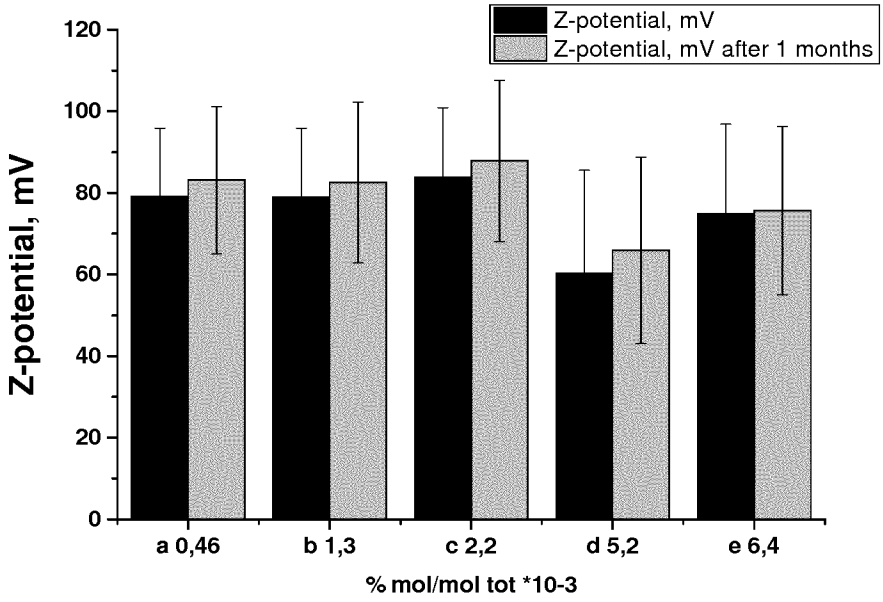

Irradiation time, min
0
10
15
20
25
30
35
40
45
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
500
550
600
650
700
750
Wavelength, nm

FIG 9


Size, nm
Size, nm after 1 months
Size, nm
0
20
40
60
80
100
120
140
160
180
a 0,46
b 1,3
c 2,2
d 5,2
e 6,4
% mol/mol tot *10-3


Z-potential, mV
Z-potential, mV after 1 months
Z-potential, mV
0
20
40
60
80
100
120
a 0,46
b 1,3
c 2,2
d 5,2
e 6,4
% mol/mol tot *10-3

