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THERANOSTIC APPLICATIONS OF FLUORESCENT LIQUID CRYSTALLINE NANOPARTICLES

Settore/i scientifico disciplinari di afferenza

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List of articles

This thesis is based on the following papers, referred to by Roman numerals in the text. The papers are appended at the end of the thesis.

- I. Drug-Loaded Fluorescent Cubosomes: Versatile Nanoparticles for Potential Theranostic Applications.**
S. Murgia, S. Bonacchi, A. M. Falchi, S. Lampis, V. Lippolis, V. Meli, M. Monduzzi, L. Prodi, J. Schmidt, Y. Talmon, C. Caltagirone, *Langmuir*, **2013**, 29, 6673-6679.
- II. Cancer-Cell-Targeted Theranostic Cubosomes.**
C. Caltagirone, A.M. Falchi, S. Lampis, V. Lippolis, V. Meli, M. Monduzzi, L. Prodi, J. Schmidt, M. Sgarzi, Y. Talmon, R. Bizzarri, S. Murgia, *Langmuir* **2014**, 30 (21), 6228–6236.
- III. Cubosome formulations stabilized by a dansyl-conjugated blockcopolymer for possible nanomedicine applications.**
S. Murgia, A. M. Falchi, V. Meli, K. Schillén, V. Lippolis, M. Monduzzi, A. Rosa, J. Schmidt, Y. Talmon, R. Bizzarri, C. Caltagirone, *Colloids and Surf. B: Biointerfaces*, **2015**, 129, 87–94.
- IV. Docetaxel loaded fluorescent liquid crystalline nanoparticles for cancer theranostics.**
V. Meli, C. Caltagirone, A. M. Falchi, V. Lippolis, M. Monduzzi, M. Obiols-Rabasa, A. Rosa, J. Schmidt, Y. Talmon, S. Murgia, *Langmuir*, **2015**, 31 (35), 9566-9575.
- V. Solvatochromic fluorescent BODIPY derivative as imaging agent in camptothecin loaded hexosomes for possible theranostic applications.**
C. Caltagirone, M. Arca, A. M. Falchi, V. Lippolis, V. Meli, M. Monduzzi, T. Nylander, A. Rosa, J. Schmidt, Y. Talmon, Sergio Murgia, *RSC Adv.*, **2015**, 5, 23443–23449.
- VI. Effects of monoolein-based cubosome formulations on lipid droplets and mitochondria of HeLa cells.**
A. M. Falchi, A. Rosa, A. Atzeri, A. Incani, S. Lampis, V. Meli, S. Murgia, *Toxicol. Res.*, **2015**, 4, 1025-1036.
- VII. Monoolein-based cubosomes affect lipid profile in HeLa cells.**
Rosa, S. Murgia, D. Putzu, V. Meli, A. M. Falchi, *Chem. Phys. Lipids*, **2015**, 191, 96-105.
- VIII. Near infra-red fluorescent cubic liquid crystalline nanoparticles for optical imaging applications *in vivo*.**
S. Biffi, L. Andolfi, C. Caltagirone, C. Garrovo, A. M. Falchi, V. Lippolis, A. Lorenzon, P. Macor, V. Meli, M. Monduzzi, M. Obiols-Rabasa, L.

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Abstract

Lipid liquid crystalline nanoparticles can find application as nanocarriers in several fields of the daily life but, very likely, the pharmaceutical arena is the most relevant. Indeed, several problems encountered in drugs administration (e.g. critical side-effects from antitumor drugs) require alternative, less invasive, but simultaneously efficient therapeutic routes to be explored. Novel fields of personalized nanomedicine are developing in this direction. One of the most interesting is theranostic, which calls for the design of platforms capable of combining therapeutic and diagnostic functionalities.

In this optic, we explored the potential of monoolein-based cubosomes and hexosomes as nanocarriers for theranostic purposes. Our work focussed on the design of lipid nanoparticles able to deliver antineoplastic drugs and imaging probes for fluorescent optical *in vitro* and *in vivo* imaging. We developed cubosome formulations loaded with antineoplastic drugs and useful for the fluorescence imaging of cells. Such formulations were also actively targeted to cancer cells and coupled with a NIR-emitting fluorophore, which was the promise for *in vivo* applications. We also investigated hexosomes with encouraging results encapsulating in their lipid matrix a BODIPY derivative with solvatochromic properties, helpful for the understanding of the dye localization.

Importantly, we reported (manuscript submitted) the first proof-of-principle for *in vivo* fluorescence optical imaging application using monoolein-based cubosomes in a healthy mouse animal model.

Finally, since relatively little is known about the interaction of cubosomes with biological systems, their effects on lipid droplets, mitochondria and lipid profile of HeLa cells were deeply studied.

This thesis is divided in two main parts. The introduction section reports on the essential background of the research field, and it is followed by the publications (published or submitted) resulting from these three years of work.

1. Surfactants and self-assembly

Surfactants are among the most versatile products of the chemical industry. They appear in a wide variety of everyday products such as the motor oils used in automobiles, pharmaceuticals, cosmetics, detergents or in industrial products, such as drilling muds used in prospecting for petroleum, or flotation agents used in beneficiation of ores.

The ability of surfactant molecules to act as detergents was known for centuries, but the mechanism through which they remove the dirt was clarified in the last century only when scientists started to become aware of surfactant self-assembly. Indeed, the first idea of a surfactant aggregate dates back to the work of J.W. McBain who, in 1913, suggested the existence of colloidal ions in sodium palmitate solutions.¹

The word surfactant derives from the contraction of the term surface-active agent and indicates a substance that, if present at low concentration in a system, is able to modify to a certain degree its surface or interfacial free energy by simply adsorption. Molecularly and structurally speaking surfactants consist of two different moieties: the lyophobic (hydrophobic) one, with very little attraction for the solvent (water), and the lyophilic (hydrophilic) one, with strong attraction for the solvent (water). This peculiar molecular organization is known as amphiphilic structure, and a practice way to quantify this duality is the hydrophilic-lipophilic balance (HLB), a number ranging between 1 and 40: the larger this number, the higher the hydrophilicity of the surfactant considered. Usually the hydrophobic group is a long-chain hydrocarbon residue, and less often a halogenated or oxygenated hydrocarbon, or siloxane chain, while the hydrophilic group is an ionic or a highly polar neutral group.

Depending on the nature of the polar group the surfactants can be classified as anionic, cationic, zwitterionic and non-ionic.² Some examples of each class are reported in figure 1.

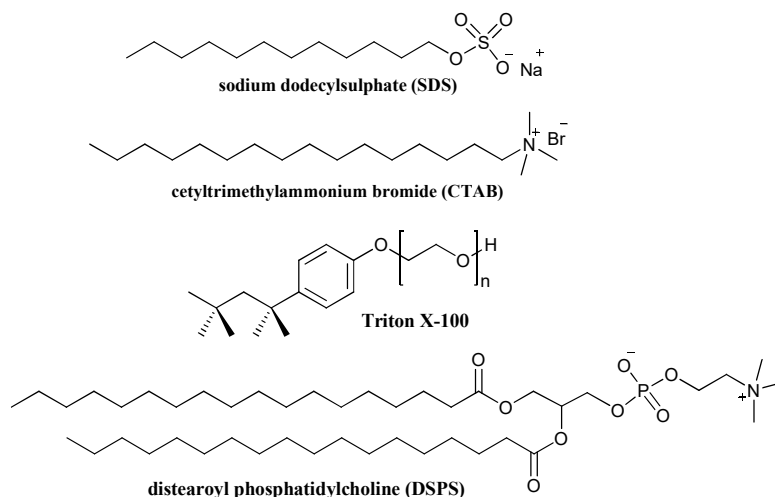


Figure 1. Examples of surfactant molecules.

The amphiphilic nature of these molecules gives them a range of properties strongly connected with two key phenomena: adsorption at interfaces and self-assembly in solution.

The adsorption at interfaces is responsible for applications in processes such as detergency, wetting, spreading and stabilization of particles, droplets and bubbles in dispersions, emulsions, and foams. The self-assembly in solution underpins the formation of nanoscale structures like micelles, microemulsions, and liquid crystals and molecular organizations easily found in biological contexts. The driving force for this phenomenon is the hydrophobic effect, which acts to minimize the interface between the hydrocarbon tails of the amphiphile, and the surrounding polar solvent.

For most surfactants aggregation occurs at a specific concentration known as the Critical Micelle Concentration (CMC), which is the lowest concentration at which aggregation in bulk solution can take place. The reason for such phenomenon is that at some critical level (related primarily to the hydrophobicity of the surfactant tail groups), it is more favourable for the surfactant tails cluster together, thus minimizing unfavourable contacts with solvent molecules. Upon reaching CMC a clear break in properties such as adsorption, surface tension or conductivity, occurs, and any further addition of surfactants will just increase the number of micelles.³ An opposite situation should also be considered, that is the reverse self-assembly in an apolar solvent. In this case the polar heads are buried inside the aggregate thus

avoiding any contact with the bulk, while the hydrophobic tails protrude outwards of the aggregate.

1.1. The Packing Parameter (P)

Micelles are the simplest self-assembled structures but, at concentration higher than the CMC, other and more topologically/morphologically complex aggregate structures may become favoured, such as elongated micelles (termed “rodlike” or “wormlike” depending on the length, see figure 2c), and other liquid crystalline phases. The interplay of weak interactions (between surfactant head groups, tail groups, and the solvent), and geometric factors, drives the propensity to form different phases and structures in solution.³

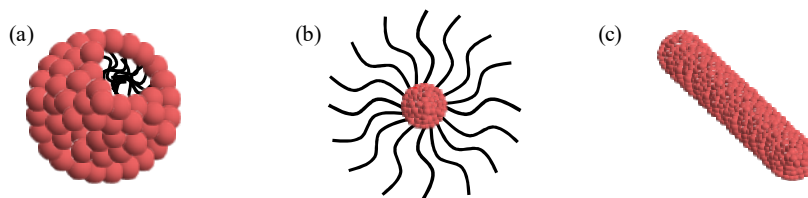


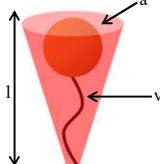
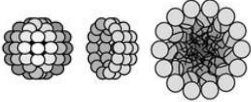
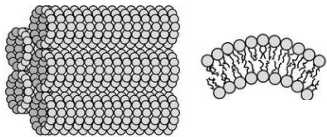
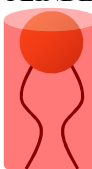
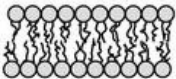
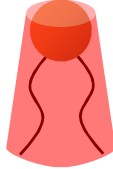
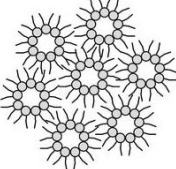
Figure 2. Structure of direct (a), inverted (b) and rod-shaped (c) micelles.

Concerning the geometric factor, between the 70s and the 80s was introduced by B. Ninham and J. Israelachvili the first conceptual idea of the surfactant shape parameter, known as packing parameter P :^{4,5}

$$P = \frac{v}{al} \quad \text{Eq. 1.1}$$

where v and l are respectively the volume and the length of the tail group, while a is the cross-sectional area of the head group. This simple factor represents the driving input for the spontaneous curvature of the polar-apolar interface (H) and allows predicting, with certain precision, which type of self-assembled aggregate will be formed (see table 1.1).

Table 1.1 Molecular shapes and relative association structures of surfactants.

Packing shape	Packing parameter (P)	Phase formed	Lipid examples
CONE 	$< 1/3$ (spheres)		Lysophospholipids (e.g. LPC, LPE, etc.)
TRUNCATED CONE 	$1/3 - 1/2$ (rods, lamellar, flexible bilayers, vesicles)		Free fatty acids (e.g. oleate, stearate, etc) and double-chained lipids with large head group areas and fluid chains
CYLINDER 	~ 1 (lamellar, planar bilayers)		Double-chained lipids with small head group areas, anionic lipids and saturated chains
INVERTED TRUNCATED CONE 	> 1 (hexagonal H_{II} , micellar reverse)		Double-chained lipids with small head group areas, non-ionic lipids and polyunsaturated chains

H is strictly correlated to P: for $P < 1$ the curvature H will be positive, for $P = 1$ H will be equal to zero, and for $P > 1$ H will be negative and inverted phases are favoured.

2. Liquid crystalline phases

When a sufficient number of micelles is formed in solution, they start to pack together (in spite of the loss of entropy) in a number of geometric arrangements strongly dependent upon the shape of the surfactant involved. The result of these arrangements are mesophases characterized by long-range orientation or translation

order typical of solid crystals, and by a mobility typical of liquids. Such mesophases are known as liquid crystalline phases. The short-range disordered arrangement of the molecules confers to liquid crystals a viscosity sometimes pretty considerable.²

Liquid crystals can be grouped into two subclasses: the thermotropics and the lyotropics. In both cases molecular shapes and mutual interactions between molecules are the driving forces for self-assembly process, but, in the thermotropic liquid crystals, the phase transitions are dependent solely from temperature, while, in the lyotropic liquid crystals, the concentration of the surfactant (and hence the presence of the solvent) is also strictly crucial.

An overview of lyotropic liquid crystals polymorphism is given below.

2.1. *Lamellar phases (L)*

The lamellar phase consists of planar surfactant bilayers stacked in a 1D lattice separated by layers of water (figure 3). They constitute the basic building block of all biological membranes. These phases are optically anisotropic and birefringent and in the polarizing microscope they show mosaic-like patterns.⁶ Different types of lamellar phases, such as L_α , L_β , L_c have been observed in lyotropic mixtures. The L_β phase differs from the L_α phase for the state of the carbonic chains. Indeed, in the L_α phase the hydrocarbon chains are molten, while in the L_β they are arranged parallel to the layer normal. Consequently, the latter looks like a gel, while the former is a more fluid phase. The L_c phase presents the chains of the surfactant molecules “frozen” into specific lattice sites, making this phase the most ordered of the three lamellar phases.⁷

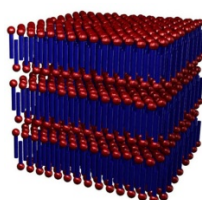


Figure 3. Schematic representation of a lamellar phase (image from the web).

2.2. Hexagonal phases (H)

The hexagonal liquid crystalline phases consist of a dense packing of cylindrical micelles arranged on a 2D hexagonal lattice. Normal (oil-in-water) hexagonal phases are denoted by H_I , while inverse (water-in-oil) hexagonal phases are denoted by H_{II} . In H_{II} phases water is contained within the cylindrical reversed micelles (figure 4). The remaining space is occupied by the non-polar chains overlapped in order to leave the cylinders close together as much as possible.⁶ The opposite configuration characterizes the H_I phases. Under a polarized light microscope hexagonal phases exhibit birefringence and their typical optical textures are smoke-like, fan-like or mosaic in appearance.

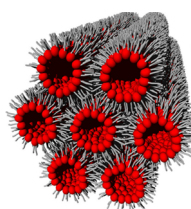


Figure 4. Schematic representation of an inverted hexagonal phase H_{II} (image from the web).

2.3. Cubic phases (I, V)

Two distinct families of cubic phases can be defined: the discrete micellar (I) and the bicontinuous (V) both having a 3D lattice arrangement. In the discrete micellar phase, micelles are packed on a cubic lattice (face-centered or body-centered), while in bicontinuous phase a curved lipid bilayer is extended in the three dimensions. Both types may be normal or inverse (I_I , I_{II} , V_I , V_{II}).

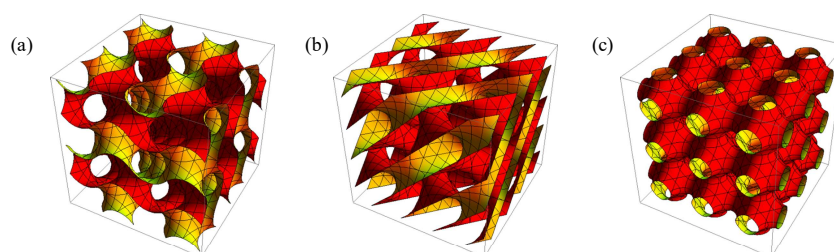


Figure 5. The three IPMS surfaces: C_G (a), C_D (b) and C_P (c). Thanks to Prof. Stefano Montaldo for the images.

In bicontinuous cubic phase the curved, triply periodic non-intersecting bilayer is folded on an infinite periodic minimal surface (IPMS) having an average curvature equal to zero everywhere. Such special organization leads to the formation of two interpenetrated and disjointed continuous water networks.

To date, three cubic bicontinuous phases based on three IPMS surfaces have been identified: the C_P (primitive, $Im3m$), the C_D (diamond, $Pn3m$) and the C_G (gyroid, $Ia3d$), showed in figure 5. The lipid bilayer is centred on the IPMS with the polar heads pointing outwards, while the water fills the labyrinth systems on each side of the surface. These phases are extremely viscous and optically isotropic, therefore no texture is visible through polarized light. The high viscosity is a consequence of the lack of shear planes within the structure that would allow a sliding movement.

2.4. *Sponge phases (L_3)*

As the bicontinuous cubic phases, the sponge phase (L_3) is constituted by a 3D lipid bilayer surrounded by two different water channels, with the only difference that the sponge phase is less ordered and can be considered to be a melt of the bicontinuous one. Figure 6 allows appreciating this difference.^{8,9}

With respect to bicontinuous cubic phases, the interface in sponge phases is highly flexible and thermal excitations lead to the break-down of the long-range order of the channel network so that the interface is no longer arranged on a lattice. Sponge phases are characterized by flow birefringence (giving anisotropic optical textures) but they are isotropic at rest.⁶

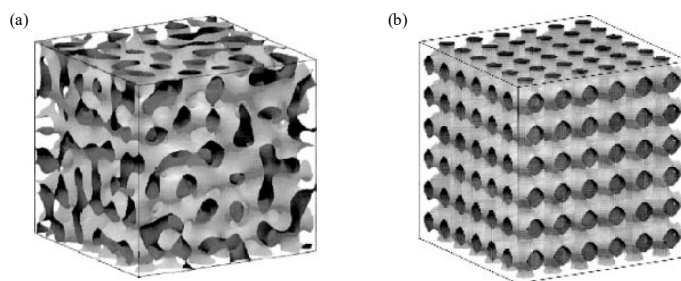


Figure 6. (a) Fragments of a disordered sponge and (b) the ordered $Im3m$ surface morphology.⁸

2.5. *Intermediate phases*

The existence of lyotropic phases with lower symmetry than those previously described has been widely proved. These phases are sometimes called “intermediate phases” and are in the middle of lamellar and bicontinuous phases. They are characterized by a smectic stack of “punctured” bilayers, together with an ordered arrangement of punctures within each bilayer, giving, like discrete micellar and bicontinuous mesophases, 3D lattices. Two polymorphs are known, the “R” (rhombohedral, space group $R3m$), containing a hexagonally close-packed array of punctures, and the (body-centred) “T” (tetragonal, space group $I422$) with a square array of punctures. Both mesophases are optically anisotropic.⁸

3. **Liquid crystalline nanoparticles**

The bulk liquid crystalline phases can be fragmented (sometimes literally and sometimes just figuratively) in submicron particles thus forming liquid crystalline nanoparticles dispersions in excess of water. The result of this process are diluted non-viscous aqueous dispersions with internal nanostructure.¹⁰

The simplest and most studied of such structures are the lamellar phase dispersions, which include liposomes or, more in general, vesicles. Liposomes consist of spherically concentric lipid bilayers (two or more) alternating with water layers, and they can be considered as dispersed particles of a L_α phase.¹¹ Liposomes have been extensively studied since they were identified and characterized by electron microscopy by Bangham in the 1960s.¹² They are still widely used as a model for cell membranes thanks to their 1D lamellar flat structure, which is the most commonly observed system in cellular contexts.

Similarly to liposomes, hexosomes (aqueous dispersions of inverse hexagonal phase) and cubosomes (aqueous dispersions of a bicontinuous cubic phase) can be prepared by dispersion formation in a multiphase region comprising a liquid crystal in coexistence with water.¹³ Within such nanoparticles, the lipid organization and dynamic properties of the original non-dispersed phases is retained.¹⁴ In 1996 and

1997 K. Larsson's group first published hexosomes' and cubosomes' cryo-TEM images (see figure 7).^{15,16}

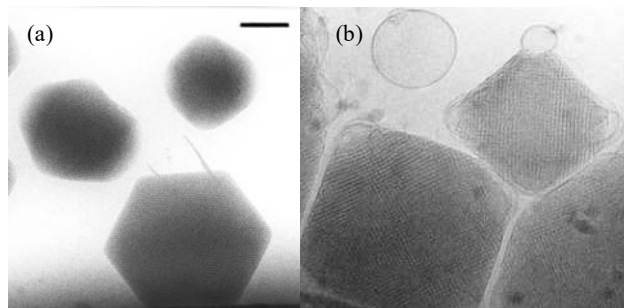


Figure 7. Cryo-TEM images of hexosomes (a) and cubosomes (b). Reprinted with permission from [Langmuir **1997**, 13 (6), 6964-6971] © 1997 America Chemical Society.¹⁶

As liposomes, hexosomes and cubosomes are particles with kinetic stability, *i.e.* they need an energetic input to form in excess of water (lyophobic colloids). However, lamellar phase is easier to disperse, thanks to the ability of the lamellae to bend into a closed structure that avoids the contact between the hydrophobic domain and water. Differently, reverse liquid crystalline phases, such as the bicontinuous cubic V_{II} and the hexagonal H_{II} , usually show a very limited stability in aqueous dispersions due to the boundary conditions imposed on the fragmented crystal.¹⁶ Indeed, to facilitate the dispersion of these phases an additional surface active component, which acts as stabilizer, is crucial. This stabilizing agent may be inclined to participate in, and sometimes change, the surfactant based liquid crystal structure as demonstrate by Landh et al. in 1994.¹⁷ Usually such molecules are polymeric stabilizers which posses poly-(ethylene oxide) (PEO) moieties, which protrude towards the water thus providing steric stabilization.