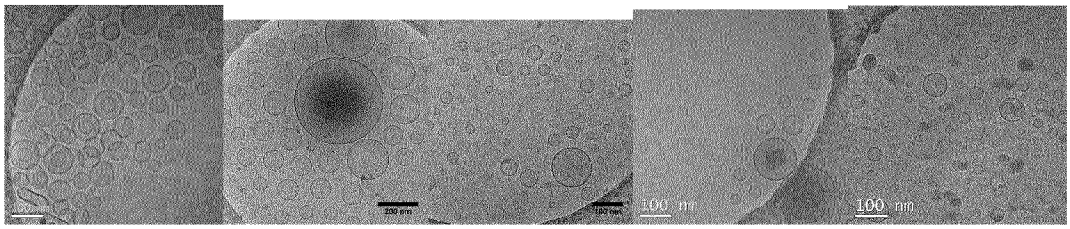

100 nm
100 nm

FIG 10

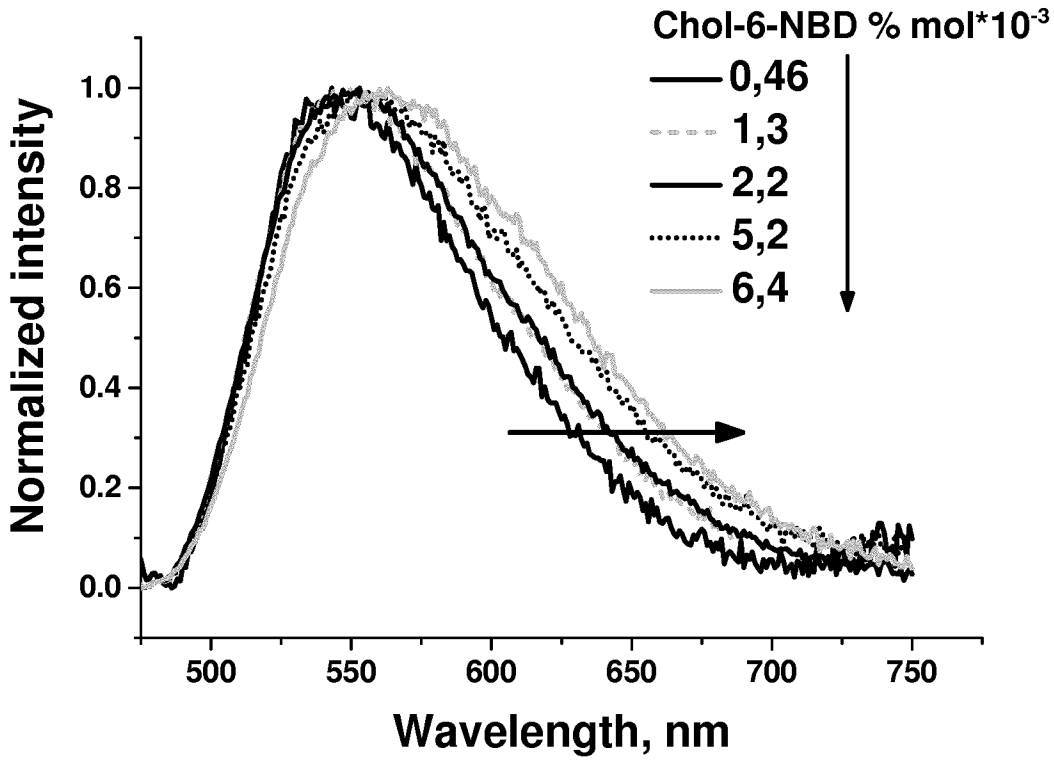
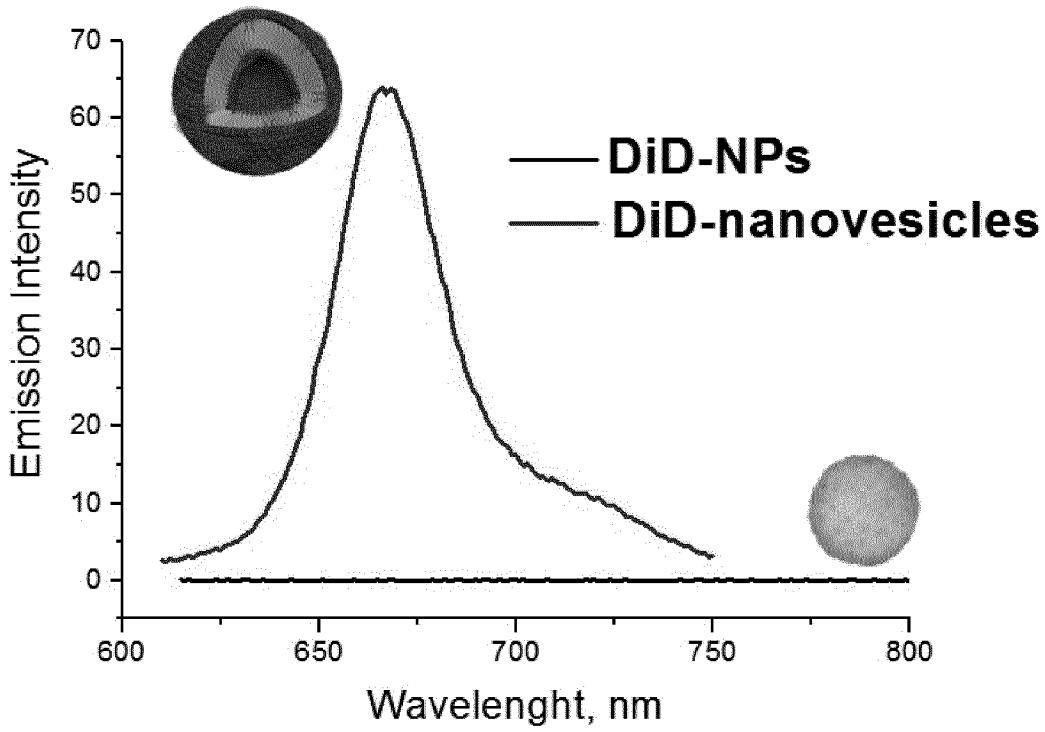

Chol-6-NBD % mol*10-3
0,46
1,3
2,2
5,2
6,4
Normalized intensity
1.0
0.8
0.6
0.4
0.2
0.0
500
550
600
650
700
750
Wavelength, nm