4. Monoolein

Glycerol monooleate, commonly known as monoolein (MO), is a non-toxic clear amber or pale yellow waxy paste widely used as emulsifying agent and as food additive since the 1950s. From a molecular structure point of view, it consists of a hydrocarbon (hydrophobic) chain (oleic acid) attached to the glycerol backbone by

an ester bond. The two remaining hydroxyl groups of the glycerol confer polar characteristics to this portion of the molecule. Its oil solubility (MO is totally insoluble in water), along with its biocompatibility and biodegradability, make it an highly suitable material in food emulsion, in cosmetic and in pharmaceutic applications.¹⁸

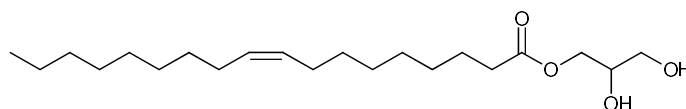


Figure 8. Chemical structure of monoolein (MO).

4.1. *MO/W phase diagram*

MO has self-assembly abilities due to its amphiphilic structure. The hydrophobic effect leads, in this case, to a very complex polymorphism in water and thus to a very complex MO/W phase diagram. The first construction of such a diagram goes back to 1965 by Lutton,¹⁹ who built the diagram on the basis of observations from polarizing optical microscopy and from the samples consistency. Lamellar, hexagonal and cubic phases were in this way named respectively as neat, middle, and viscous isotropic phases, while the different types of phase are not distinguished. From 1979 until 1983 intensive investigations on this system were carried out by Larsson and co-workers^{20,21} and by Lonlgey and McIntosh,²² throughout NMR and X-ray diffraction techniques. At this stage the different bicontinuous cubic phases were finally identified.

The first nearly complete MO/W phase diagram was mapped out by Hyde *et al.* in 1984.²³ The temperature range studied was 20 °C – 110 °C, while the system composition range from 0% (w/w) to at least 40% (w/w) in water. In 1996 Briggs *et al.* improved the temperature range going down to 0 °C. They found notable agreement with Hyde, except for some significant differences on phases boundaries. Furthermore the authors introduced some useful comments on water channel radius of *Pn3m* cubic phase.²⁴

Finally in 2000 Qiu and co-workers²⁵ extended the temperature range until -15 °C and the water content up to 50% (w/w), starting from dry and going to fully hydrated

samples. They spent much effort to ensure equilibrium phase behaviour in all the temperature range, giving particular attention to sub-zero degrees. By this way it was established that phases below 20 °C are metastable, *i.e.* they are not in equilibrium. The phase diagram determined by Qiu et al. is shown in figure 9.

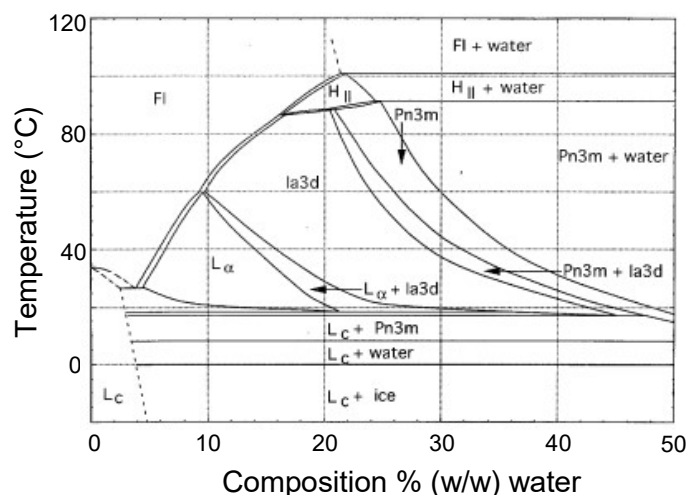


Figure 9. Phase diagram of MO-W system at ambient temperature. Reprinted from *Biomaterials*, 21, H. Qiu, M. Caffrey, The phase diagram of the monoolein/water system: Metastability and equilibrium aspects, 223-224, ©2000, with permission from Elsevier.²⁵

In the lower part of the diagram (low temperatures and low water content) a lamellar crystalline region (L_c) can coexist with more than one polymorph. At low water content, but higher temperature, the L_c phase melts into a fluid isotropic phase (FI) identified in later study as inverse micellar phase (L_2). Further increasing the water content a lamellar liquid crystalline phase (L_α) takes place, while increasing the temperature the L_α phase melts again into the L_2 phase. Bicontinuous cubic phases are shown at higher water contents in the form of two different type of phases, $Ia3d$ (C_G , gyroid type) and $Pn3m$ (D, double diamond type), while, increasing the temperature from bicontinuous cubic phases, a phase transition occurs into an inverse hexagonal phase H_{II} , and into the inverse micellar phase L_2 at the end. A peculiar characteristic of the MO/W diagram (and of monoglyceride/water systems in general) is the liquid crystal + water excess region, where, above a certain percentage of solvent, water and a structured phase can coexist separately, thus indicating the maximum solubilisation capacity of that phase for water. In particular

the bicontinuous cubic phase $Pn3m$ can solubilise more water than H_{II} and L_2 phases (respectively 40%, 25% and 20%).⁹

4.2. *Applications of MO*

As already hinted in the third paragraph, to date MO find useful application in several areas, ranging from pharmaceuticals, food, cosmetics and agriculture. The reasons of this variety of applications can be found in the physicochemical properties of MO, in its high biodegradability and biocompatibility, and in the really significant interest over the years towards its phase behaviour. It has been shown to be a versatile material since it can be included in many different formulations.

Pharmaceutical applications of monoolein attracted great attention since it can be employed as emulsifier, solubilizer, oral/parenteral/periodontal drug delivery, storage system for the protection of macromolecules susceptible to degradation, or as colloidal carrier system. Furthermore, thanks to its amphiphilic nature, MO is able to deliver both lipophilic and hydrophilic drugs.

5. Soft matter and drug delivery

In the last decades considerable amount of research effort has been devoted to the development of new drugs with the aim to improve therapeutics. Such drugs often have an optimum concentration range above which they are toxic and below which they are ineffective, and the rapid clearance from the blood circulation, due to their low molecular weight (usually under 500 Da), leads to drug concentrations below the efficient therapeutic range in a very short time. This unfavourable situation brings to a poor pharmacokinetic and an inappropriate biodistribution of the drug within the organism.

Furthermore, the administration of molecularly dispersed drugs (“free drugs”) is the reason of an indiscriminate volume distribution of the drug itself with unwelcome accumulation in healthy tissues and thus to sometimes severe side effects. Indeed, many agents which have been shown to be highly effective *in vitro* may be relatively toxic, or at best ineffective, when administered *in vivo*.²⁶⁻²⁸ Moreover a considerable

fraction of newly developed drugs has low (or at all) solubility in water and thus it would be useless in parenteral administration.

To date an efficient approach which allows to overcome these problems is the encapsulation of drugs in colloidal systems which can act as nanovectors. Such strategy implies that the biodistribution of the drug will be determined by that of the vector, which will be completely different, mainly because of the size of this delivery device, larger than that of the drug itself. To date, a wide variety of materials have been used to build nanoparticles for drug delivery, ranging from polymers to lipids and also to metals. Some very desirable requirements of such materials are biocompatibility and biodegradability, which should underpin their “digestion” once administered. The drug release from the matrix may occur either via diffusion process (depending upon the geometry, the porosity and the tortuosity of the matrix pores) or by its degradation.²⁷

We will focus particularly on soft materials based nanoparticles, whose components can belong mainly to two molecular classes: lipids (like phospholipids and glycerides) and polymeric compounds.

Up to now the less appealing drug delivery technology belonging to soft matter consists on micellar systems of small surfactant molecules. This tendency is mostly due to their markedly higher CMCs which lead to lower stability of the aggregates at high dilution states (those encountered in a hypothetical parenteral administration) and thus to shorter retention times of the drug.^{26,29}

With respect to small surfactant micelles their polymeric counterpart have much lower CMCs and lower rate of dissociation, features which make them more suitable in drug vectorization.³⁰ Polymeric micelles have dimensions of several tenths of nanometres with quite narrow size distribution. Each block of the block-copolymers, that constitute these micelles, has a specific role, that can be structural, thus guiding the carrier formation, or functional, thus conferring to the aggregate special abilities.²⁶ Some examples of block-copolymers used are poly(caprolactone), poly(aspartate), and poly(lactate).

Close to polymeric micelles are polymersomes, structurally similar to liposomes: their bilayered membrane, enclosing an aqueous compartment, allows them to host both hydrophilic and hydrophobic drugs.

Lipids, if compared with small surfactants and polymers, are the most physiological option for drug delivery thanks to their biocompatibility and biodegradability. Liposomes, hexosomes and cubosomes, already mentioned, are highly appropriate for such use. Their corresponding bulk phases (lamellar, reverse bicontinuous cubic and reverse hexagonal) have, instead, very limited utilization in the pharmaceutical arena because of their high viscosity and difficult handling. As polymersomes, also liquid crystalline nanoparticles can deliver both hydrophilic and hydrophobic drugs and be extremely stable upon dilution.

Liposomes are proven candidates for drug delivery and they have already been successfully marketed: Doxil, Caelys and Myocet are liposomal systems which are used for Kaposi's sarcoma, ovarian cancer and multiple myeloma.³¹ A number of new liposomal formulations are still involved in pre-clinical and clinical trials.

With respect to liposomes at least three others abilities may be ascribed to hexosomes and cubosomes, making them truly attractive devices in drug delivery applications. These are: (i) high mechanical rigidity, (ii) structural stability and (iii) large interfacial surface area per unit of volume. Nevertheless, beyond this promising features liposomes, hexosomes and cubosomes suffer of a serious drawback, which actually is across-the-board to the most of nanoparticles: they need stabilization against the rapid clearance from the bloodstream by plasma proteins, and such stabilization is usually achieved with a brush of neutral hydrophilic flexible polymers. Most of the times these flexible polymers are poly-(ethylene oxide) (PEO) groups protruded outwards of the particles, in the aqueous environment.³² For hexosomes and cubosomes the stabilizer is also strictly necessary for their steric stabilization against aggregation in aqueous environment.

Another type of lipid-based drug delivery system is represented by the solid lipid nanoparticles (SLN). These particles are made by a core of solid lipids (usually glycerides) surrounded by an emulsifier shell that stabilizes the nanocarrier against

aggregation phenomena. SLN can mainly deliver hydrophobic drugs into their lipid cores.

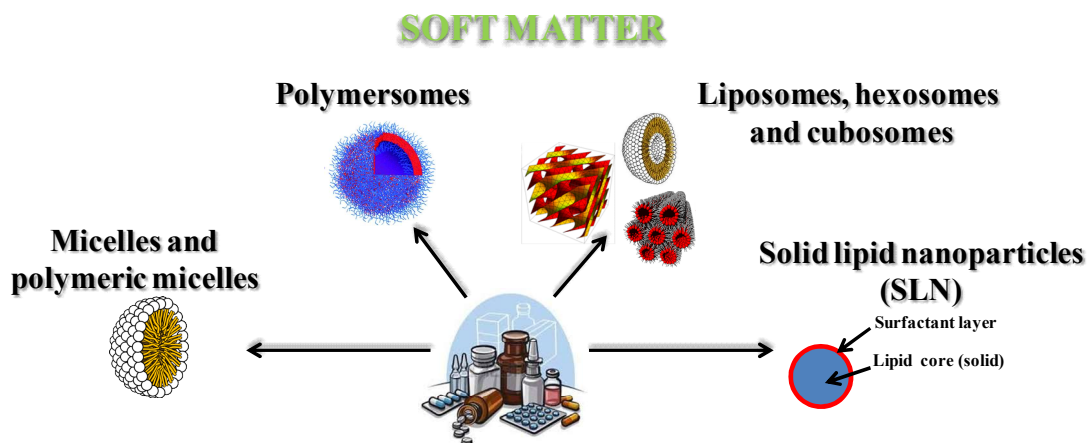


Figure 10. Schematic representation of soft matter nanoparticles for drug delivery.

Hard matter and therapeutic applications

Not only organic and totally biodegradable materials can be employed to produce systems with therapeutic applications. There is a huge research focussed on inorganic nanostructures for therapy, of which magnetic nanoparticles, gold nanoparticles and mesoporous materials constitute the main examples. The presence of a drug is not strictly necessary, for some of these systems, to exert a therapeutic functionality. For instance, the ability of magnetic and gold nanoparticles to produce heat under a specific stimulus (a magnetic field or a laser radiation, respectively) may be useful in cancer treatment by hyperthermia. This is a particular anticancer therapy in which heat is used to induce cell death, when a temperature of at least 42 °C is achieved.¹⁰² Differently, mesoporous materials may be drug loaded by means of functionalization of silanol groups present on their surface. These materials are, in fact, made by silica which was previously templated by supramolecular assemblies of surfactants.¹⁰³

At this point, before exploring the basic concepts of theranostic, some attention should be paid to nanomedicine definition. From the previous discussion emerged that, in the last decades, a great interest was addressed and countless successes were achieved in drug delivery applied nanotechnology. Therefore nanomedicine field received, and still it is receiving, strong contributions from researchers all over the world. It has found large application in the treatment of critical diseases such as cancer, neurodegenerative, inflammatory and autoimmune diseases. To date, a very

challenging area of nanomedicine is the personalized nanomedicine, which calls for the design of nanocarriers that allow for treatment protocols tailored to each specific patient.

6. Theranostic

To face the great incidence of cancer, neurological, and cardiovascular diseases in the world mortality, new therapeutic and diagnostic strategies are needed, strategies which could allow for an early detection and treatment. For this purpose, a new platform of personalized nanomedicine is developing, called theranostic (figure 11). It combines in the same entity therapeutic and diagnostic functions. Within this field are designed new multi-functional nanoparticles in which the therapeutic and the diagnostic agents are integrated, instead to be administrated separately as diagnostic probes and pharmacologically active molecules (situation in which would be necessary to take into account their differences in biodistribution). Indeed, it would be greatly advantageous if the circulating time and the biodistribution of a nanomedicine system could be visualized non-invasively *in vivo* in real time, post-injection, thanks to the employment of an imagine technique deriving from the type of imaging agent used.^{33,34}

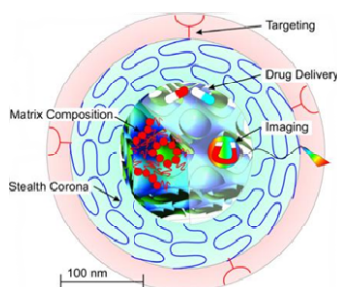


Figure 11. Schematic representation of a nanoparticles for theranostic purposes. Reprinted from Journal of Colloid and Interface Science, 393, X. Mulet, B.J. Boyd, C. J. Drummond, Advances in drug delivery and medical imaging using colloidal lyotropic liquid crystalline dispersions1-20, ©2013, with permission from Elsevier³⁵

6.1. *Imaging modalities*

In the field of molecular imaging the exploitation of specific molecular probes and contrast agents allows for the “visualization, characterization and measurement of biological process at molecular and cellular levels in human or other living system”.³⁶ Referred to soft matter nanoparticles, the imaging agents, as well as the drug, can be included within them or located on their surface.³⁷

The main imaging techniques include: magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), radionuclide-bases imaging (positron emission tomography, PET, and single-photon emission computed tomography, SPECT), and optical imaging (OI). In the following sections each modality will be discussed in more detail.

6.1.1. *Magnetic Resonance Imaging (MRI)*

MRI is widely used in hospitals around the world since it received FDA (Food and Drug Administration) approval for clinical use. At the basis of this technique there is the precession of water hydrogen nuclei if a magnetic field is applied. Following the application of radio frequency pulses and magnetic field gradients, the relaxation process, through which they return to their original aligned state, can be exploited to produce images. The local differences in spin relaxation kinetics along the longitudinal (spin-lattice relaxation time, T_1) and the transverse (spin-spin relaxation time, T_2) planes, of the main magnetic field applied, generate the contrast in the images obtained. Paramagnetic agents allow for an increasing of this contrast by shortening the relaxation parameters T_1 and T_2 . These contrast agents can be tagged onto small molecules, macromolecules or nanoparticles. MRI has good spatial resolution but suffers from low sensitivities. In the area of soft matter nanoparticles, a special role is assigned to the chelates of paramagnetic metals, such as the transition element Mn^{2+} and the lanthanide Gd^{3+} . Examples of biocompatible and biodegradable materials employed in the manufacturing of nanoparticles are polysaccharides and phospholipids. In polysaccharides, due to the presence of many hydroxyl groups it is easy to link Gd^{3+} -chelates through ester bonds.³⁸ Differently, in

liposomes constituted by phospholipids, the water-soluble Gd^{3+} -chelates can be hosted inside the aqueous interior.³⁹

6.1.2. *Radionuclide-based imaging (PET and SPECT)*

Both Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) imply the use of radiotracer and the detection of γ -rays by a camera. Examples of single photons emitter elements are $^{99\text{m}}\text{Tc}$, ^{123}I and ^{111}In , while examples of positron emitter elements are ^{18}F , ^{64}Cu and ^{124}I . These radioisotopes can be used to labelled molecular imaging probes that will be linked to soft matter drug delivery systems. The images acquired have a low background signal and need low amplification since the γ -rays possess energies in the range of megavolt. SPECT radionuclides are simple to prepare, and usually have a longer half-life than those for PET. In literature is plenty of examples of soft matter drug delivery system that exploit SPECT and PET as imaging modality.^{40,41}

6.1.3. *Computed tomography (CT)*

CT is one of the most used clinical diagnostic tools. It measures the absorption of X-rays as they pass through the tissues. The different degree of the attenuation from tissues, which is at the basis of the images production, depends upon the electron density of the tissues themselves.³⁷

CT contrast agents have, typically, low molecular weight and therefore they suffer from a rapid clearance. Thus, nanoparticles containing electron dense elements, such as iodine, have been proposed as CT contrast agents. Liposomes containing iodinated molecules in high concentration have been formulated.⁴²

6.1.4. *Ultrasound imaging (US)*

US is another very common clinical imaging technique. It is cheap, fast, relatively simple and safe. Images are obtained thanks to the back reflection, from internal organs, of sound waves produced by a high frequency sound waves emitting transducer, placed against the skin.

Contrast agents, such as gas-filled microbubbles, are used to enhance the ultrasound signal. Their major drawback is that they dissolve and collapse within seconds after

injection into the blood-stream. Up to now, several strategies have been developed to overcome this problem: microbubbles were encapsulated within soft matter nanoparticles made by surfactants, lipids or polymers.⁴³⁴⁴

6.1.5. *Optical imaging (OI)*

OI is a non-ionizing, non-invasive technique that is acquiring great importance in early diagnosis and in treatment of specific diseases. It utilizes specific probes that, if excited by a radiation, emits light at lower energies than the incident. The possibility offered by this imaging modality to avoid ionizing radiations is of crucial importance, because allows to expose patients to repeated treatments over time. Fluorescence (and phosphorescence) based imaging techniques are very sensitive, versatile and easy to use and, remarkably, the instrumentation required is relatively cheap (if compared to that of some other imaging techniques). The main problem of OI is that, respect to the modalities previous discussed, it suffers from poor penetration capacity, caused by attenuation of photon propagation in living tissue. Moreover, it has a poor signal to noise ratio due to tissues autofluorescence. These not trivial problems can be overcome by using radiations whose wavelength is in the NIR region (650-950 nm). In this “biological window” deeper penetration and minimization of the background effect can be achieved.⁴⁵

Two main kinds of fluorescent imaging may be identified: the first involves imaging due to intrinsically fluorescent (bio)chemical species (NADH in tissues or chlorophyll in all kinds of plants), the second includes imaging based on samples (or cells) made fluorescent for the presence of synthetic fluorescent probes, labels, nanoparticles or nanosensors.

Talking about bioimaging based on synthetic fluorescent probes and nanoparticles, it is possible to differentiate between three techniques: i) the most simple concerns the use of a strong fluorophore or fluorescent nanoparticles internalized into cells with the only aim of make them (or tissues) fluorescents. These fluorophores or nanomaterials do not exhibit affinity for any specific site. ii) The second case is the “targeted bioimaging”: fluorophores and nanomaterials have been properly functionalized with receptors, ligands or antibodies able to recognize their

counterparts so that specific domains or sites are detected. iii) The third technique requires the use of probes and nanomaterials with sensing capability. By this way (bio)chemical species, which are not intrinsically fluorescent, can be imaged.⁴⁶

6.2. *Targeting strategies*

Among the new personalized strategies for an accurate and early-stage detection of diseases there are targeted therapies based on the utilization of passive and active methods of accumulation of the bioactive molecules (drugs) in the sites/targets of interest. The developing of such approaches should promote an enhanced intratumoral drugs concentration, with a consequent limited toxicity to healthy tissues.

6.2.1. *Passive targeting*

The volume distribution throughout the body of a molecularly dispersed drug is absolutely indiscriminate between healthy and ill tissues. Differently, as already mentioned in the fifth paragraph, a drug encapsulated in a nanovector could undergo accumulation in the site of interest. Focussing on cancer disease, the first feature of nanoparticles that confers them targeting abilities is their size. An ideal window of dimensions can be defined by setting the lower size threshold as the minimum size required to avoid a rapid clearance from the kidney glomerula, which is 5-6 nm. An upper size boundary is harder to specify, but it is mostly determined by the need to avoid a rapid elimination by the RES (Reticulo Endothelial System). The RES is part of the immune system, and includes phagocytic cells like monocytes and macrophages that are localized in the spleen, lymph nodes, and Kupffer cells in the liver. Specifically, the smaller the nanomaterials, the harder their elimination by the RES. In addition, according to the Enhanced and Permeability Retention (EPR) effect, macromolecules and nanoparticles are able to accumulate in tumour tissues (at concentrations five to ten times higher than in normal tissue within 1–2 days) because of the great permeability of their blood vessels: the altered angiogenesis of a tumour mass leads to non-mature, tortuous, and (most important) fenestrated vascularisation. These gaps can space from 380 to 780 nm depending on the type of

tumour, and, beyond the promoted accumulation in tumour tissues, they lead to an increased retention time of macromolecules or nanoparticles once sequestered within the tumour, as consequence of a more difficult extracellular diffusion. To date, most drug delivery systems for cancer are being designed based on these principles.^{35,47,48}

6.2.2. *Active targeting*

Targeting cancer cells by means the EPR effect is not feasible in all cases because the degree of altered angiogenesis can vary with tumour type and status. Active targeting is the alternative path to overcoming these limitations. This strategy provides for the employment of targeting moieties, attached onto the nanoparticles surface, which can induce receptor-mediated uptake of nanoparticles and then drug release right inside the target tissue. To achieve a high efficiency of binding and internalization of the colloidal dispersions, receptors should be expressed exclusively (or at least mostly) on target cells with respect to normal ones.

When designing the targeting moiety of a nanoparticle several factors need to be taken into account. Some of these include the configuration of the targeting moieties, where and how they are anchored to the nanovector, their density for unit of surface area, and their influence on the stability of the colloidal system.

Antibodies, peptides, aptamers and small molecules that interact with receptors are commonly attached to nanomaterials for active targeting.

Antibody-based targeting exploits the recognition mechanism between an antigen and its antibody to trigger the nanocarrier to the site of interest. In the last decades the efficiency of this targeting strategy has been widely demonstrated and chemical modifications of monoclonal antibodies (mAbs) have advanced their clinical utility.

Antibodies are often used as their fragments, however the use of the whole antibody with the presence of its two binding sites gives rise to a higher binding activity. A further advantage of intact antibodies is their high stability during long-term storage.^{49,50}

Wei and Gao *et al.* developed multifunctional polymer vesicles formed by carboxy-terminated poly(-lactic-co-glycolic, PLGA) acid, loaded with magnetic nanoparticles

as imaging agent and with docetaxel as chemotherapy agent. They decorated PLGA-based polymeric nanoparticles with prostate stem cell antigen's (PSCA, over-expressed in prostate cancer tissues) antibody as the targeting moiety. Nanoparticles designed in such a way facilitated significantly the specific delivery of docetaxel to prostate tumor cells.⁵¹

Zhai *et al.* actively targeted phytantriol-based cubosomes and hexosomes with epidermal growth factor receptor (EGFR) antigen binding fragment, via the maleimide-thiol reaction.⁵² They observed the retention of the ligand binding affinities to the EGFR target, and the preservation of the size and of the internal lipid nanostructure of the particles.

In spite of the huge effort spent on monoclonal antibodies development they still show some limitations: they are large and complex molecules which require a noteworthy engineering at molecular level to be effective; they need expensive manufacturing and from batch to batch exist significant variations that limit their efficiency as targeting molecules.

Peptides and antibody fragments have been developed to overcome some of these shortcomings and are now under clinical development. Due to their small size, low immunogenicity, ease of manufacture at small costs, and excellent quality control, peptides are very attractive as targeting molecules. Nevertheless they can show some drawbacks too, such as low target affinity (with respect to antibodies) and susceptibility to proteolytic cleavage.⁵³

Beyond antibodies and peptides much effort was spent to design targeting agents like aptamers. They are small nucleic acid ligands (single stranded DNA, RNA or unnatural oligonucleotides) able to fold into unique structures that bind specific targets with high affinity and specificity. Furthermore they are non-immunogenic and easy to isolate.

Aptamers can bind a wide variety of targets, such as intracellular proteins, transmembrane proteins, soluble proteins and carbohydrates, and some of them have also been developed to bind specifically to receptors on cancer cells.

Since the smaller the size of the species the more attractive will be its use as targeting agent, small molecules such as folic acid and carbohydrates are extremely suitable for this task.

Folic acid has been widely studied as targeting moiety. It is a water-soluble vitamin (B6) essential in humans for rapid cell division and growth, especially during embryonic development. Folate receptors are homogeneously overexpressed in many cancer cells and thus they are able to interact with high affinity with folate acid conjugates present onto nanocarriers surface mediating their cellular uptake by endocytosis.

Lectins are carbohydrate-binding proteins, macromolecules that are highly specific for sugar moieties (lactose, galactose, mannose). Their presence on cell surface membrane represents an interesting opportunity for targeted delivery of nanocarriers. However, to compensate for the weak binding affinity of carbohydrates, multiple or multivalent molecules should be conjugated to the nanocarrier itself.

7. Cubosomes and hexosomes: a state-of-art

Some lipids (or lipid mixtures) are able to form lyotropic liquid crystalline structures thermodynamically stable in the presence of excess of water. Hexosomes and cubosomes are two of the products of the retention of such liquid crystalline structures into nanoparticles. Respectively, they consist of 2D inverse hexagonal phase or 3D inverse bicontinuous cubic phase aqueous dispersions. As specified earlier, the non-dispersed phases whose they belong to, are very viscous and therefore difficult to handle, for example, in intravenous administration.^{35,54}

Differently, these non-lamellar inverse mesophase possess low viscosity, much more suitable for pharmaceutical applications. Compared to liposomes, hexosomes and cubosomes show several advantages. Some of them are already reported in paragraph 5, but at least another one deserves to be noticed: their internal nanostructure permits the loading of different type of molecules, including hydrophobic, hydrophilic, and amphiphilic ones (similarly to liposomes) but these

agents can be loaded in hexosomes and cubosomes to higher extent because of the higher hydrophobic portion per unit of volume with respect to liposomes. This is a key advantage for the solubilisation of poorly water-soluble drugs in colloidal formulation. Despite these desirable features, drug delivery investigation on hexosomes and cubosomes are still in their infancy if compared with liposomes.

In 1979 Patton and Carey⁵⁵ observed cubosomes for the first time during fats (triglycerides) digestion. In that case the cubic phase was stabilized in presence of micellar solutions of bile salts.

They monitored a fat digestion by means of visible light microscopy, demonstrating that the final product of the digestion sequence is a viscous isotropic (non-birefringent) phase. This phase was hypothesized to be cubic, and composed by a MO/W mixture stabilized by a lamellar envelope of bile salt and monoglycerides. Larsson in 1989 clarified the structure of those nanoparticles as bicontinuous, and in 1996 and 1997 his group first published hexosomes and cubosomes cryo-TEM images.^{15,16} Later, in 1999, Baglioni *et al.* used atomic force microscope to obtain the first AFM images of such colloidal dispersions,⁵⁶ while, investigating the ¹³C NMR relaxation rates of the various MO carbon atoms, Monduzzi *et al.* found that cubosomes retain the lipid organization and the dynamic properties of the original bulk (non-dispersed) phases from which they belong to.¹⁴ Monoolein, phytantriol, monolinolein and monoleiadin is a limited list of the most common lipids with propensity to generate lyotropic non-lamellar liquid crystalline phases.⁵⁷ Anyway, it has been taken into account that several variables can determine the arrangement adopted by these amphiphiles. Temperature, pressure, purity of lipids used, pH and ionic strength may affect the phase behaviour of these systems. The two most common used lipids for hexosomes and cubosomes production are MO and phytantriol (PHYT), and their phase diagrams were very well explored.^{25,58} It should be noted that the PHYT phase diagram has shown to be sensibly compromised from small level of impurities in the lipid.⁵⁹

Preparation methods of hexosomes and cubosomes are generally based on a high-energy emulsification procedure (ultrasonication, high-pressure homogenization or

high-speed shearing) and require the presence of a stabilizer. Basically, these methods can belong to the bottom-up class or to the top-down one.

Bottom-up approaches involve the dilution followed by homogenization of the mixture of stabilizer and lipid in water. Spicer *et al.* in 2001⁶⁰ developed a novel process for producing cubosomes. The key of this procedure is the inclusion of a hydrotrope (molecule with amphiphilic character but incapable of displaying surfactant phase behaviour), the ethanol, that exhibits a “salting-in” behaviour by increasing the solubility of lipids otherwise insoluble in water. Exploiting both these approaches (with or without hydrotrope) the nanoparticles are formed by nucleation in the aqueous solution.^{61–63} It is important to observe that the presence of a hydrotrope, besides being useful for the production of attractive nanoparticles dispersion, may also represent a problem in more-than-one-component system, since the single components may have different solubility in the hydrotrope. Furthermore, the presence of a hydrotrope in the final preparation may cause *in vivo* issues such as toxicity.

First step within the top-down approach is the hydration of the amphiphile. By this way its bulk phase is produced, and then significant levels of energy should be applied to break it up. The formation of a stable, sub-micron, dispersion of liquid crystalline phases is thus achieved with the help of the omnipresent stabilizer agent. In principle, the top-down method allows for the entrapment of hydrophilic drugs (oligonucleotides, peptides, and proteins) that, otherwise, will be hardly introduced throughout the bottom-up approach. An alternative path for introducing water-soluble additives (such as DNA or RNA), is the addition of charges in the lyotropic liquid crystalline nanoparticles, by the incorporation of ionized lipids. For instance, negative or positive charge can be added to nanoparticles through the use of phosphatidylserine or dioleoyltrimethylammonium propane (DOTAP) respectively.⁶⁴

The stabilization of discrete colloidal nanoparticles like hexosomes and cubosomes, with the aim of avoiding their aggregation in excess of water, and thus to promote their long-term stability, can be achieved by electrostatic or steric effect, or a combination of both. Although several stabilizers were investigated over these last

decades, which are able to accomplish this role, non-ionic block copolymers are the most used to impart steric stabilization even at low concentration.

In principle, two fundamental features should be considered for the choice of the stabilizing agent: the volume and density of the stabilization layer and its adhesion strength onto the nanoparticle surface. The most diffuse molecular moiety chosen for this function is poly(ethylene glycol) or poly(ethylene oxide) (PEO), and the poloxamer 407 (PEO₉₉–PPO₆₇–PEO₉₉), commercially known as Pluronic F127 is the most diffuse block copolymer for this purpose.^{16,65,66,67}

DSPE-PEG2000,^{68,69} β -casein,⁷⁰ hydroxypropyl methyl cellulose acetate succinate and poly(ethylene oxide)⁷¹ stearate were found to be efficient in stabilizing hexosomes or cubosomes.

A key role of the stabilization layer is to create a stealth barrier able to reduce undesirable biological interactions with immune system, responsible for the clearance of the nanoparticles. The PEO barrier was discovered to be truly effective in reducing the absorption by opsonins (serum protein) by enabling the nanoparticles to evade phagocytosis.⁷² An explanation of such behaviour is that the more hydrophilic will be the exposed surface of nanoparticles, the lower will be the opsonisation rate.⁷³

Despite the capability of cubosomes and hexosomes of delivering both therapeutic and diagnostic agents,⁷⁴ the scenario of these lyotropic liquid crystalline nanoparticles is still mostly focussed on their characterisation, while investigations of their multi-functionality are fully inadequate if compared with those pertaining to liposomes. Nevertheless, there are two commercial ventures, Camurus® and Phosphagenics®, specialised in the development of lyotropic liquid crystalline phases for bioactive delivery.

The bioactive compound can be loaded in the drug delivery carrier at different stages of the preparation, and its localisation will depend on its inherent properties, specifically, on its lipophilicity, amphiphilicity, and size. Depending on these properties the drug may be located in the water domain, in the lipidic domain or at the interface between them.

The hydrophilic drugs are hardly loaded in a lyotropic liquid crystalline dispersed matrix without considerable modification of the matrix composition itself. Despite these complications, Kwon and co-workers in 2010 made attempts in this direction, by preparing cubosome formulations for the dermal administration of hinokitiol and water soluble extracts of Korean barberry.^{75,76}

Because of their difficult administration, poorly water-soluble drugs reached much more attention from the drug delivery audience. An interesting example is curcumin, a potent therapeutic molecule with antitumor activity, poor bioavailability, pH sensitivity, and poor tissue absorption,⁷⁷ that, similarly to other lipophilic molecules of pharmaceutical interest, was successfully encapsulated in hexosomes and cubosomes.⁷⁸ Also oral and ophthalmic administration have been explored for drug loaded cubosomes.^{79–81}

When using a bioactive molecule to be located in the lipid bilayer of liquid crystalline nanoparticles, it should be taken into account that these molecules may somewhat alter the bilayer curvature and so, to some extent, induce a phase change.⁸²

Loading of proteins and peptides into liquid crystalline phases is a goal of great interest in therapeutic and targeting applications. The main problem when using these macromolecules is their relatively fragile nature (denaturation events are just around the corner), which should be considered during preparation and characterization of colloidal dispersions. However, considerable effort has been spent in this direction. Angelova and co-workers studied the interaction of PEGylated hexosomes with cationic proteins, demonstrating that, depending on concentration and amphiphilicity, proteins may lead to either preservation or dramatic changes in the inner structure of the hexosomes. From the same group in-depth investigations were, and still are, focussed on the arena of proteocubosomes.^{83–86}

A crucial part in the design of a lyotropic liquid crystalline nanoparticles, if a theranostic use is expected, is their association with a tracking element, useful to provide spatial and temporal information about the localisation of the colloidal dispersions (both *in vitro* and *in vivo*), and to, eventually, allow for an early

diagnosis of a disease. As reported in the paragraph 6.1 several techniques, and thus several types of imaging agents, are available for this purpose. MRI is a non-invasive technique commonly exploited in the clinic, and several times MRI contrast agents were loaded into the internal nanostructures of hexosome and cubosome nanoparticles. Muir *et al.* incorporated a nitroxide lipid into the matrix of liquid crystal nanoparticles, discovering a great enhancement of relaxivity when a $Pn3m$ space group cubic phase is present, if compared with the hexagonal phase that appeared at high nitroxide loadings.⁸⁷ Liu and co-workers incorporate Gd^{3+} -oleate into cubosomes to assess their performance.⁸⁸

Computed tomography (SPECT/CT) is another attractive imaging technique. Nilsson *et al.* developed PHYT/oleic acid (OA) hexosome radiolabelled via surface chelation of technetium-99m (^{99m}Tc).⁸⁹

With respect to the imaging techniques discussed above, the field of optical imaging by means fluorescent lyotropic liquid crystalline nanoparticles is still in its early life. Some examples of such colloidal system, probed with fluorophore, can be found in the recent literature. Aleandri *et al.* developed cubosomes functionalized with biotin as target agent and loaded with paclitaxel. Their cubosomes were labelled with a hydrophobic fluorescent lipid to follow their cellular uptake in HeLa cells.⁹⁰ In 2012 Wu *et al.* designed odorranalectin targeted cubosomes for brain delivery, and labelled them with coumarin-6, a lipophilic fluorophore, to investigate their brain distribution *in vivo*.⁹¹ Other fluorescent cubosome dispersions were prepared, but none of them were used with theranostic purposes.⁹²⁻⁹⁴ A special mention deserves an innovative hybrid formulation constituted by hydrophobic Fe_3O_4 nanoparticles entrapped within the lipid matrix of cubosomes, the so-called magnetocubosomes. Remarkably authors found that these biocompatible nanoparticles are responsive to a low frequency alternating magnetic field, which allows a faster release of model drugs confined in the cubic phase.⁹⁵

The knowledge of the cytotoxicity of non-lamellar lipid dispersions is a critical point when developing these systems for pharmaceutical applications. This aspect may be influenced by size, concentration, composition (PHYT-based or MO-based nanoparticles), internal phase (hexosomes and emulsified microemulsions seem to

induce less cytotoxic activity) and it is strongly dependent on proliferation and embryonic origin of the cells chosen for testing. Haemolytic activity and interaction with blood component (plasma) were also examined.^{96–100}

In the light of all these knowledge on lyotropic liquid crystalline nanoparticles, collected in decades of dedication from researchers all over the world, we developed our colloidal dispersions of MO-based cubosomes and hexosomes. A little amount of oleic acid (OA) was employed as additive to induce a phase transition, at room temperature, from inverse bicontinuous cubic phase ($Pn3m$) to inverse hexagonal phase (H_{II}) for hexosomes formation. As stabilizer we chose Pluronic F108 instead of the widespread Pluronic F127 (see chemical structure in figure 12). It possess a number of hydrophilic moieties higher than F127, and a hydrophobic one lower than F127, thus, its HLB value is higher than that of F127. Its stabilization power was found to be superior to F127 right for this reason.¹⁰¹

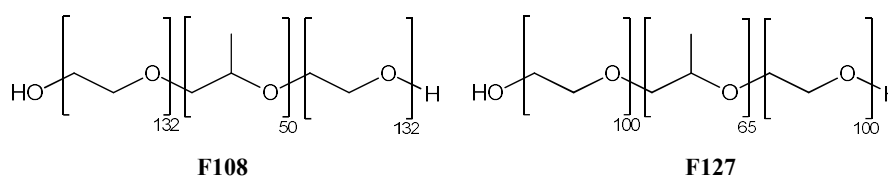


Figure 12. Chemical structure of Pluronics.

Thinking about possible applications of hexosomes and cubosomes in the antitumor therapy we loaded them with several antineoplastic drugs (structures are reported in figure 13) and we labelled them with fluorescent molecules. The latter were, sometimes, modified *via* synthetic approaches with two different goals: i) for a better and less invasive encapsulation within the lipid bilayer (which is the case of the modification of fluorescein, dansyl and cyanine with long hydrocarbon chains), and ii) to avoid, as much as possible, the interactions between the fluorophore and the lipid matrix (which is the case of Pluronic F108-D and F108-R).

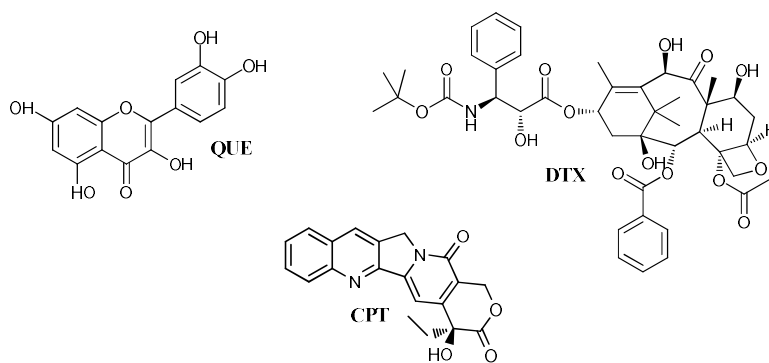


Figure 13. Chemical structure of antitumor drugs used: quercetin (QUE), camptothecin (CPT) and docetaxel (DTX).

Conclusions

We explored the potential of MO-based non-lamellar liquid crystalline nanoparticles, namely cubosomes and hexosomes, as theranostic tools in cancer treatments.

We proved that they allow for the encapsulation of antineoplastic poorly water-soluble drugs (quercetin, camptothecin, and docetaxel) and that the presence of such drugs within the lipid bilayer did not affect the nanostructural and morphological characteristics of the nanoparticles. Furthermore, we observed the superior cytotoxic ability of docetaxel towards HeLa cells when embedded in the cubosomes matrix with respect to its use as a free drug.

Similarly, we demonstrated that these nanocarriers can be simultaneously coupled with fluorescent imaging agents still preserving the original physicochemical features of pristine cubosomes and hexosomes nanoparticles. In addition, also the photophysical properties of the imaging agents, critical for the fluorescence optical imaging, are retained in the colloidal systems. Sometimes, these properties were extremely helpful for understanding the fluorophore localization in the nanoparticle (solvatochromism).

In the frame of possible applications in cancer treatments, we made cubosomes and hexosomes actively targeted by decorating their surface with folic acid. Thanks to this modification the uptake of targeted cubosomes in HeLa cells considerably increases with respect to pristine cubosomes.