FIG 11


Emission Intensity
70
60
50
40
30
20
10
0
600
650
700
750
800
Wavelenght, nm
DiD-NPs
DiD-nanovesicles

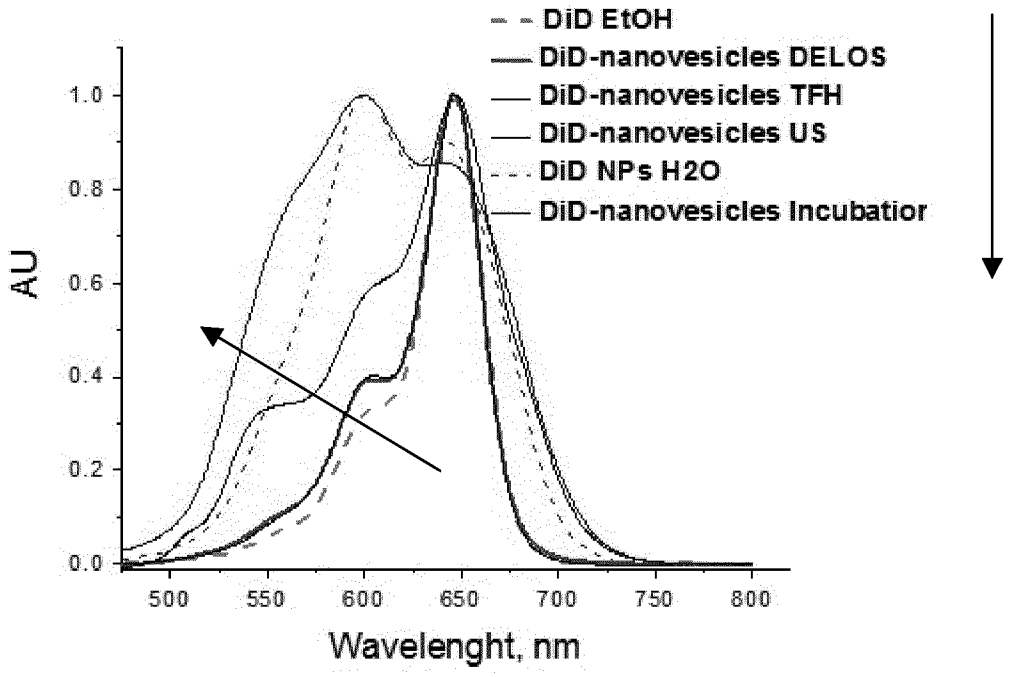
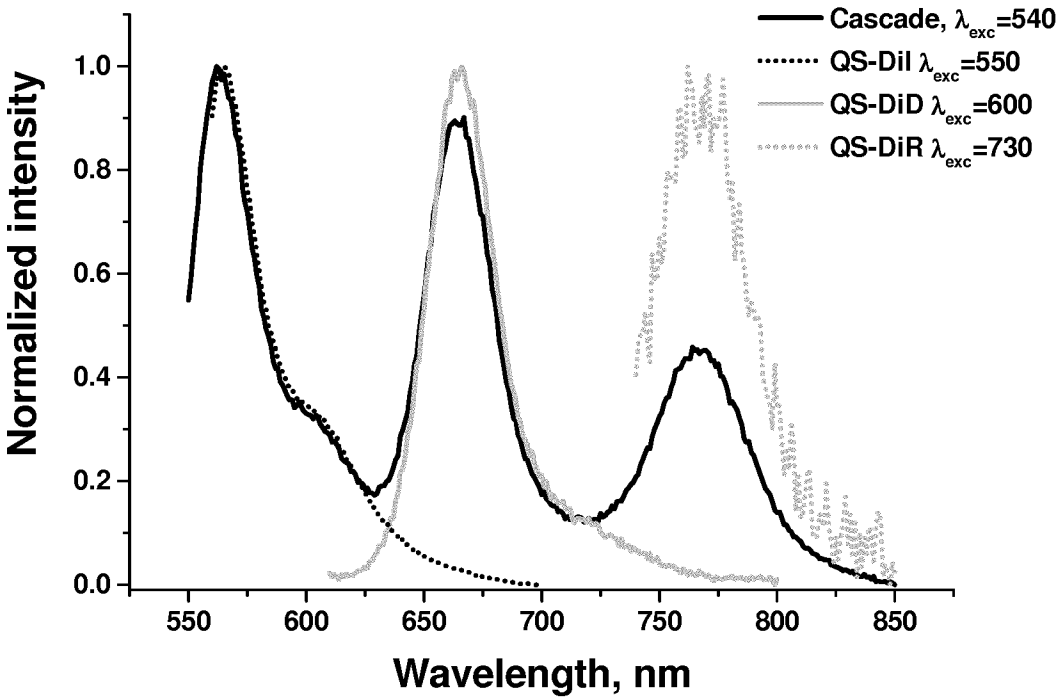