A detailed study of changes occurring in HeLa cells, when exposed to treatment with MO-based cubosomes, was conducted. Such investigation is of primary importance within the context of biomedical applications. It was observed that the uptake of cubosome formulations induced modification of the cell lipid profile, lipid droplet accumulation, mitochondrial hyperpolarization and mitochondrial ROS generation. Remarkably, these modifications did not cause significant toxicity in HeLa cells.

The first proof-of-principle for *in vivo* fluorescence optical imaging application using MO-based cubosomes in a healthy mouse animal model was here successfully shown. With the aid of a modified cyanine, we obtained important results about the

biodistribution *in vivo* of cubosomes upon intravenous injection. In particular, we noted that the fluorescent cubosomes were rapidly (within six hours) taken up by the liver. Furthermore, haemolytic analysis revealed that, at the concentration used for the *in vivo* experiment (about 50 µg/mL of monoolein), the cubosomes formulation did not induce lysis of human erythrocytes.

All these results strongly encourage further investigation of non-lamellar lipid liquid crystalline nanoparticles in thereanostic nanomedicine, and for pharmaceutical applications in general.

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Drug-Loaded Fluorescent Cubosomes: Versatile Nanoparticles for Potential Theranostic Applications.

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Drug-Loaded Fluorescent Cubosomes: Versatile Nanoparticles for Potential Theranostic Applications

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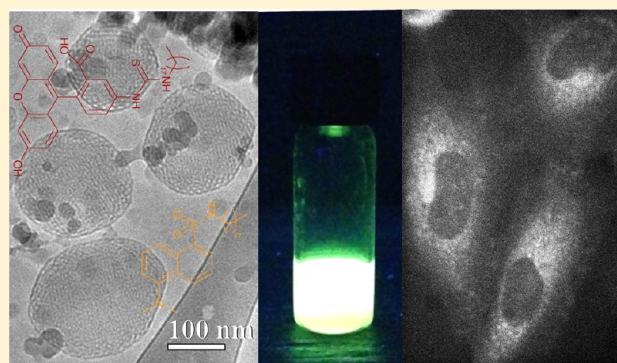
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Supporting Information

ABSTRACT: In this work, monoolein-based cubosomes were doped with two fluorescent probes, namely, fluorescein and dansyl, properly modified with a hydrocarbon chain to increase their encapsulation efficiency within the monoolein palisade. The same nanocarriers were also loaded with quercetin, a hydrophobic molecule with potential anticancer activity. Particularly, the cubosomes doped with the modified fluorescein probe were successfully exploited for single living cell imaging. The physicochemical and photophysical characterizations reported here, along with the well-known ability of cubosomes in hosting molecules with pharmaceutical interest, strongly encourage the use of these innovative fluorescent nanocarriers for theranostic purposes.



■ INTRODUCTION

Application of nanotechnology to medicine (nanomedicine) holds promise to deeply reform the diagnostic and therapeutic tools available in clinical practice.^{1,2} Indeed, nanotechnology offers a unique possibility to improve both imaging and drug delivery techniques. To reach this goal, a myriad of nanocarriers, belonging to soft and hard matter, have been engineered and tested for their physicochemical, pharmaceutical, and cytotoxic features. Relevant examples include liposome formulations,^{3–6} polymeric,^{7–11} gold, or iron oxide nanoparticles, as well as dendrimers and quantum dots.¹² Nanoparticles were originally developed to transport separately pharmaceutically active or imaging agents to the pathological sites with therapeutic or diagnostic scopes. Recently, both of these aspects were merged into single platforms, thus forming theranostic nanomedicine, an emerging cross-discipline, where chemistry, biology, pharmacy, and medicine meet.¹³ Therefore, within the same dosage, theranostic nanoparticles may (actively or passively) deliver both drugs and imaging probes to the site of disease, overcoming the differences in biodistribution and selectivity of these two distinct entities and reducing side effects.^{14–20} Although many kinds of nanoparticles have been proposed for theranostic applications,^{21–26} cubosomes have until now been neglected. Cubosomes are lipid-based nanoparticles sterically stabilized by pluronics^{27–30} that can be envisaged as aqueous dispersions of bicontinuous cubic liquid-crystalline phases. Briefly, they are constituted of curved, triply

periodic non-intersecting bilayers folded on an infinite periodic minimal surface having cubic symmetry and organized to form two disjointed continuous water channels.³¹ Cubosomes, which can be regarded as the nonlamellar analogue of liposomes, show high mechanical rigidity and structural stability and may be loaded with both hydrophobic and hydrophilic drugs (including proteins),³² taking advantage also of their huge interfacial surface area per unit of volume. Indeed, with respect to liposomes, cubosomes possess a greater hydrophobic volume that, in principle, allows encapsulating higher amounts of poor water-soluble drugs. Moreover, they are mostly prepared using monoolein, a monoglyceride that can be easily biodegraded by lipases.^{33,34} Due to these features, cubosomes are often suggested as drug delivery systems useful in parenteral administration (i.e., all routes of drug administration different from mouth).³² However, they possess further appealing features that could be exploited in theranostic nanomedicine. Indeed, they are sufficiently large to avoid a rapid clearance from the bloodstream while their nanostructure is enclosed within a PEG corona that, similarly to stealth liposomes, should protect cubosomes against opsonisation (opsonins are proteins that bind to foreign particles, allowing phagocytic cell recognition) and consequent clearance from the bloodstream.¹²

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With the aim of exploring the potentiality of aqueous cubosome dispersions for theranostic applications, in this work, we decorated cubosomes with two modified fluorophores and loaded this kind of nanoparticle with a therapeutic dose of quercetin (3,3',4',5,7-pentahydroxyflavone). Two commercially available fluorophores, namely, fluorescein and dansyl, were functionalized with a C18 hydrocarbon chain to give, respectively, F1 and F2 (Figure 1). Particularly, quercetin is a

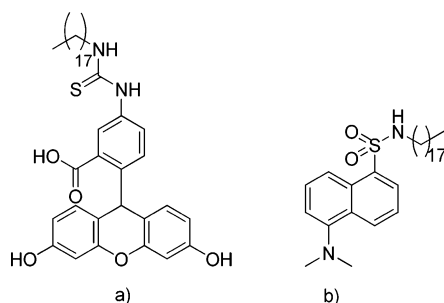


Figure 1. Fluorophores F1 (a) and F2 (b).

polyphenolic compound widely distributed in plants that, similarly to many other hydrophobic drugs, has a limited clinical use due to its poor water solubility with a consequent very low bioavailability. Quercetin exerts multiple pharmacological effects. Among these, a potent *in vivo* antioxidant activity, induction of apoptosis, modulation of cell cycle, antimutagenesis, inhibition of angiogenesis, and anti-inflammatory effects are well-recognized. Moreover, quercetin is considered to be an anticancer drug since some studies have shown its inhibitory effects on tumor growth.^{35–37}

MATERIALS AND METHODS

Chemicals. Monoolein (MO, 1-monooleoylglycerol, RYLO MG 19 PHARMA, glycerol monooleate; 98.1 wt %) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. Pluronic F108 (PEO₁₃₂–PPO₅₀–PEO₁₃₂), quercetin, fluorescein isothiocyanate isomer I (90%), dansyl chloride (≥95%), octadecylamine (≥99%), sodium sulfate (≥99.0%), potassium carbonate (99%), acetonitrile (≥99.9%), diethyl ether (≥99.5%), dichloromethane (≥99%), and tetrahydrofuran (≥99%) were purchased from Sigma-Aldrich. Distilled water passed through a Milli-Q water purification system (Millipore) was used to prepare the samples.

Sample Preparation. Cubic phases were prepared by weighing the appropriate amounts of components (MO, water, quercetin, and fluorophore) into glass tubes ($L \approx 0.5$ cm) that were immediately sealed, centrifuged at 2300 rpm at 25 °C, and allowed to equilibrate at 25 °C for 1 week. Cubosomes, empty or doped with the fluorophore molecule, were prepared by dispersing the appropriate amount of MO in a solution of Pluronic F108 using an ultrasonic processor UP100H by Dr. Hielscher, cycle 0.9, amplitude 90%, for 10 min. To obtain fluorescent cubosomes, the fluorophore was dispersed in the melted monoolein with the help of an ultrasonic bath before dispersion in Pluronic F108. The sample volume was usually 4 mL with approximately 96.4 wt % of water, 3.3 wt % of MO, and 0.3 wt % of Pluronic F108. The percentage of the fluorophore was 2.5×10^{-3} and 2.8×10^{-3} wt % for F1 and F2, respectively. The same procedure was followed when the fluorescent cubosomes were also loaded with quercetin. The percentage of added quercetin was 6.4×10^{-6} wt %.

Light Microscopy. The homogeneous liquid-crystalline phases were observed by optical microscopy (Zeiss Axioplan II) in polarized light at 25 °C.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Vitrified specimens were prepared in a controlled environment vitrification system (CEVS) at 25 °C and 100% relative humidity. A

drop of the sample was placed on a perforated carbon film-coated copper grid, blotted with filter paper, and plunged into liquid ethane at its freezing point. The vitrified specimens were transferred to a 626 Gatan cryo-holder and observed at 120 kV acceleration voltage in an FEI Tecnai T12 G2 transmission electron microscope at about −175 °C in the low-dose imaging mode to minimize electron-beam radiation damage. Images were digitally recorded with a Gatan US1000 high-resolution CCD camera.

Ultrafiltration and Dialysis. Ultrafiltrated cubosomes were carried out by means of Amicon Ultra 0.5 mL centrifugal filters (RC 100 kDa, 7000 rpm/10 min). After quercetin was loaded, cubosome dispersion was purified from the non-encapsulated drug by dialysis as follows: 2 mL was loaded into a Spectra/Por membrane (12–14 kDa MW cutoff; Spectrum Laboratories Inc., USA) and dialyzed against water (1000 mL) for 2 h (by replacing the water after 1 h) at 5 °C. Drug loading efficiency ($E\%$), expressed as percentage of the drug amount initially used, was determined by UV–vis spectroscopy after disruption of cubosomes with methanol. Quercetin content was quantified at 373 nm using a Thermo Nicolet Evolution 300 UV–vis spectrophotometer.

Dynamic Light Scattering (DLS). Particle size and ζ -potential determinations of the nanoparticles were performed with a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK) at a temperature of 25 ± 0.1 °C. Samples were backscattered by a 4 mW He–Ne laser (operating at a wavelength of 633 nm) at an angle of 173°. Diluted samples (1:50) were housed in disposable polystyrene cuvettes of 1 cm optical path length with water as solvent. At least two independent samples were taken, each of which was measured from three to five times. The width of DLS hydrodynamic diameter distribution is indicated by PDI (polydispersion index).

Photophysical Measurements. Cubosome solutions were diluted with Milli-Q water and ultrafiltered before performing the photophysical measurements. The emission and excitation spectra were recorded with an Edinburgh FLS920 equipped photomultiplier Hamamatsu R928P. The same instrument connected to a PCS900 PC card was used for the TCSPC (time-correlated single photon counting) experiments (excitation laser $\lambda = 405$ nm). Fluorescence intensities were corrected for inner filter effects according to standard methods.³⁸ All fluorescence anisotropy measurements were performed on an Edinburgh FLS920 equipped with Glan-Thompson polarizers. Anisotropy measurements were collected using an L-format configuration, and all data were corrected for polarization bias using the G-factor. Low-volume quartz cuvettes with an optical path length of 1.0×0.2 cm were used to reduce the scattering effects from the cubosome solutions.

Small-Angle X-ray Scattering (SAXS) Experiments. Small-angle X-ray scattering was recorded with a S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). Cu K α radiation of wavelength 1.542 Å was provided by a GeniX X-ray generator, operating at 50 kV and 1 mA. A 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels of width 54.0 μ m was used for detection of scattered X-rays in the small-angle region. The working q range ($\text{\AA}^{-1}$) was $0.003 \leq q \leq 0.6$, where $q = 4\pi \sin(\theta) \lambda^{-1}$ is the scattering wave vector. A stainless steel sample holder with thin polymeric sheet (Bratfolie, Kalle) windows enclosing a few milligrams of the sample was used for cubic phases, while thin-walled 2 mm glass capillaries were used for cubosome dispersions. The diffraction patterns for cubic phases and cubosomes were recorded, respectively, for 30 min and 7 h. The lattice parameter a of the cubic phases was determined using the relation $a = d(h_2 + k_2 + l_2)^{1/2}$ from linear fits of the plots of $1/d$ versus $(h_2 + k_2 + l_2)^{1/2}$, where $d = 2\pi/q$ (q is the measured peak position) and h , k , and l are the Miller indices. Water channel radii were calculated using the relation $R_w = [(A_0/2\pi\chi)^{1/2}a] - L$, where L is the lipid length value (17 Å), a is the lattice parameter obtained from the SAXS analysis, and A_0 and χ are the surface area and the Euler characteristic of the IPMS geometries ($Ia3d$, $A_0 = 3.091$, $\chi = -8$; $Pn3m$, $A_0 = 1.919$, $\chi = -2$). To minimize scattering from air, the camera volume was kept under vacuum during the measurements. Silver behenate ($\text{CH}_3-(\text{CH}_2)_{20}-\text{COOAg}$) with a d

Table 1. Photophysical Properties of F1 and F2 in the Bulk, Loaded Inside the Cubosomes (Aqueous Solution, pH 7 after Ultrafiltration), in Basic Aqueous Solution (F1), and in Ethanol Solution (F2)

		epsilon ($\text{M}^{-1} \text{cm}^{-1}$)	exc ^a ($\lambda_{\text{max/nm}}$)	em ^b ($\lambda_{\text{max/nm}}$)	r ^c	τ^d (ns)
F1	bulk cubic phase		485	544	0.2	3.9
	cubosomes (aqueous dispersion)		484	517	0.3	3.8
	H ₂ O buffered pH 9	89000 ($\lambda = 500 \text{ nm}$)	494	517	0.0	3.0
F2	bulk cubic phase		333	500	0.1	12.7
	cubosomes (aqueous dispersion)		336	520	0.1	12.8
	EtOH	4200 ($\lambda = 340 \text{ nm}$)	333	507	0.0	13.3

^aExcitation spectra (F1, $\lambda_{\text{em}} = 520 \text{ nm}$; F2, $\lambda_{\text{em}} = 510 \text{ nm}$). ^bEmission spectra (F1, $\lambda_{\text{exc}} = 470 \text{ nm}$; F2, $\lambda_{\text{exc}} = 340 \text{ nm}$). ^cEmission anisotropy (F1, $\lambda_{\text{exc}} = 470 \text{ nm}$; F2, $\lambda_{\text{exc}} = 340 \text{ nm}$). ^dAverage lifetime (F1 and F2, laser source $\lambda_{\text{exc}} = 405 \text{ nm}$).

spacing value of 58.38 Å was used as a standard to calibrate the angular scale of the measured intensity.

Cell Cultures and Treatment. Mouse 3T3 fibroblasts (ATCC collection) were grown at 37 °C in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL⁻¹), and streptomycin (100 µg mL⁻¹) (Invitrogen) in a 5% CO₂ incubator at 37 °C. Cells were grown in 35 mm dishes, and experiments were carried out 2 days after seeding when cells had reached 80–90% confluence. Nanoparticle formulations were added to the cells at a concentration of 1:1000 (2 µL of sample in 2 mL of medium) and incubated at 37 °C for 3 h. For live cell imaging, the sample suspension was replaced with fresh serum-free medium before the imaging session.

Fluorescence Microscopy. Fluorescent microscopy observations of the cells were made using a Zeiss (Axioskop) upright microscope (Zeiss, Oberkochen, Germany) equipped with a filter system of 470 ± 20 nm for excitation and 535 ± 40 nm for emission, a 40×/0.75 NA water immersion objective, and a HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome cooled CCD camera (QICAM, Qimaging, Canada) and analyzed with Image Pro Plus software (Media Cybernetics, Silver Springs, MD). The fluorescence images of cubosomes in a diluted aqueous solution mixed with ethylene glycol (see Figure 4) were obtained with an inverted microscope (Olympus IX71) equipped with a Basler CCD color camera (SC A640-70gs) and using as excitation light the 488 nm laser emission of an Ar⁺ ion laser (Melles Griot, IMA1-Multiwavelength, 43 series ion laser).

RESULTS AND DISCUSSION

Here, we explored monoolein (MO)-based cubosomes doped with two hydrophobically modified fluorophores and loaded with quercetin as nanoparticles for theranostic applications. First, the two fluorophores F1 and F2 (see Figure 1) were synthesized by refluxing octadecylamine with fluorescein isothiocyanate isomer I in THF (in the case of F1) or with dansyl chloride in MeCN in the presence of K₂CO₃ (in the case of F2), to give the desired thiourea (F1) or sulfonamide (F2) in 35.5 and 74.5% yields, respectively.

Indeed, hydrophobic derivatization is a common approach to improve encapsulation of different probes within lipid nanostructures.³⁸ Here, it should be noticed that fluorescein cannot be easily dispersed within cubosomes. Actually, through an optical microscope, we observed crystals of this fluorophore in bulk cubic phases even at very low concentration. Therefore, to facilitate the accommodation of the fluorophores within the MO palisade while preserving the original cubosome nanostructure, fluorescein and dansyl were functionalized with a C18 hydrophobic chain to obtain the fluorophores F1 and F2 (Figure 1). Moreover, as fluorescein and dansyl are slightly water-soluble, while F1 and F2 are highly hydrophobic, the adopted approach should allow increased encapsulation efficiency in the nanoparticles.

To evaluate the capacity of the MO bicontinuous cubic phase to host the fluorophores, F1 and F2 were initially encapsulated in a MO/W (water) = 70/30 (wt %) *Ia3d* (gyroid) cubic bulk phase rather than in the cubosome dispersion. The fluorophore concentration was kept below a threshold of 0.1 wt % to avoid structural alterations of the original cubic nanostructure. The MO/W = 70/30 cubic samples loaded with F1 and F2 were observed with an optical microscope under polarized light to check the absence of undissolved crystals, while their fluorescence was confirmed by exposing the cubic bulk phase to ultraviolet light. Noticeably, F1 and F2 show rather strong fluorescence in the bicontinuous bulk cubic phases, with an emission maximum at 544 and 500 nm and a high anisotropy value of 0.2 and 0.1, respectively (see Table 1). Since fluorescein undergoes deactivation by self-quenching processes,^{39,40} this is additional proof that the F1 fluorophore is dispersed within the MO matrix rather than self-aggregated.

Results from this preliminary study demonstrated that the optimal fluorophore concentration in the MO/W = 70/30 *Ia3d* cubic bulk phase was approximately 0.05 and 0.06 wt % for F1 and F2, respectively. At this concentration, no crystals were observed and strong fluorescence was detected. The preservation of the cubic nanostructure was assessed by SAXS. The diffraction pattern showed at least five Bragg peaks with relative positions in a ratio of $\sqrt{6}:\sqrt{8}:\sqrt{14}:4:\sqrt{20}$, which are characteristic of the *Ia3d* space group (Figure S1 in Supporting Information). The lattice parameter and the water channel radii extracted from the SAXS diffraction patterns were found to be 131.1 ± 0.3 and 15.5 Å for F1 and 130.4 ± 0.4 and 15.3 Å for F2. These structural parameters are almost identical to those found in the absence of the fluorophores. It is worth recalling here that, upon increasing the water content, the *Ia3d* phase evolves toward a different bicontinuous cubic phase characterized by a *Pn3m* (double diamond) space group. The aqueous dispersions of MO bicontinuous cubic phases (cubosomes) should possess this type of inner nanostructure since the *Pn3m* phase can coexist with an excess of water.

According to a bottom-up approach, fluorescent cubosomes were prepared by dispersing a melted mixture of MO and F1 or F2 in a pluronic F108 (PF108) solution via sonication. In this study, PF108 was chosen since it demonstrated superior ability in cubosome formulation.²⁷ Cubosome dispersions were prepared at the composition MO/W/PF108 = 3.3/96.4/0.3 (wt %), while the amount of F1 or F2 was, respectively, 2.5×10^{-3} and 2.8×10^{-3} wt %. Cubosomes were then characterized for particle size and morphology, as well as for their inner structure and photophysical properties. The cryo-TEM image of F1-doped cubosomes is reported in Figure 2. Several nanoparticles with inner cubic structure are clearly discernible. On the basis of repeated observations, the sample under

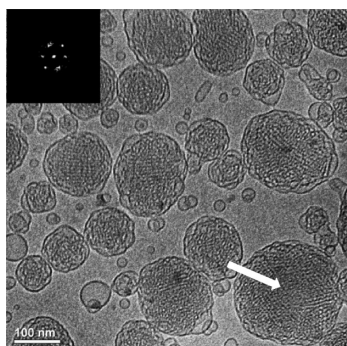


Figure 2. Cryo-TEM image of MO-based cubosomes loaded with F1. Fast Fourier transformation of the domain indicated by the arrow is shown in the inset.

investigation was found to be a dispersion of cubosomes having a diameter in the range of 100–200 nm.

The optical diffractogram obtained by the fast Fourier transform (inset in Figure 2) of the cryo-TEM image suggests the presence of an inner cubic arrangement, as it shows the typical motifs of the $\{111\}$ reflection of a $Pn3m$ phase.⁴¹ Differently, when F2 was added to the formulation along with the expected cubosomes, a new kind of nanoparticles was observed (Figure 3). In other words, the insertion of the F2

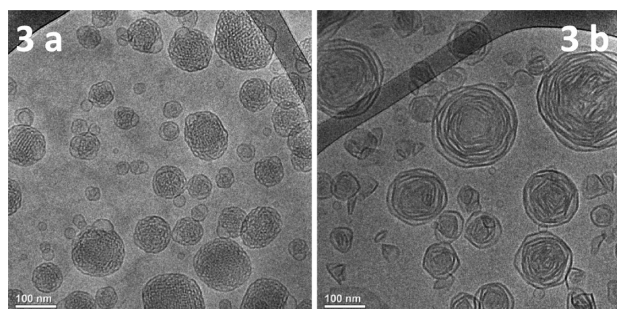


Figure 3. Cryo-TEM image of MO-based sample loaded with F2: (a) cubosomes and (b) rose-like nanoparticles.

fluorophore within the MO palisade causes a profound alteration of the surfactant packing, thus originating the new nanostructure having a rose-like morphology (Figure 3b). Such “roses” coexist with the ordered cubic phase dispersion, representing about 15% of the dispersed phase (as a rough estimate). In some cases, transition between the cubic and the rose-like morphology can be observed (see Figure S2).

The inner cubic structure of the cubosomes was confirmed by SAXS. The diffraction pattern shows at least three Bragg peaks with relative positions in a ratio of $\sqrt{2}:\sqrt{3}:2$, which are characteristics of the $Pn3m$ phase (Figure S3). For empty or variously loaded cubosomes, Table 2 reports the structural parameters from the SAXS analysis along with mean diameters, polydispersion indexes, and ζ -potentials obtained from dynamic light scattering (DLS). Also note that, in striking agreement with cryo-TEM images, DLS analysis certifies very similar nanoparticles’ dimensions, besides a quite low PDI. Concerning the F2-doped sample, given the high dilution, the characteristic pattern (if any) of the rose-like nanostructure was not discernible in the SAXS pattern.

The presence of free dye in the aqueous dispersion of cubosomes doped either with F1 or F2 was avoided by

Table 2. Lattice Parameter (a), Water Channel Radius (r_w), Mean Diameter (D_{av}), Polydispersion Index (PDI), and ζ -Potential of the Cubosome Nanoparticles Empty or Loaded with Various Cargos

cubosome cargos	a (Å)	r_w (Å)	D_{av} (nm)	PDI	ζ -potential (mV)
F1	98 ± 1	21.3 ± 0.8	161 ± 2	0.17	-29.4 ± 0.6
	101 ± 1	22.4 ± 0.8	148 ± 2	0.12	-26.6 ± 0.6
F2	98 ± 1	21.2 ± 0.8	148 ± 2	0.14	-24.5 ± 0.1
quercetin	98 ± 1	21.3 ± 0.8	161 ± 3	0.15	-25.6 ± 0.9
F1 + quercetin	99 ± 1	21.6 ± 0.8	160 ± 2	0.19	-27.6 ± 0.2
F2 + quercetin	96 ± 1	20.3 ± 0.8	159 ± 2	0.12	-22.8 ± 0.3

purifying samples via ultrafiltration. Their absence was checked through fluorescence optical microscopy observations, as exemplified in Figure 4. Particularly, to decrease the

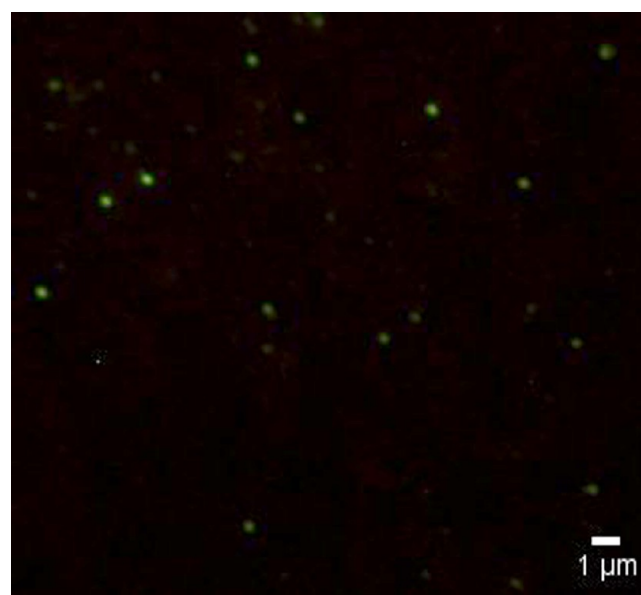


Figure 4. Fluorescence microscopy image of F1-doped cubosomes (see the text).

cubosomes’ diffusion on the glass microscopy slide, the ultrafiltrated aqueous solutions of F1- and F2-doped cubosomes were diluted with ethylene glycol before the pictures were taken. If some free dye was molecularly dispersed in the aqueous phase, the image shown in Figure 4 should have appeared as a more or less homogeneous bright field. On the contrary, the fluorescent spots on a dark background observed in Figure 4 represent an additional proof that F1 is completely encapsulated within the nanoparticles. The same holds for F2.

The photophysical properties of an aqueous dispersion (pH 7) of cubosomes are very similar to those of F1 dissolved in a basic aqueous solution at pH 9 (where F1 is soluble), with a maximum of the emission band at 517 nm, an excitation band that overlaps that of F1, as shown in Figure 5, and a slightly longer averaged excited state lifetime (3.8 ns for F1 within the cubosomes and 3.0 ns for F1 in aqueous solution at pH 9; see Table 1).

These results are expected since the energetics of the π – π^* excited state of the fluorescein unit is known to be independent of the polarity of the environment. A very interesting result

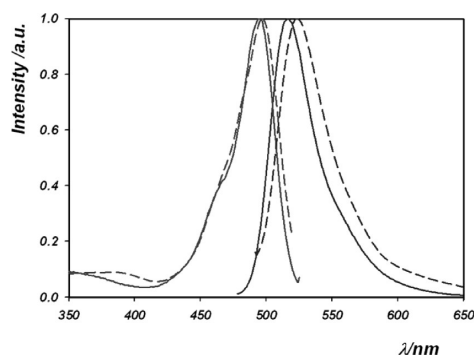


Figure 5. Normalized emission (λ_{exc} 470 nm) and excitation (λ_{em} 540 nm) spectra of MO-based cubosomes doped with F1 in an aqueous dispersion at pH 7 (dashed lines) and F1 in an aqueous dispersion buffered at pH 9 (solid line).

emerged from fluorescence anisotropy measurements. The observed value of 0.3 clearly indicates that F1 is inserted into a large structure with a reduced mobility since the anisotropy of freely mobile fluorescein is negligible. This conclusion is supported by the photophysical characterization of MO-based cubosomes doped with the dansyl derivative F2. Indeed, dansyl is known to have photophysical properties that strongly depend on the solvent polarity. We found that F2-doped cubosomes show an emission maximum at 520 nm and an excited state lifetime of 12.8 ns (see Figure S4 and Table 1).

These results are very similar to those observed for F2 in ethanol (507 nm and 13.3 ns) and very different from those reported for dansyl derivatives in water solution (560 nm and 2.9 ns in water; 500 nm and 13.4 ns in ethanol).⁴² These results also confirm that F2 is embedded within the nanoparticles and give some information on its localization. Indeed, these data suggest that the fluorescent moiety of F2 is located in the same region of the MO polar heads, experiencing interactions with the MO OH groups.

On the basis of the cryo-TEM analysis, which in the case of the F2-doped cubosome dispersion evidenced the presence of nanoparticles having an inner structure different from cubic, only the cellular uptake of MO-based cubosomes doped with F1 was investigated. 3T3 fibroblast cells were incubated in the presence of nanoparticles for 3 h and imaged with fluorescent microscopy. These observations were made immediately after replacing the nanoparticle suspension in the medium with fresh particle-free medium. Diffuse fluorescence was detected in the cytosol and, particularly, in the perinuclear region of living cells (Figure 6). Therefore, as confirmed by the intracellular fluorescence detection, we can conclude that F1 penetrates into the cells via the cubosome carrier.

Finally, the F1-doped cubosome dispersion was loaded with quercetin, a polyphenolic molecule endowed of anticancer activity. The calculated loading efficiency (see the Materials and Methods section) was 80%. SAXS and DLS results reported in Table 2 demonstrate that the presence of quercetin does not significantly alter the inner nanostructure of the cubosome, as well as their size and charge. Interestingly, although pluronic-stabilized cubosomes are basically uncharged, they display a negative ζ -potential, as reported by other authors.^{43,44} The origin of this negative charge is still unclear, but preferential adsorption of hydroxyl ions at the oil–water interface, as in case of oil droplets in water, has been proposed to explain the observed values of ζ -potential.⁴⁴

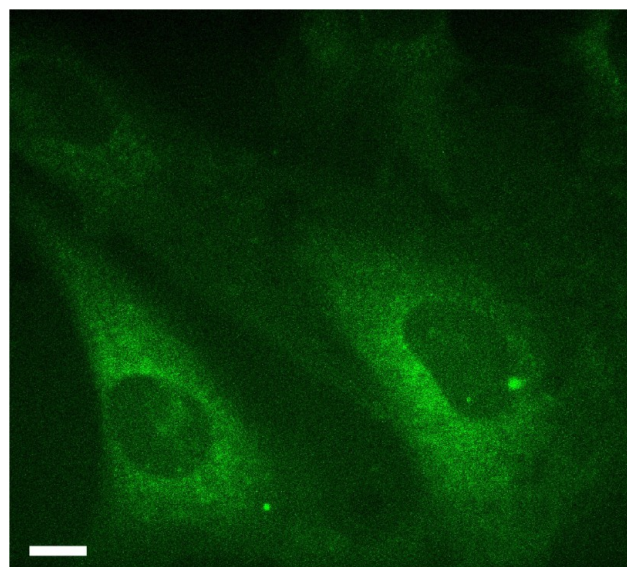


Figure 6. Fluorescence image of viable 3T3 fibroblast cells after treatment with F1-loaded MO-based cubosomes.

CONCLUSIONS

Cubosomes are often proposed as suitable drug nano-carriers,^{29,32,45–47} and the ability of bicontinuous cubic phases to host molecules with pharmaceutical interest has been proved numerous times.^{48,49} In this paper, we demonstrated that MO-based cubosomes, still retaining the characteristics given by their peculiar inner nanostructure, can efficiently and easily be coupled with properly designed fluorophores and loaded with an anticancer drug. Therefore, the physicochemical and photophysical measurements, along with the single living cell imaging analysis here reported, make these innovative fluorescent nanocarriers a candidate for theranostic applications.

ASSOCIATED CONTENT

Supporting Information

Additional figures as described in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Cancer-Cell-Targeted Theranostic Cubosomes.

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Cancer-Cell-Targeted Theranostic Cubosomes

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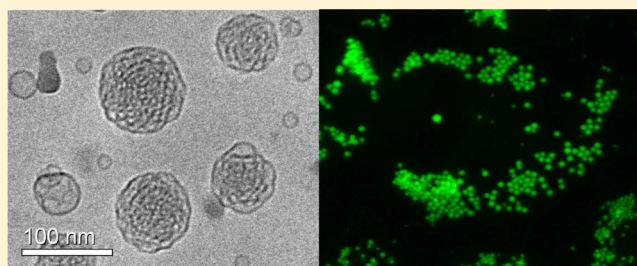
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S Supporting Information

ABSTRACT: This work was devoted to the development of a new type of lipid-based (cubosome) theranostic nanoparticle able to simultaneously host camptothecin, a potent anticancer drug, and a squaraine-based NIR-emitting fluorescent probe. Furthermore, to confer targeting abilities on these nanoparticles, they were dispersed using mixtures of Pluronic F108 and folate-conjugated Pluronic F108 in appropriate ratios. The physicochemical characterization, performed via SAXS, DLS, and cryo-TEM techniques, proved that aqueous dispersions of such cubosomes can be effectively prepared, while the photophysical characterization demonstrated that these nanoparticles may be used for in vivo imaging purposes. The superior ability of these innovative nanoparticles in targeting cancer cells was emphasized by investigating the lipid droplet alterations induced in HeLa cells upon exposure to targeted and nontargeted cubosomes.



INTRODUCTION

The main goal of nanomedicine is probably the improvement of diagnostic and therapeutic tools available in clinics. Therefore, considerable efforts have been made to develop the physicochemical and biological characteristics of the nanocarriers engineered to encapsulate drugs or imaging agents with pharmaceutical purposes. Since nanocarriers may be formulated using an extensive range of organic and inorganic materials, these efforts resulted in the assembly of a huge number of "soft" and "hard" nanocarriers, such as polymeric,^{1–5} silica,^{6,7} or iron oxide nanoparticles,^{8–10} liposomes,^{11–13} dendrimers,^{10,14,15} and quantum dots.^{16–18} Recently, the development of single platforms able to simultaneously transport and release pharmaceutically active and imaging agents has led to so-called theranostic nanoparticles. Besides, the latter usually contain a targeting ligand as a third component to specifically direct both the therapeutic and diagnostic agents to the pathological site. In principle, these multifunctional nanocarriers should fulfill a number of requisites that include the controlled (co)delivery of poorly water-soluble (multiple) drugs, the selective targeting of malignant cells for minimizing side effects, and the in-progress evaluation of the treatment efficacy via molecular detection.^{19–29}

The accumulation of nanoparticles in tumor tissues passively occurs as a result of aberrant tumor angiogenesis that forms nonmature, leaky capillaries. Indeed, the primary consequences

of the defects in tumor vessels are great permeability to colloidal macromolecules or nanoparticles in comparison to physiological vessels and reduced lymphatic drainage of these colloids from the interstitial space of tumors, a phenomenon called the enhanced permeation and retention (EPR) effect.³⁰ Conversely, nanoparticles may be actively targeted to tumor tissues employing cancer-cell-specific ligands such as folic acid. The popularity of this ligand has mainly grown because cancer cells frequently overexpress folate receptors (which is the case for ovary, breast, brain, kidney, head, neck and other tumors) and because of its high binding affinity, ease of modification, stability during storage, and low cost.^{31,32}

Fluorescent imaging is one of the most powerful techniques in clinical diagnosis³³ for monitoring biological processes in living systems due to its characteristics of high spatial and temporal resolution. Contrast agents such as Gd³⁺ complexes³⁴ and fluorodeoxyglucose³⁵ are widely used in MRI (magnetic resonance imaging) and PET (positron emission tomography). In the last few decades, a profusion of fluorescent probes has been developed by synthesis chemists. However, most of them can hardly be used for in vivo applications because the majority of the conventional fluorophores emit in the UV–vis region.

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Indeed, in this portion of the electromagnetic spectrum the absorption of biomolecules (water, hemoglobin, etc.), the autofluorescence of the cells, and the light scattering are high, causing limited tissue penetration as well as low signal-to-noise ratios, resulting in unsuitability for in vivo imaging. Conversely, dyes active in the NIR (near infrared) region of the spectrum (650–900 nm) have attracted much attention because of minimum photodamage to biological samples, deep tissue penetration, and minimum interference from background autofluorescence by biomolecules in living systems.^{36–38} Different classes of NIR-emitting fluorophores have been developed such as phthalocyanines, cyanines, and squaraines. In particular, the latter³⁹ are the dicondensation products of electron-rich molecules with squaric acid and are characterized by a sharp and intense low-energy absorption associated with strong fluorescence in solution.

Among the various nanocarriers engineered for theranostic nanomedicine, cubosomes have been only recently proposed.⁴⁰ Basically, these are aqueous dispersions of lipid-based bicontinuous liquid-crystalline phases typically stabilized by Pluronics.^{41,42} Therefore, the inner nanostructure of the nanoparticles is constituted of curved, triply periodic non-intersecting bilayers folded on an infinite periodic minimal surface of cubic symmetry and organized to form two disjointed continuous water channels.^{43,44}

Throughout this article it will be shown that cubosome nanoparticles coloaded with both an NIR fluorescent probe and an anticancer drug can be prepared and effectively stabilized by mixtures of F108 and folate-conjugated F108 Pluronics that give these nanoparticles superior targeting ability toward HeLa cancer cells with respect to traditional cubosomes.

■ EXPERIMENTAL SECTION

Chemicals. Monoolein (MO, 1-monooleoylglycerol, RYLO MG 19 PHARMA, glycerol monooleate, 98.1 wt %) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. Pluronic F108 (PEO₁₃₂–PPO₅₀–PEO₁₃₂), folic acid (≥97%), camptothecin (≥90%), 2,4-di-3-guaiazulenyl-1,3-dihydroxycyclobutenediylum dihydroxide bis(inner salt), acetonitrile (≥99.9%), diethyl ether (≥99.5%), *N,N'*-carbonyldiimidazole (CDI, ≥ 97%), ethylenediamine, (≥99.5%), *N*-hydroxysuccinimide (NHS, 98%), *N,N'*-dicyclohexylcarbodiimide (DCC, ≥ 99.5%), and triethylamine (≥99.5%) were purchased from Sigma-Aldrich. Distilled water passed through a Milli-Q water-purification system (Millipore) was used to prepare the samples.

Nuclear Magnetic Resonance Experiments. ¹H NMR measurements were performed on a Bruker Avance 300 MHz (7.05 T) spectrometer at an operating frequency of 300.131 MHz at 25 °C. A standard variable-temperature control unit with an accuracy of ±0.5 °C was used. Chemical shifts for ¹H NMR are reported in parts per million (ppm), with coupling constants reported in Hertz (Hz). The following abbreviations are used for spin multiplicity: s, singlet; d, doublet; t, triplet; and m, multiplet. A selection of the characteristic ¹H signals are reported in the synthesis of F108-CDI, F108-NH₂, and F108-FA Pluronics.

Synthesis of Folate-Conjugated Pluronic F108. *Synthesis of CDI-Activated Pluronic F108 (F108-CDI).* To a solution of Pluronic F108 (2.01 g, 0.14 mmol) in dry acetonitrile (10 mL), a solution of a great excess of *N,N'*-carbonyldiimidazole (CDI, 0.26 g, 1.6 mmol) in dry acetonitrile (5 mL) was added dropwise during a period of 45 min. The mixture was kept stirring at room temperature under a nitrogen atmosphere for 6 h. Then, the solution was concentrated in a rotary evaporator and washed with diethyl ether (3 × 10 mL) to remove unreacted CDI. The product was dried under vacuum and collected as a white powder. Yield: 93% (1.890 g, 0.13 mmol); mp: 54 °C. ¹H NMR (300 MHz, DMSO-*d*₆, 298 K): δH 1.03 (d, *J* = 6 Hz; 3H × 50, –CH₃ of PPO), 3.47–3.54 (m, 3H × 50, 4H × 264, –CH₂–

CH(CH₃)–O– of PPO and –CH₂–CH₂–O– of PEO), 7.50–7.65 (m, 2H × 2, protons of the imidazole moiety), 8.25 (s, 1H × 2, proton of the imidazole moiety). IR (solid state, cm^{–1}) ν = 2885 (s), 1743 (w).

Synthesis of NH₂-Terminated Pluronic F108 (F108-NH₂). To a solution of F108-CDI (1.010 g, 0.068 mmol) in dry acetonitrile (10 mL), 0.78 mL of ethylenediamine was added. The reaction mixture was kept stirring for 24 h at room temperature under a nitrogen atmosphere. The solvent and unreacted ethylenediamine were removed under vacuum. The crude product was precipitated with diethyl ether and collected as a beige powder. Yield: 81% (0.813 g; 0.055 mmol); mp: 56 °C. ¹H NMR (300 MHz, DMSO-*d*₆, 298 K): δH 1.03 (d, *J* = 6 Hz, 3H × 50, –CH₃ of PPO), 2.65–2.75 (m, 2H × 2 protons adjacent to the terminal amine), 2.96 (q, *J* = 6 Hz, 2H × 2 protons adjacent to the amide bond), 3.47–3.54 (m, 3H × 50, 4H × 264, –CH₂–CH(CH₃)–O– of PPO and –CH₂–CH₂–O– of PEO), 7.17 (t, *J* = 6 Hz, 1H × 2, amidic NH). IR (solid state, cm^{–1}) ν = 2885 (s), 1716 (w).