AU
1.0
0.8
0.6
0.4
0.2
0.0
500
550
600
650
700
750
800
Wavelenght, nm
DiD EtOH
DiD-nanovesicles DELOS
DiD-nanovesicles TFH
DiD-nanovesicles US
DiD NPs H2O
DiD-nanovesicles Incubatior

FIG 12


Cascade, λexc=540
QS-DiI λexc=550
QS-DiD λexc=600
QS-DiR λexc=730
Normalized intensity
1.0
0.8
0.6
0.4
0.2
0.0
550
600
650
700
750
800
850
Wavelength, nm

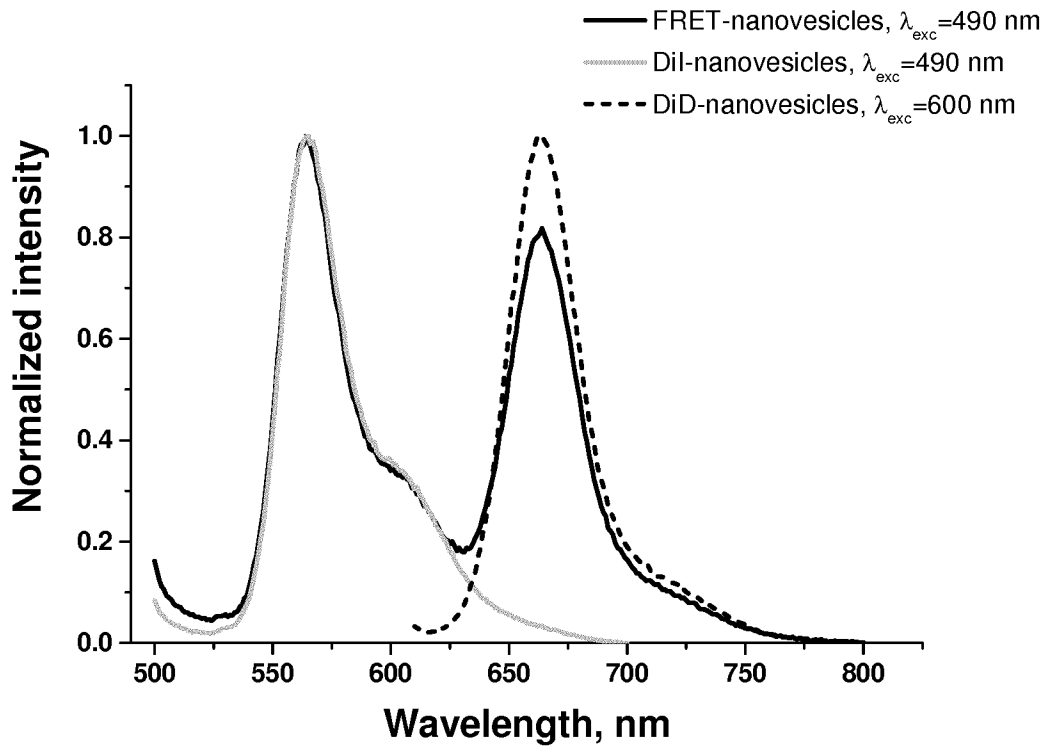
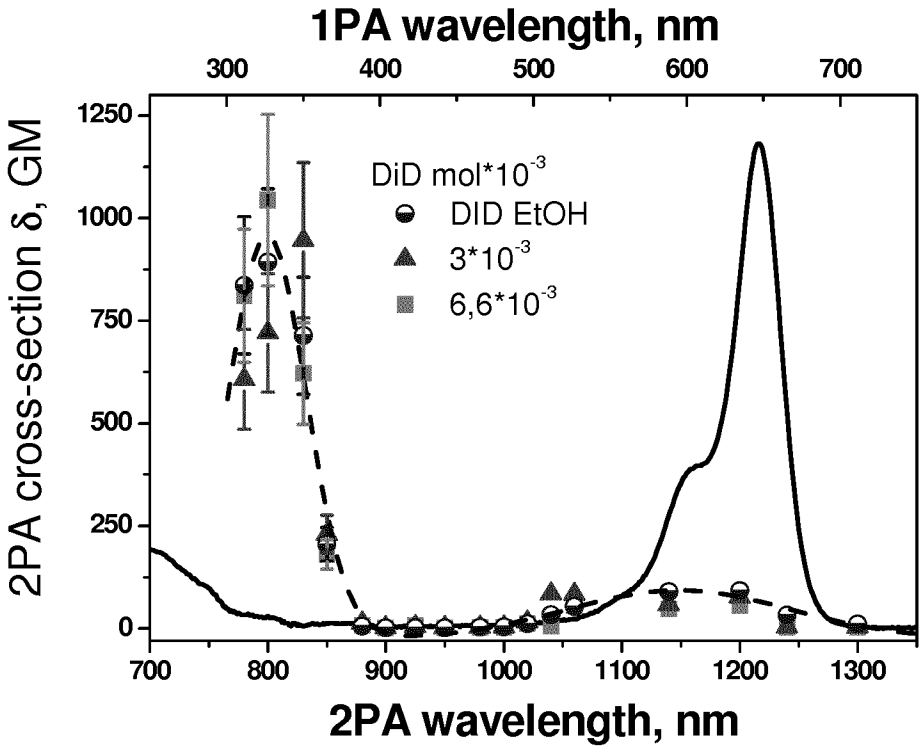