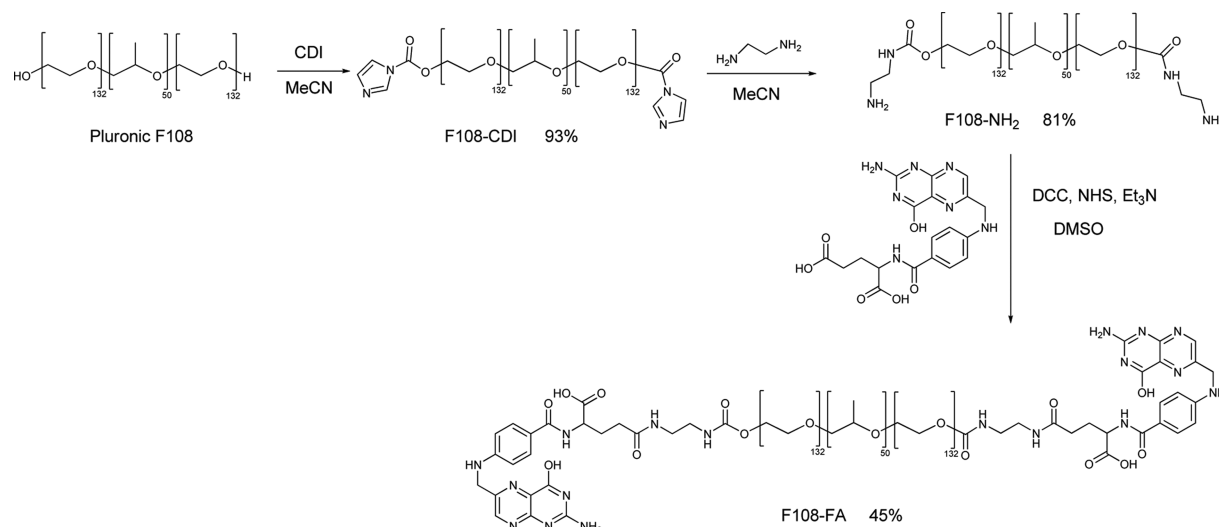
Synthesis of Folate-Conjugated Pluronic F108 (F108-FA). F108-NH₂ (0.498 g, 0.034 mmol), *N*-hydroxysuccinimide (NHS, 0.033 g, 0.29 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC, 0.055 g, 0.27 mmol), and folic acid (FA, 0.053 g, 0.12 mmol) were dissolved in 10 mL of DMSO in the presence of triethylamine (50 μ L). The reaction mixture was stirred for 24 h at room temperature under a nitrogen atmosphere. Twenty milliliters of deionized water was added to the reaction mixture to precipitate DCU (dicyclohexylurea), and then the mixture was centrifuged and the supernatant was dialyzed (molecular weight cutoff of the dialysis tube was 14 kDa) for 3 days against deionized water, which was changed every 3–6 h. The resulting product was lyophilized for 3 days in order to remove all of the residual water. Yield: 45% (0.230 g, 0.015 mmol); mp: 145 °C. ¹H NMR (300 MHz, DMSO-*d*₆, 298 K): δH 1.03 (d, *J* = 6 Hz; 3H × 50, –CH₃ of PPO), 3.47–3.54 (m, 3H × 50, 4H × 264, –CH₂–CH(CH₃)–O– of PPO and –CH₂–CH₂–O– of PEO), 6.63 (d, *J* = 9 Hz, 2H × 2 of the phenilic ring), 6.88 (t, *J* = 7.5 Hz, 1H × 2, aryl NH), 7.10–7.30 (m, 1H × 2, amidic NH from the precursor), 7.50–7.80 (m, 2H × 2, of the phenilic ring), 7.80–8.10 (m, 1H × 2, amidic NH), 8.66 (s, 1H × 2, pteridinic CH). IR (solid state, cm^{–1}) ν = 2885 (s), 1608, 1642, 1689, 1723 (w).

Sample Preparation. Monoolein-based cubosomes were prepared and stabilized by dispersing the appropriate amount of MO in water solutions of different F108/F108-FA mixtures (100:0, 90:10, 80:20, 60:40, 50:50, 30:70, and 0:100 wt %/wt %) using an UP100H ultrasonic processor developed by Hielscher, cycle 0.9, amplitude 90%, for 10 min. Doped cubosomes were obtained by dispersing the fluorophore and the drug in the melted monoolein with the help of an ultrasonic bath before dispersion in the stabilizer solution. The sample volume was usually 4 mL with approximately 96.4 wt % water, 3.3 wt % MO, and 0.3 wt % Pluronics mixture. Fluorophore and camptothecin quantities were, respectively, 2.8×10^{-4} and 4.0×10^{-4} wt %.

Dialysis and Drug-Loading Efficiency (E%). After drug loading, cubosome dispersions were purified from the nonencapsulated drug by dialysis as follows: 2 mL was loaded into a dialysis tubing cellulose membrane (14 kDa MW cutoff, purchased from Sigma-Aldrich) and dialyzed against water (1000 mL) for 2 h (by replacing the water after 1 h) at 5 °C. The drug-loading efficiency, expressed as a percentage of the initially used amount of drug, was determined by UV–vis spectroscopy after the disruption of cubosomes with acetonitrile. Camptothecin and fluorophore contents were quantified respectively at 363 and 755 nm using a Thermo Nicolet Evolution 300 UV–vis spectrophotometer.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Vitrified specimens were prepared in a controlled-environment vitrification system (CEVS) at 25 °C and 100% relative humidity. A drop of the sample was placed on a perforated carbon film-coated copper grid, blotted with filter paper, and plunged into liquid ethane at its freezing point. The vitrified specimens were transferred to a 626 Gatan cryo-holder and observed at a 120 kV acceleration voltage in an FEI Tecnai T12 G² transmission electron microscope at about –175

Scheme 1. Reaction Scheme Adopted for the Synthesis of F108-FA



°C in low-dose imaging mode to minimize electron-beam radiation damage. Images were digitally recorded with a Gatan US1000 high-resolution CCD camera.

Dynamic Light Scattering (DLS). Particle size and ζ -potential determinations of the nanoparticles were performed with a ZetaSizer Nano ZS (Malvern Instruments, Malvern, U.K.) at a temperature of 25 ± 0.1 °C. Samples were backscattered by a 4 mW He–Ne laser (operating at a wavelength of 633 nm) at an angle of 173°. Diluted samples (1:50) were housed in disposable polystyrene cuvettes of 1 cm optical path length with water as the solvent. At least two independent samples were taken, each of which was measured three to five times. The autocorrelation function (ACF) of scattered light intensity was converted to the ACF of the scattered electric field. From this last quantity, the software supplied by the producer evaluates the z -averaged hydrodynamic diameter (D_{av}) and the variance of the intensity-weighted size distribution function through cumulant analysis (second order) and subsequent application of the Stokes–Einstein equation. The intensity-weighted size distribution function, i.e., the fraction of the light intensity scattered by particles of different size, was also recovered by taking the inverse Laplace transform of the ACF using the software implemented by the manufacturer. In all cases, the intensity-weighted size distribution functions were monomodal with mean and variance indistinguishable from those recovered through cumulant analysis. The hydrodynamic diameter distribution is indicated by the PDI (polydispersion index).

Small-Angle X-ray Scattering (SAXS) Experiments. Small-angle X-ray scattering was recorded with a S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). Cu K α radiation of wavelength 1.542 Å was provided by a GeniX X-ray generator, operating at 50 kV and 1 mA. A 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels of width 54.0 μ m was used for the detection of scattered X-rays in the small-angle region. The working q range ($\text{\AA}^{-1}$) was $0.003 \leq q \leq 0.6$, where $q = 4\pi \sin(\theta)/\lambda$ is the scattering wave vector. For the analysis, thin-walled 2 mm glass capillaries were filled with the cubosome dispersions. The diffraction patterns of cubosomes were recorded for 2 h. The lattice parameter a of the cubic phases was determined using the relation $a = d(h^2 + k^2 + l^2)^{1/2}$ from linear fits of the plots of $1/d$ versus $(h^2 + k^2 + l^2)^{1/2}$, where $d = 2\pi/q$ (q is the measured peak position) and h , k , and l are the Miller indices. Water channel radii were calculated using the relation $R_w = [(A_0/2\pi\chi)^{1/2}a] - L$, where L is the lipid length value (17 Å), a is the lattice parameter obtained from the SAXS analysis, and A_0 and χ are the surface area and the Euler characteristic of the IPMS geometries ($Pn3m$, $A_0 = 1.919$, $\chi = -2$). To minimize scattering from air, the camera volume was kept under vacuum during the measurements. Silver behenate ($\text{CH}_3-(\text{CH}_2)_{20}-\text{COOAg}$) with a d

spacing value of 58.38 Å was used as a standard to calibrate the angular scale of the measured intensity.

Photophysical Measurements. Cubosome solutions were diluted with Milli-Q water. UV–vis absorption spectra were recorded at 25 °C with a PerkinElmer Lambda 45 spectrophotometer. Quartz cuvettes with an optical path length of 1 cm were used. Corrected fluorescence emission and excitation spectra (450 W Xe lamp) were obtained with an Edinburgh Instruments FLS920 modular UV–vis–NIR spectrofluorimeter equipped with a Hamamatsu R928P P photomultiplier tube (for the 500–850 nm spectral range) and an Edinburgh Instruments Ge detector (for the 800–1600 nm spectral range). The same instrument connected to a PCS900 PC card was used for the TCSPC (time-correlated single-photon counting) experiments (excitation laser $\lambda = 405$ nm). Corrections for instrumental response, inner filter effects, and phototube sensitivity were performed.⁴⁵ All fluorescence anisotropy measurements were performed on an Edinburgh FLS920 equipped with Glan-Thompson polarizers. Anisotropy measurements were carried out by an L-format configuration, and all data were corrected for polarization bias using the G factor.

Cell Culture and Treatments. Human carcinoma cell line HeLa (ATCC collection) was grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL^{-1}), and streptomycin (100 $\mu\text{g mL}^{-1}$) (Invitrogen) in a 5% CO_2 incubator at 37 °C. Cells were seeded in 35 mm dishes, and experiments were carried out 2 days after seeding, when cells had reached 90% confluency. Cubosomes (1:500, 4 μL in 2 mL of free-serum medium) were added to the cells and incubated at 37 °C for 2, 4, and 24 h. For live cell imaging, after replacing the particle suspension with fresh serum-free medium, cells were loaded with fluorescent probes, and after the incubation time, the probes were washed out before the imaging session. Cells were supravitaly stained with the following probes: 300 nM Nile red (NR) (9-diethylamino-5H-benzo[*a*]phenoxazine-5-one) for 15 min and 650 nM Hoechst 33258 (HOE) for 30 min. Vehicles were DMSO for NR and water for HOE. Stock solutions were 1000-fold concentrated not to exceed the 0.1% concentration of vesicles in the medium. Nile red was from Fluka (Buchs, SG, Switzerland); Hoechst was from Sigma-Aldrich (St. Louis, MO, USA).

Fluorescence Microscopy. Light microscopy observations were made with a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with 10 $\times$, 20 $\times$, and 40 $\times$ /0.75 NA water-immersion objectives and an HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome-cooled CCD camera (QICAM, Qimaging, Canada) with variable exposure. For the observation of the HOE

Table 1. Space Group, Lattice Parameter (a), Water Channel Radius (r_w), Mean Diameter (D_{av}), Polydispersion Index (PDI), ζ Potential, and Stability of the Cubosome Nanoparticles Stabilized with Different F108/F108-FA Ratios (Wt %)^a

F108/F108-FA	space group	a (Å)	r_w (Å)	D_{av} (nm)	PDI	ζ potential (mV)	stability (weeks)
100/0	<i>Pn3m</i>	99 ± 2	22 ± 1	133 ± 1	0.11	−28 ± 2	>24
90/10	<i>Pn3m</i>	95 ± 1	20 ± 1	135 ± 3	0.13	−20 ± 1	>24
80/20	<i>Pn3m</i>	97 ± 2	21 ± 1	139 ± 3	0.11	−29 ± 1	>24
60/40	<i>Pn3m</i>	100 ± 1	22 ± 1	142 ± 1	0.13	−29 ± 1	18
50/50	<i>Pn3m</i>	93 ± 1	19 ± 1	151 ± 1	0.13	−23 ± 1	18
30/70	<i>Pn3m</i>	99 ± 1	22 ± 1	159 ± 2	0.12	−25 ± 1	2
0/100	<i>Pn3m</i>	96 ± 1	20 ± 1	168 ± 1	0.13	−25 ± 1	2
80/20 + drug	<i>Pn3m</i>	102 ± 1	23 ± 1	145 ± 2	0.14	−23 ± 1	>24
80/20 + drug + dye	<i>Pn3m</i>	102 ± 1	23 ± 1	141 ± 1	0.12	−25 ± 1	>24

^aErrors are reported as mean ± SD.

probe, the filters were ex 360 ± 20 nm and em 460 ± 25 nm. For NR, the filters were ex 470 ± 20 nm and em 535 ± 40 nm for nonpolar lipids and ex 546 ± 6 nm and em 620 ± 60 nm for total lipids. The adopted filters allowed a virtually complete separation of the emissions and the simultaneous observation of NR and HOE probes. Image alignments were obtained with Image Pro Plus software.

Lipid Droplet Staining and Quantification. Cells were seeded in 35 mm dishes and cultured in serum-containing media. After nanoparticle treatments, cells were stained with NR and HOE. NR is an ideal probe for the detection of lipids, as it exhibits high affinity, specificity, and sensitivity to the degree of hydrophobicity of lipids. The latter feature results in a shift of the emission spectrum from red to green in the presence of polar and nonpolar lipids, respectively.⁴⁶ For this reason, in staining living cells with NR, cytoplasmic membranes are stained red, whereas neutral lipids of lipid droplets are stained green. Fluorescent images of NR-stained cells were acquired at 10× and 40× magnifications of a fluorescence microscope. Measurements, made on a single-cell basis, gave the amount of lipid droplet content, evaluated by the fluorescence intensity of NR-green emission. The IOD (integrated optical density) per cell was calculated using Image ProPlus software (Media Cybernetics, Silver Springs, MD).

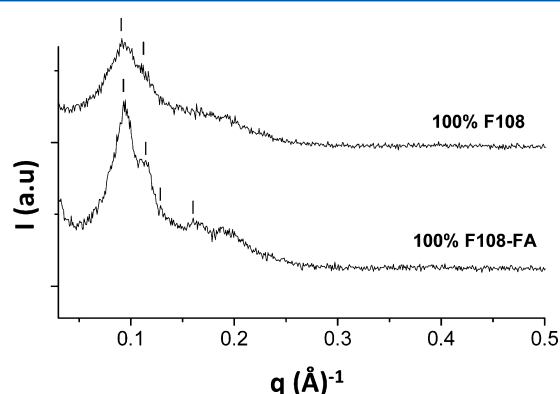
Confocal Microscopy. Measurements were performed on mounted slides by a Leica TCS SP5 SM (Leica Microsystems, Mannheim, Germany) inverted confocal microscope. For Hoechst (nuclear stain), excitation was provided by a 405 nm 40 MHz pulsed laser diode (Picoquant, Berlin, Germany), whereas the 458 nm laser line of a cw Ar laser was used to excite the dye. In both cases, the typical excitation average power was in the 100–800 μ W range, when measured after the objective. Hoechst emission was collected at 420–480 nm by the internal monochromator of the microscope interfaced with a photomultiplier detector. Dye emission was filtered at wavelengths >680 nm by an interferential filter (Semrock, Rochester, NY, USA) and collected by one avalanche photodiode detector (Picoquant, Berlin, Germany). The confocal pinhole was set to a 1 Airy size; a 100× 1.4 NA objective was used for all measurements. The line-scanning speed was 400–700 Hz in standard acquisition mode.

RESULTS AND DISCUSSION

Cubosomes Characterization. Here, the use of mono-olein (MO)-based cubosomes as nanoparticles for the active targeting of cancer cells was investigated. For this purpose, the surfactant used to stabilize cubosomes (Pluronic F108, F108) was conjugated to folate⁴⁷ (Scheme 1), a targeting ligand which displays a high affinity for the folate receptor overexpressed on the tumor cell membrane. Briefly, F108, initially activated with CDI, was reacted with ethylenediamine to obtain amine-terminated F108, and the resulting F108-NH₂ was finally reacted with NHS, DCC, Et₃N, and folic acid (see Supporting Information for characterization details). The purity of F108-FA and then the absence of unreacted F108-NH₂ were

confirmed by a thin-layer ninhydrin assay. Furthermore, after the pH value was decreasing to 2, there was no precipitation of unreacted folic acid.⁴⁸

Subsequently, mixtures of F108 and its folate-conjugated counterpart were used to stabilize the aqueous cubosome dispersions. At first, the physicochemical characterization of cubosome nanoparticles stabilized with different F108/F108-FA ratios was performed by SAXS and DLS. The results are summarized in Table 1. As can be seen from the SAXS data, nanoparticles retained the *Pn3m* structure found in traditional cubosomes (i.e., those prepared with 100% F108), with no significant variations in the lattice parameter, even after the complete substitution of F108 with F108-FA. Conversely, the DLS analysis indicated that the amount of F108-FA used to stabilize nanoparticles strongly affected the mean particles size, which increased from 133 nm (100% F108) to 168 nm (100% F108-FA). Although a slight increase in the PDI was detected upon decreasing the F108/F108-FA ratio, independently by the presence of the folate-conjugated Pluronic, the various solutions consisted mainly of homogeneous cubosome dispersions. The nanoparticles growth was also confirmed by SAXS experiments because while increasing the amount of F108-FA the profile of the quasi-Bragg peaks became sharper (Figure 1). Indeed, the absence of well-defined Bragg peaks in

**Figure 1.** SAXS patterns of cubosome dispersions stabilized by F108 and F108-FA Pluronic. Relevant peaks are indicated.

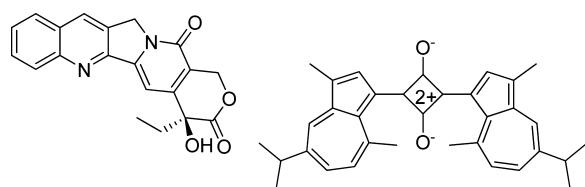
SAXS patterns of cubosome dispersions is typically related to the very small nanoparticle size (in which the peak width is inversely proportional to the crystalline domain size). Therefore, the quasi-Bragg peaks' appearance in the sample containing 100% F108-FA represents an additional proof of the increased size of the nanoparticles.

Concerning the measured ζ -potential values (reported in Table 1), they were found to be in the range of -20 to -30 mV, almost equivalent to those of nontargeted cubosomes (-28 mV), with some fluctuations that appeared independently of the F108/F108-FA ratio. Nevertheless, the stability over time was found to depend greatly on the F108-FA content because samples prepared with a higher percentage of F108-FA exhibited a reduced shelf life, as demonstrated by the observed phase separation (Table 1).

The role of Pluronics in this kind of formulation consists of stabilizing the obtained nanoparticles against coalescence or coagulation basically via mixing and entropic effects.² Therefore, the reported experimental findings suggest that the conjugation of the folate to the terminal end of the PEO moieties greatly modifies the original stabilizing properties of F108, thus producing the observed alteration in cubosome formulations when F108 is gradually replaced by F108-FA. Indeed, it is likely that such a conjugation destabilizes the delicate balance between the PEO–solvent and PEO–PEO interactions, on the basis of the mixing effect, thus leading to the observed changes in size and stability of the cubosomes. In other words, the regular increase in the nanoparticles' hydrodynamic radii as well as the reduced stability and the increased PDI of the cubosome dispersions while the F108-FA content is increased is strong evidence of the less effective dispersant and stabilizing properties of F108-FA with respect to those of F108.

Given the potential biomedical application of these formulations and on the basis of the stability tests, the cubosome dispersion stabilized with mixture F108/F108-FA = 80/20 (wt %) was selected for loading with the anticancer drug camptothecin (Scheme 2), which, along with its analogues, has been demonstrated to be effective against a broad spectrum of tumors.^{49–51}

Scheme 2. Chemical Structure of Camptothecin (Left) and NIR Dye 2,4-Di-3-guaiazulenyl-1,3-dihydroxycyclobutenediylum Dihydroxide Bis(inner salt) (Right)



SAXS and DLS revealed that inclusion of camptothecin (a highly hydrophobic drug) within the lipid bilayer basically did not alter the cubosome nanostructure. The morphology of these nanoparticles was observed by cryo-TEM (Figure 2). Several nanoparticles with inner cubic structure are clearly discernible, along with the vesicular structures often seen in cubosome formulations. By means of repeated observations, the sample that was investigated was found to be a dispersion of cubosomes having a diameter of 100–200 nm, in good agreement with the dimensions obtained by DLS. It is worth noticing that all of the size distributions obtained from DLS appeared to be monomodal with mean sizes ranging from 133 to 168 nm. At variance, an inspection of TEM images reveals the coexistence of large cubosomes with small unilamellar vesicles. This is because the ACF (Experimental Section) measured in DLS reflects the intensity-weighted size distribu-

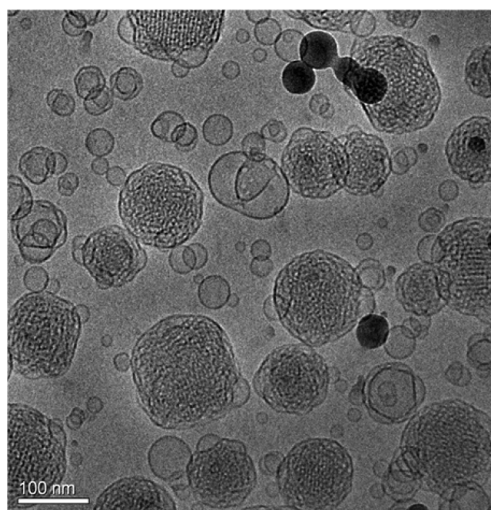


Figure 2. Cryo-TEM image of MO-based cubosomes containing both the drug and the dye and stabilized by the mixture F108/F108-FA = 80/20.

tion function, i.e., the fraction of the light intensity scattered by particles of different sizes. Since the efficiency of light scattering increases dramatically with particle size, such a size distribution is heavily biased toward large particles, and the presence of large cubosomes fully dominates the correlogram. Cryo-TEM experiments performed on targeted cubosomes not containing the drug (data not shown) revealed that they possess an identical nanostructure with respect to those loaded with camptothecin and the dye. The loading efficiencies ($E\%$) for the drug and the fluorophore were found to be 71 and 95%, respectively.

NIR Fluorescent Probe Characterization. The absorption spectrum of an aqueous suspension of cubosomes loaded with both camptothecin and NIR dye 2,4-di-3-guaiazulenyl-1,3-dihydroxycyclobutenediylum dihydroxide bis(inner salt) (Scheme 2) did not show any characteristic band because the turbidity of the suspension originated strong light scattering. However, a shoulder at around 760 nm ascribable to the dye could be observed (data not shown). When excited at 340 nm, an unstructured emission band at 428 nm (Figure 3a) with an excited-state lifetime of 0.6 ns, which can be assigned to camptothecin,^{52–54} was observed. Moreover, an emission band with a maximum at 938 nm was observed when exciting the sample at 418 nm (Figure 3b). The fluorescence anisotropy measurement at 938 nm ($r = 0.1$) suggested that the dye has reduced mobility, indicating that it is enclosed in the nanoparticle. This conclusion is also supported by the insolubility of the dye in pure water. (The absorption and emission spectra of an aqueous solution of the dye did not show any of the characteristic bands of the fluorophore.)

Lipid Droplet Evaluation. The selective cellular uptake of targeted cubosomes was investigated via IOD analysis by observing the lipid droplet (LD) formation in HeLa cells after treatment with the monoolein-based nanoparticles following a previously developed protocol.¹² LDs are cytoplasmic organelles consisting of a hydrophobic core of neutral lipids (such as triglycerides and cholesterol esters) encased by a phospholipid monolayer harboring a set of enzymes and regulatory proteins, which catalyze the metabolically controlled synthesis and mobilization of fat stores.⁵⁵ LD formation is a regulated cellular process that culminates in the compartmentalization of

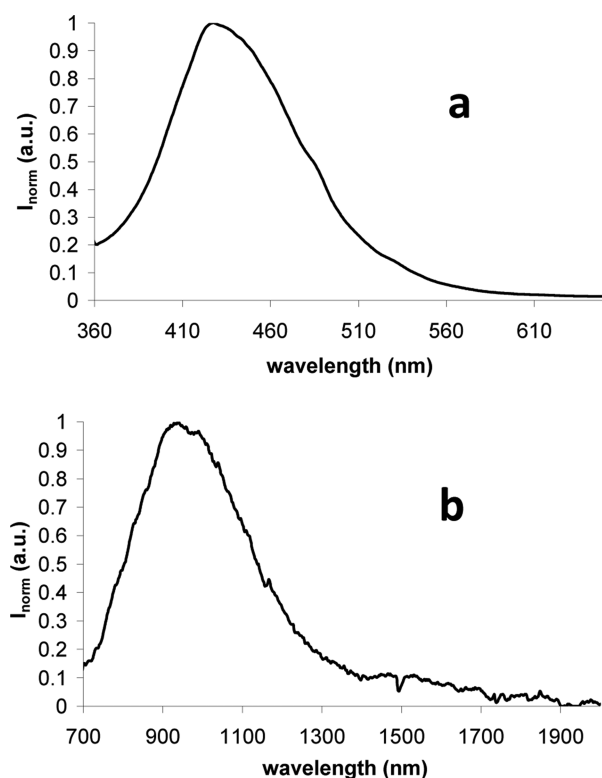


Figure 3. Normalized emission of an aqueous dispersion of targeted cubosomes (stabilized with the mixture F108/F108-FA = 80/20) loaded with camptothecin and the NIR-emitting dye. (a) Emission band of camptothecin at 428 nm (λ_{exc} = 340 nm); (b) emission band of the dye at 938 nm (λ_{exc} = 418 nm).

lipids, when long-chain unsaturated fatty acids, such as oleic acid, are available from exogenous sources inside the cells.⁵⁶ Indeed, after entering the cells, unsaturated fatty acids are rapidly activated, esterified, and stored as triglycerides inside the lipid droplets. Therefore, detecting changes in LD formation and size may be useful in differentiating between targeted and traditional cubosome uptake.

Within this scope, HeLa cells, used as model cancer cells since they express high levels of folate receptors,⁵⁷ were exposed at different times to both types of cubosomes coloaded with NR and HOE probes to identify LDs and nuclear morphology, respectively. The IOD per cell value of nontreated control cells was set to 100%, and values of cubosome-treated cells were expressed as a percentage of control. Results are reported in Figure 4a,b.

At a short incubation time (2 h) with targeted cubosomes, the size and number of LDs, detectable as punctuated green fluorescent cytoplasmic structures dispersed throughout the cytoplasm, did not show significant differences in comparison to treated nontargeted cubosomes and untreated control cells. On the contrary, statistically significant differences were observed after 4 h of incubation. In those cells, a noteworthy increase in the IOD per cell of 1.6-fold ($p < 0.05$) was estimated with targeted cubosomes, in comparison to the 2 h treatment and 1.8-fold ($p < 0.001$) increase in comparison to that of nontargeted cubosomes. In contrast, the latter did not show significant differences in comparison to the 2 h treatment. At a longer exposure time (24 h), both nanoparticles were effectively internalized. However, treatment with targeted cubosomes caused a 1.2-fold increase ($p < 0.05$) in the IOD/

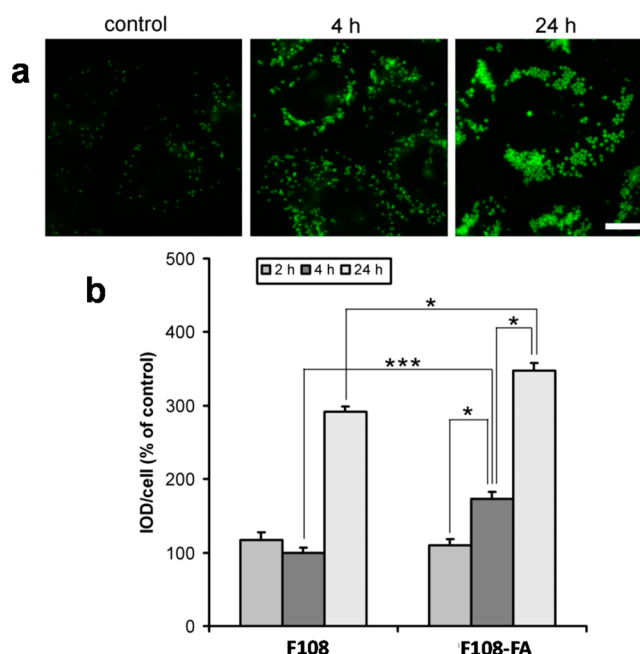


Figure 4. Lipid droplet accumulation induced in HeLa cells by cubosome treatment. (a) Representative color images of nontreated HeLa cells (control) and cells exposed to the monoolein-based cubosomes dispersed through a 80/20 mixture of F108/F108-FA at 4 and 24 h of incubation time. Scale bar = 20 μm . (b) Results of lipid droplet formation at different incubation times. Data are expressed as the mean $\pm$ SD from at least two independent experiments. Statistically significant differences are indicated by * ($p < 0.05$) and *** ($p < 0.0001$) via the t test.

cell in comparison to that of nontargeted nanoparticles (2.9- and 3.5-fold increases ($p < 0.0001$) in traditional and FA-containing cubosomes, respectively, versus the control). At this time, LDs appeared as fluorescent bright spots with a globular shape that were more numerous and larger in a remarkable way (1 to 2 μm diameter). Importantly, neither a sign of morphological damage nor chromatin condensation (Hoechst positive nuclei) was observed after both types of nanoparticle treatment (data not shown).

Confocal Microscopy. HeLa cells treated with dye-unloaded (control) or dye-loaded cubosomes were imaged by confocal microscopy after fixation with 4% paraformaldehyde for 10 min at room temperature (Figure 5). Note that chromatin was also stained with the Hoechst marker to identify the positions of cell nuclei (Figure 5b,e). Since the dye is infrared-emitting, fluorescence collection at >680 nm was obtained by using a single-photon avalanche photodiode with 30 and 5% photon sensitivities at 700 and 900 nm, respectively.

An inspection of control cells reveals that some autofluorescence is produced in the IR region of fluorescence collection upon excitation at 458 nm (Figure 5c). However, dye-loaded cells display significant differences in the morphology and distribution of emitters (Figure 5f). In fact, the dye-treated cells display a bright, cell-wide emission background together with large emitting perinuclear spots, while both features are nearly absent in control cells. (Note that panels c and f share the same fluorescence pseudocolor scale, i.e., the intensity differences are real.) Overall, these findings are consistent with an internalization of the dye mediated by cubosome nanocarriers.

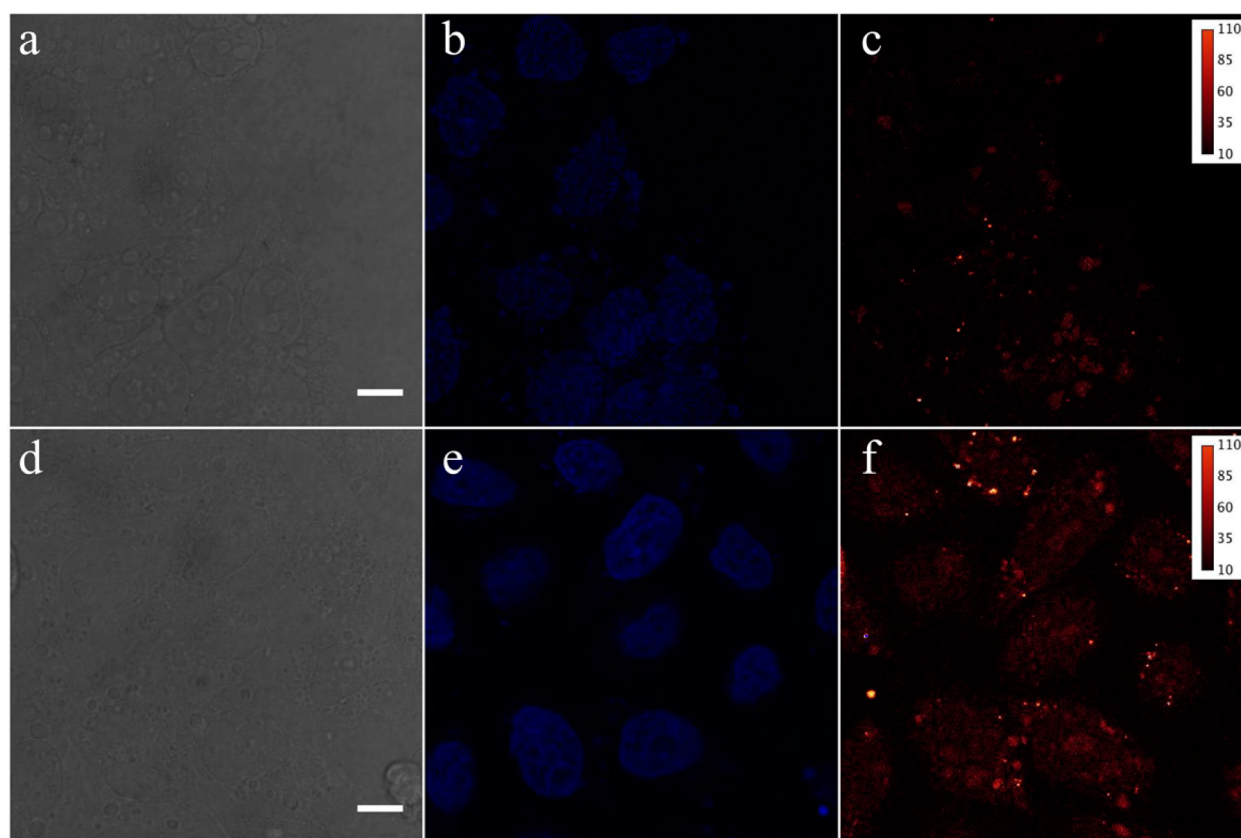


Figure 5. Fluorescence microscopy images of HeLa cells treated with dye-free (a–c) or dye-loaded cubosomes (d–f). (a, d) Transmission micrographs of cells; scale bar = 10 μm . (b, e) Fluorescence micrographs collected in the 420–480 nm interval where nuclear marker Hoechst emits. (c, f) Fluorescence micrographs collected at >680 nm where the dye emits; both images display fluorescence according to the same pseudocolor intensity scale (inset).

CONCLUSIONS

Recent works suggested the possibility of using cubosomes in theranostic nanomedicine.^{40,58,59} Here, the potential use of cubosomes as theranostic nanoparticles also having targeting properties was explored. The physicochemical investigations proved that these nanoparticles, still retaining their inner nanostructure, can be effectively stabilized by mixtures of Pluronic F108 and folate-conjugated F108. Furthermore, it was demonstrated that camptothecin, a potent anticancer drug, and an NIR-emitting fluorescent probe (a squaraine) can be successfully loaded within the cubosomes. Finally, the cellular uptake of these innovative nanocarriers was monitored on HeLa cells. Following the indication given by the cell lipid droplets in terms of their increase in number and growth in size, the superior performance of the cubosomes stabilized through the F108 and folate-conjugated F108 Pluronic mixtures was confirmed. Indeed, even if at longer treatment times (24 h) both targeted and nontargeted cubosomes were effectively internalized and differences were partially leveled out, results collected at short incubation time (4 h) clearly evidenced that the conjugation of Pluronic with folate speeds the cubosomes uptake as a result of receptor–ligand interactions.

On the whole, the results presented here validate cubosomes as a useful platform for theranostic nanomedicine.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the folate-conjugated Pluronic F108 synthesis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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III

Cubosome formulations stabilized by a dansyl-conjugated blockcopolymer for possible nanomedicine applications.

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Cubosome formulations stabilized by a dansyl-conjugated block copolymer for possible nanomedicine applications

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ABSTRACT

We present here an innovative, fluorescent, monoolein-based cubosome dispersion. Rather than embedded within the monoolein palisade, the fluorescent imaging agent, namely dansyl, was conjugated to the terminal ethylene oxide moieties of the block copolymer Pluronic F108. We discuss the physicochemical and photophysical properties of this fluorescent Pluronic and of a cubosome formulation stabilized by a mixture of dansyl-conjugated and non-conjugated Pluronic, also including an anticancer drug (quercetin). Furthermore, we performed biocompatibility tests against HeLa cells to assess internalization and cytotoxicity features of this nanoparticles aqueous dispersion. Cryo-TEM, SAXS, and DLS analysis, proved the bicontinuous cubic inner nanostructure and the morphology of this fluorescent cubosome dispersion, while photophysical measurements and biocompatibility results basically validate their potential use for theranostic nanomedicine applications.

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1. Introduction

Recent advances in nanomedicine resulted in the engineering of a number of nanoparticle systems based on hard and soft matter, able to simultaneously transport therapeutics and act as diagnostic agents. The new cross-discipline originating from these advances is known as theranostic nanomedicine [1–4]. With the purpose of enhancing the efficacy of the formulation, while reducing side effects, theranostic nanoparticles are designed for delivery to the site of disease, and within the same dosage, both pharmaceutically active molecules and imaging probes. This goal is often achieved by coating the surface of theranostic nanoparticles with a targeting ligand to specifically address the nanocarrier and its cargo to the pathological site. Additionally, this strategy of administration

should allow to overcome the differences in biodistribution and selectivity of the two distinct entities represented by the drug and the diagnostic agent [5,6].

Monoolein (MO, glycerol monooleate) is a polar lipid that in water, besides a reverse micellar and a lamellar phase, originates two cubic bicontinuous liquid-crystalline phases, namely, the gyroid (C_G , space group $Im3d$) and the double diamond (C_D , space group $Pn3m$), constituted by a curved, triply periodic, and non-intersecting MO bilayer, folded to form two disjointed continuous water channels [5–9]. The C_G and the C_D phases were virtually found ubiquitous in nature [10], and several papers were devoted to investigate the biological role of these nanostructures [10–13]. Remarkably, dispersion agents like Pluronics, nonionic poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymers, can be used to formulate these liquid-crystalline phases with cubic symmetry into stable colloidal dispersions in water, known as cubosomes [14,15]. It deserves noticing that colloidal dispersions characterized by a cubic inner structure are also formulated using other kinds of polar lipids (e.g., phytantriol or mixtures of soy phosphatidylcholine and glycerol dioleate), and dispersants (e.g., D- α -tocopheryl

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poly(ethylene glycol) 1000 succinate, or Polyoxyethylene (20) sorbitan monooleate) [16,17].

Cubosomes were only recently indicated as candidates for applications in theranostic nanomedicine [15,18,19]. Particularly, we demonstrated that cubosomes can be at the same time loaded with fluorophore probes (UV-visible or NIR emitting) and anticancer drugs (such as quercetin or camptothecin), and that they can be formulated with a mixture of Pluronic F108 (PF108) and folic acid-conjugated PF108 to make them cancer cell-specific targeted [20,21]. Among the numerous appealing features that can be exploited in theranostic nanomedicine, we can list the high structural stability and mechanical rigidity of cubosomes (due to their semi-rigid periodicity) they possess with respect to liposomes [22], their biodegradability (they are mainly prepared using monoolein, a biocompatible and biodegradable monoglyceride generally recognized as safe, GRAS, by the FDA) [23], and the possibility of carrying both hydrophobic and hydrophilic drugs. Importantly, their size fits perfectly that required in cancer therapy for the exploitation of the enhanced permeation retention (EPR) mechanism, while their PEG corona should prevent opsonization of these nanoparticles, thus slowing clearance from the bloodstream by the reticuloendothelial system [15,24]. Furthermore, in comparison with other lipid-based nanoparticles such as liposomes, cubosomes possess a huge interfacial surface area per unit of volume. Worth mentioning, *in vitro* investigations reported that components of the human plasma interact with the monoolein-based cubosomes provoking a cubic-to-hexagonal phase transition of the nanostructure [25,26]. According to SAXS experiments, such transition can be recorded few minutes after contact of the cubosomes with plasma, and it is completed after 1 h. Yet, an *in vivo* investigation of monoolein-based cubosomes loaded with a MRI contrast agent performed in rats, evidenced that the formulation, characterized by a half-life of about 90 min, was well tolerated, and successfully provided MRI contrast *in vivo* [18]. Therefore, independent of structure of the nanoparticles that finally reach the target tissue, the originally administered cubosome formulation was effective in delivering the imaging agent.

The formulation of functionalized cubosome dispersions is not trivial. Indeed, similarly to other self-assembled nanoparticles, cubosomes suffer of an intrinsic fragility. Therefore, even small amounts of additives used, for example, to impart them the desired features for nanomedicine application, may disturb the local surfactant dynamics, thus altering the original surfactant effective packing parameter with a consequent collapse of the cubic matrix [15,27]. In other words, additive loading may induce the transition toward other kinds of nanostructured dispersions, or to phase separation.

An innovative approach to confer cubosomes fluorescent properties while avoiding the impact of the imaging probe on the lipid bilayer was developed here. Particularly, the fluorophore, namely dansyl, was conjugated to the hydrophilic moieties of the PF108 used to stabilize the formulation. Given its solvatochromic properties, such a fluorophore was chosen to obtain further information on the nanostructure. In this paper, we discuss the physicochemical, photophysical, and HeLa cells internalization, and cytotoxic characteristics of this new cubosome formulation, also loaded with the anticancer drug quercetin.

2. Materials and methods

2.1. Chemicals

Monoolein (MO, 1-monooleoylglycerol, RYLO MG 19 PHARMA, glycerol monooleate; 98.1 wt %) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. The nonionic

poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymer Pluronic PF108 with a block length composition of EO₁₃₂–PO₅₀–EO₁₃₂ and an average molar mass of 14,600 g/mol, DMSO, Nile Red, MTT, quercetin, dansyl chloride ($\geq 95\%$), potassium carbonate (99%), acetonitrile ($\geq 99.9\%$), diethyl ether ($\geq 99.5\%$), and dichloromethane ($\geq 99\%$) were purchased from Sigma-Aldrich. Distilled water passed through a Milli-Q water purification system (Millipore) was used to prepare the samples.