FRET-nanovesicles, λexc=490 nm
DiI-nanovesicles, λexc=490 nm
DiD-nanovesicles, λexc=600 nm
Normalized intensity
1.0
0.8
0.6
0.4
0.2
0.0
500
550
600
650
700
750
800
Wavelength, nm

FIG 13


1PA wavelength, nm
300
400
500
600
700
2PA cross-section δ, GM
1250
1000
750
500
250
0
DiD mol*10-3
DID EtOH
3*10-3
6,6*10-3
700
800
900
1000
1100
1200
1300
2PA wavelength, nm

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/069190

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/22
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/164072 A1 (LINDER CHARLES [IL] ET AL) 28 June 2012 (2012-06-28) the whole document In particular: Examples 1-29; Claims 1-30. ----- -/--	1-15

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

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2017	10/10/2017

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/069190

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FENART L ET AL: "EVALUATION OF EFFECT OF CHARGE AND LIPID COATING ON ABILITY OF 60-NM NANOPARTICLES TO CROSS AN IN VITRO MODEL OF THE BLOOD-BRAIN BARRIER", JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, US, vol. 291, no. 3, 1 January 1999 (1999-01-01), pages 1017-1022, XP000938176, ISSN: 0022-3565 the whole document In particular: Title; Abstract; Materials and methods section.	1-10, 13-15
A	US 2012/253191 A1 (ZHENG GANG [CA] ET AL) 4 October 2012 (2012-10-04) the whole document	1-15
A	S. GRINBERG ET AL: "Novel Cationic Amphiphilic Derivatives from Vernonia Oil: Synthesis and Self-Aggregation into Bilayer Vesicles, Nanoparticles, and DNA Complexants", LANGMUIR, vol. 21, no. 17, 1 August 2005 (2005-08-01), pages 7638-7645, XP055335415, US ISSN: 0743-7463, DOI: 10.1021/la050091j the whole document	1-15
X	Ingrid Cabrera Puig ET AL: "Nanovesicle-bioactive conjugates to be used as nanomedicines, prepared by a one-step scalable method using CO 2 -expanded solvents", , 26 October 2013 (2013-10-26), XP55407641, Retrieved from the Internet: URL:https://ddd.uab.cat/pub/tesis/2013/hdl_10803_129149/icp1de1.pdf the whole document In particular: pages 15-23, sections 1.1.3 and 1.2; pages 28-37, sections 1.3.3 and 1.3.3.1 as well as tables, 1.1 and 1.2, figures 1.17-1.24; page 228, section 6.	1-15

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Information on patent family members

International application No
PCT/EP2017/069190

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