2.2. Synthesis of dansyl-conjugated F108 (PF108-D)

K₂CO₃ (4 mg, 2.9×10^{-2} mmol) and dansyl chloride (6.3 mg, 2.32×10^{-2} mmol) were added to a solution of PF108–NH₂ (86 mg, 5.82×10^{-3} mmol) in anhydrous MeCN (5 ml), and the resulting mixture was refluxed under N₂ atmosphere overnight. The resulting turbid pale yellow solution was dried and the residue was dissolved in dichloromethane, filtered, and evaporated, under reduced pressure. The yellow oil obtained was washed with diethyl ether to eliminate excess dansyl chloride. The resulting yellow sticky oil was purified by dialysis (14 kDa MW cutoff) against deionized water, changed every 3–6 h for 3 days. The resulting product was lyophilized for 3 days to remove all of the residual water. Yield: 68% (60 mg, 3.94 mmol); mp: 49 °C. ¹H-NMR (400 MHz, DMSO-d₆, 298 K): δ 1.03 (d, $J=4$ Hz; 3H $\times$ 50, –CH₃ of PPO), 2.83 (s, 3H $\times$ 4, –N(CH₃)₂ of the dansyl moieties) 3.48–3.55 (m, 3H $\times$ 50, 4H $\times$ 264, –CH₂–CH(CH₃)–O– of PPO and –CH₂–CH₂–O– of PEO), 4.50–4.65 (m, 1H $\times$ 2, sulphonamidic –NH), 7.10–7.30 (m, 1H $\times$ 2, amidic –NH), 7.7–8.2 (m, 6H $\times$ 2, aromatic protons).

2.3. Sample preparation

Monoolein-based cubosomes were prepared and stabilized by dispersing the appropriate amount of MO in water solutions of a 65/35 mixture of PF108/PF108-D using an ultrasonic processor UP100H by Dr. Hielscher (100 W, 30 kHz), at 90% of the maximum power with pulses of 1 s interrupted by 1 s breaks for 10 min. Doped cubosomes were obtained by dispersing the drug in the melted monoolein with the help of an ultrasonic bath before mixing with the Pluronic solution. The sample volume was usually 4 ml with approximately 96.4 wt% of water, 3.3 wt% of MO, and 0.3 wt% Pluronics mixture. Quercetin percentage was 3.3×10^{-4} wt%. SAXS experiments were performed on samples prepared with 92.8 wt% of water, 6.6 wt% of MO, and 0.6 wt% of Pluronic mixture.

2.4. Dialysis and drug loading efficiency

After loading with quercetin, the cubosome dispersion was purified from the non-encapsulated drug by dialysis: 2 ml were loaded into a dialysis tubing cellulose membrane (14 kDa MW cutoff) and dialyzed against water (1000 ml), for 2 h (by replacing the water after 1 h) at 5 °C. Drug loading efficiency ($E\%$), expressed as percentage of the drug amount initially used, was determined by UV–vis spectroscopy after disruption of cubosomes with methanol. Quercetin content was quantified by a Thermo Nicolet Evolution 300 UV–vis spectrophotometer at 373 nm.

2.5. Cryogenic transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) experiments were performed to visualize the cubosome nanoparticles. Vitrified specimens were prepared in a controlled environment vitrification system (CEVS) at 25 °C and 100% relative humidity. A drop (3 μ l) of the sample was placed on a perforated carbon film-coated copper grid, blotted with filter paper, and plunged into

liquid ethane at its freezing point. More details about the methodology can be found elsewhere [28]. The vitrified specimens were transferred to a 626 Gatan cryo-holder and observed at 120 kV acceleration voltage in an FEI Tecnai T12 G² transmission electron microscope at about -175°C in the low-dose imaging mode to minimize electron-beam radiation-damage. Typical electron exposures were 10 to 15 $\text{e}^{-}/\text{\AA}^2$. Images were digitally recorded with a Gatan US1000 high-resolution ($2\text{k} \times 2\text{k}$ pixels) CCD camera, using the DigitalMicrograph software.

2.6. Dynamic light scattering

For the determination of the size of the nanoparticles, the block copolymer micelles and unimers, as well as the ζ -potential of the nanoparticles, dynamic light scattering (DLS), and electrophoretic mobility measurements, respectively, were performed at either 25°C or 50°C ($\pm 0.1^{\circ}\text{C}$) using ZetaSizer Nano ZSP (Malvern Instruments Ltd., Worcestershire, U.K.), which is equipped with a 10 mW helium–neon laser (operating at a wavelength of 633 nm). With this instrument, the DLS measurements are carried out at a fixed scattering angle $\theta = 173^{\circ}$ using a backscattering technique. The electrophoretic mobility measurements are performed at $\theta = 17^{\circ}$ using the M3-PALS (phase analysis light scattering) technique to detect the particle movement, which is used to calculate its electrophoretic mobility and from which the ζ -potential of the nanoparticles can be estimated [29,30]. The samples were put in disposable polystyrene cuvettes of 1 cm optical path length with Milli-Q water as solvent and the two types of measurements were thereafter conducted one after another, (DLS first). The cubosome nanoparticle samples were diluted 1:50 times prior to the measurements. The DLS measurements on the pure PF108 and PF108-D block copolymer solutions were performed without dilution at a concentration of 2.9×10^{-4} , and 3.8×10^{-4} M, respectively. The measured time correlation functions of the scattered intensity were analyzed using the software available in the Malvern instrument (CONTIN for PF108 and PF108-D block copolymer solutions) to obtain the intensity-weighted size distributions based on the apparent hydrodynamic diameter, d_H , which is calculated from the Stokes–Einstein relation $d_H = kT/6\pi\eta_0 D$, where D is the translational collective diffusion coefficient, obtained from the decay time of the correlation function, and η_0 is the viscosity of water. The reported d_H values correspond to an average of between three to five measurements. The DLS data were also analyzed using the cumulant method where the Z-average (intensity-weighted) d_H is calculated from the first cumulant from the second order analysis [31,32]. The polydispersity index (PDI) defined as the second cumulant, i.e., the width of the size distribution, divided by the first cumulant squared was obtained in the cumulant analysis. For all systems and both techniques, at least two independently prepared samples were investigated.

2.7. Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering was recorded with a S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). Cu K α radiation of wavelength 1.542 Å was provided by a GeniX x-ray generator, operating at 50 kV and 1 mA. A 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels, 54.0 μm wide was used for detection of scattered X-rays in the small-angle region. The working q range ($\text{\AA}^{-1}$) was $0.003 \leq q \leq 0.6$, where $q = 4\pi \sin(\theta)/\lambda$ is the scattering wave vector. For the analysis, thin-walled 2 mm glass capillaries were filled with the cubosome dispersions. The diffraction patterns of cubosomes were recorded for 3 h. The lattice parameter a of the cubic phases was determined using the relation $a = d(h^2 + k^2 + l^2)^{1/2}$ from linear fits of the plots of $1/d$ versus $(h^2 + k^2 + l^2)^{1/2}$, where $d = 2\pi/q$ (q is the measured

peak position) and h , k , and l , are the Miller indices. Water channel radii were calculated using the relation $R_w = [(A_0/2\pi\chi)^{1/2}a] - L$ [33], where L is the lipid length value (17 Å), a is the lattice parameter obtained from the SAXS analysis, and A_0 and χ are the surface area and the Euler characteristic of the infinite periodic minimal surface geometries ($Pn3m$, $A_0 = 1.919$, $\chi = -2$; $Im3m$, $A_0 = 2.345$, $\chi = -4$).

2.8. Photophysical characterization

Cubosome dispersions were diluted with Milli-Q water (1:6) and ultrafiltered with 450 μm filter before performing the photophysical measurements. The emission and excitation spectra were recorded with a PerkinElmer LS 55 spectrofluorimeter. The fluorescence quantum yield on PF108-D was determined by using the quinine sulphate dissolved in H_2SO_4 0.1 N as the reference standard ($\Phi_{\text{ref}} = 0.5654$). The absorption spectra were recorded on a Thermo Nicolet Evolution 300 spectrophotometer.

2.9. Cell culture

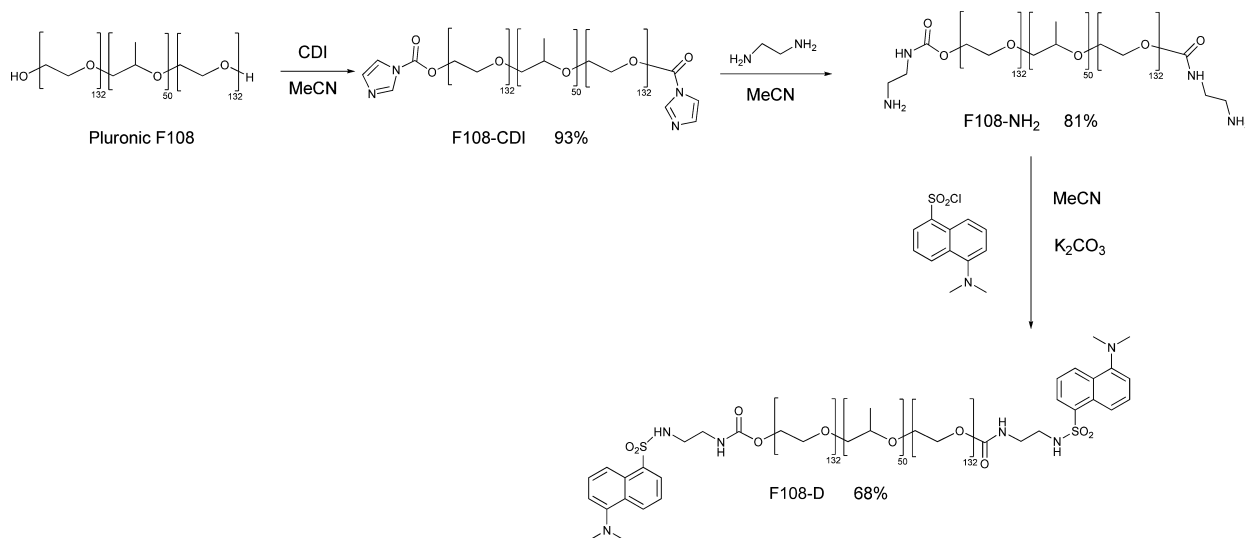
Human carcinoma HeLa cell line (ATCC collection) was grown in Phenol Red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U ml^{-1}), and streptomycin ($100 \mu\text{g ml}^{-1}$) (Invitrogen) in a 5% CO_2 incubator at 37°C . Cells were seeded in 35 mm dishes, and experiments were carried out 2 days after seeding, when cells had reached 90% confluency. Cubosomes (1:500, 2 μl of formulation in 1 ml of medium) were added to the cells and incubated at 37°C for 4 h. Live cell imaging experiments were performed as follows. The cubosome suspension was initially replaced with fresh serum-free medium, and then cells were loaded with the fluorescent probe Nile Red (NR). The fluorescent probe was washed out before the imaging session.

2.10. Fluorescence microscopy

Cells were stained with 300 nM NR (9-diethylamino-5H-benzo[a]phenoxazine-5-one) for 30 min. Microscopy observations were made with a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with $10\times$, $20\times$, and $40\times/0.75$ NA water-immersion objectives and an HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome-cooled CCD camera (QICAM, Qimaging, Canada) with variable exposure. For the observation of the NR probe, the filters were ex 470 ± 20 nm and em 535 ± 40 nm for nonpolar lipids, and ex 546 ± 6 nm and em 620 ± 60 nm, for total lipids. Image alignments were obtained with Image Pro Plus software (Media Cybernetics, Silver Springs, MD).

2.11. Lipid droplet quantification

Cells, after nanoparticle treatments, were stained with NR. The latter is an ideal probe for the detection of lipids, as it exhibits high affinity, specificity, and sensitivity, to the degree of hydrophobicity of lipids. The latter feature results in a shift of the emission spectrum from red to green in the presence of polar, and nonpolar lipids, respectively. For this reason, in staining live cells with NR, cytoplasmic membranes are stained red, whereas neutral lipids of lipid droplets are stained green. Measurements, made on a single-cell basis, concerned the amount of lipid droplet content, evaluated by the fluorescence intensity of NR-green emission. The IOD (integrated optical density) per cell was calculated using the Image ProPlus software.



Scheme 1. Reaction scheme for the synthesis of F108-D.

2.12. Cytotoxic activity: MTT assay

The cytotoxic effect of nanoparticle formulations was evaluated in HeLa cells by the MTT assay. HeLa cells were seeded in 24-well plates at density of 3×10^4 cells/well in 500 μ l of serum-containing media. Experiments were carried out 2 days after seeding when cells had reached 90% confluence. Cubosomes were added to the cells at a concentration of 1:500 (2 μ l of sample in 1 ml of serum-free medium) and incubated at 37 °C for 4 h. A 50 μ l portion of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml in H₂O), was then added and left for 2 h at 37 °C. The medium was aspirated, 500 μ l of DMSO was added to the wells, and color development was measured at 570 nm with an Infinite 200 auto microplate reader (Infinite 200, Tecan, Austria). The absorbance is proportional to the number of viable cells. All measurements were performed in quadruplicate and repeated at least three times. Results are shown as percent of cell viability in comparison with non-treated control cells.

2.13. Statistics

Statistical analysis was carried out with Excel (Microsoft Co., Redmond, WA) or Graph Pad INSTAT software (GraphPad software, San Diego, CA). Results were expressed as a mean $\pm$ standard deviation (SD) of two independent experiments involving duplicate analyses for each sample. Statistically significant difference was evaluated by two sample *t*-tests with $p < 0.05$ as a minimal level of significance, or using one-way analysis of variation (One-way ANOVA) and the Bonferroni Post Test.

3. Results

3.1. Fluorescent cubosome formulation and physicochemical characterization

The block copolymer Pluronic F108 (PF108) was synthetically modified as recently reported in the literature [20], by reaction with ethylenediamine after activation with CDI (carbonyldiimidazole) to obtain PF108-NH₂. The aminated derivative was then reacted with commercially available dansyl chloride in anhydrous MeCN in the presence of K₂CO₃ as a base (Scheme 1). The sulfonamide derivative PF108-D was obtained in 68% yield. To avoid significant alterations of the pristine cubosome characteristics, the fluorescent block copolymer PF108-D was used to stabilize the formulation in

mixture with the non-conjugated PF108. Preliminary tests indicated this mixture to be adequate in the ratio PF108/PF108-D=65/35. The cryo-TEM images of the monoolein colloidal dispersion stabilized with such a mixture (cubosomes-D), encapsulating an anticancer drug, namely quercetin, are shown in Fig. 1. Quercetin loading efficiency (*E*%) was found to be 80%.

The cryo-TEM direct images of the nanoparticle dispersions reveal a colloidal dispersion consisting of nanoparticles of a diameter varying in the range from about 50 to 300 nm, characterized by a highly ordered inner nanostructure consistent with that expected for cubosomes. From a morphological point of view, along with many cubic-shaped nanoparticles, numerous round-shaped cubosomes were also observed. Moreover, as previously reported in the literature, cryo-TEM images also show several small unilamellar vesicles as well as nanoparticles where the so-called inter-lamellar attachments can be clearly seen. [34]

SAXS was used to confirm the cubic symmetry of the nanoparticles. To improve the signal-to-noise ratio, these experiments were performed on samples formulated with 7.2 wt% of the dispersed phase (MO + PF108/PF108-D mixture, see also paragraph 2.3). The diffraction patterns reported in Fig. 2 revealed the coexistence of two phases identified through at least two Bragg peaks with relative positions in a ratio of $\sqrt{2}:\sqrt{3}$, which we indexed as the (110) and (111) reflections of a double diamond inverse bicontinuous cubic phase (*Pn3m* crystallographic space group), and three Bragg peaks with relative positions in a ratio of $\sqrt{2}:\sqrt{4}:\sqrt{6}$, which we indexed as the (110), (200), and (211) reflections of a primitive inverse bicontinuous cubic phase (*Im3m* crystallographic space group) [34]. Structural parameters from SAXS measurements, such as the lattice parameter (*a*) and the water channel radius (*r_w*), are reported in Table 1.

DLS results reported in Table 1 showed that the average hydrodynamic diameters of the nanoparticles are in good agreement with that estimated from cryo-TEM images, and the size polydispersity (*i.e.*, the PDIs) and the ζ -potential do not significantly diverge from those reported for similar formulations [20,21].

Formulations here investigated display a negative charge although they are constituted by nonionic molecules. This fact deserves some comments. To exclude the possibility that the negative charge might be ascribed to the presence of MO impurities, a cubosome formulation was prepared using pure (>99%) MO from Sigma Aldrich. This formulation was characterized by an average diameter of the nanoparticles of 133 ± 1 nm and a negative zeta-potential of -16 ± 1 mV. Moreover, also cubosomes formulations

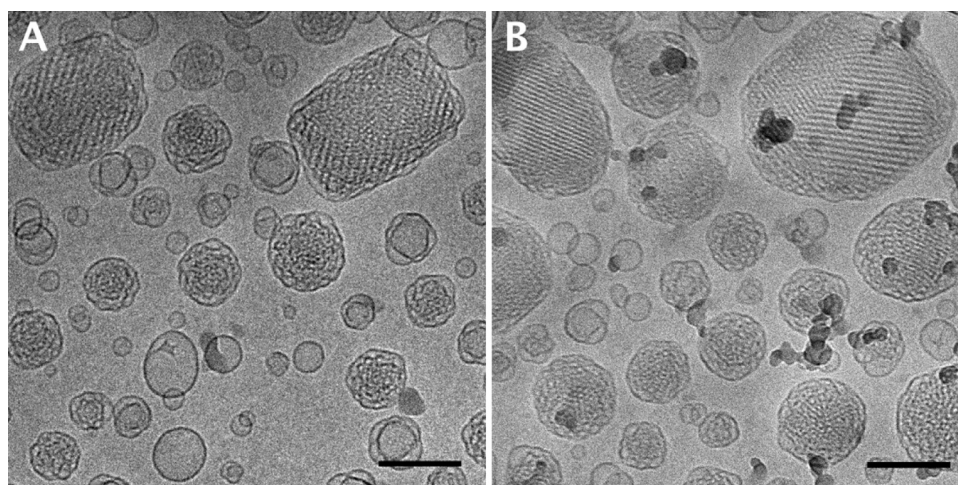


Fig. 1. Cryo-TEM image of monoolein-based cubosome nanoparticles empty (A) or containing the anticancer drug quercetin (B) and prepared using a mixture of PF108 and PF108-D in the ratio 65/35. Small dark globules in B are frost particles. Bars correspond to 100 nm.

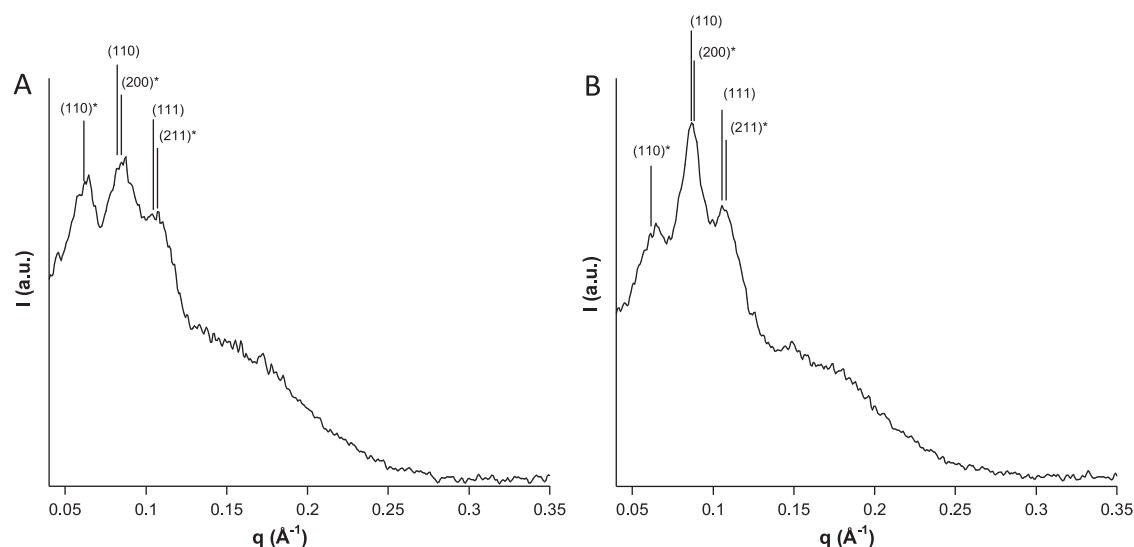


Fig. 2. SAXS diffractograms of monoolein-based cubosome nanoparticles either empty (A) or loaded with quercetin (B) and prepared using a mixture of PF108 and PF108-D in the ratio 65/35. Experiments were performed on samples formulated with 7.2 wt% of the dispersed phase (see also the text).

prepared using phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) show a negative zeta-potential (about -9 mV) [35]. It should be noticed that, differently from monoolein, this polar lipid does not contain ester bonds that can be hydrolyzed to form a carboxylic acid. Consequently, also the presence of small amounts of acids due to monoolein hydrolysis products (though possible) cannot fully justify the measured zeta-potentials in all these different systems. On the other hand, hydroxide ions adsorption (a phenomenon often observed at the oil/water interface), originating a polarized water layer surrounding the outer surface of the nanoparticles, could explain the observed negative charge [36,37].

As expected, cryo-TEM, SAXS, and DLS analysis confirmed that the presence of the fluorophore molecules in the external

polyethylene oxide corona of the cubosomes did not modify the supramolecular morphological features of the colloidal dispersion. Furthermore, after encapsulation of quercetin within the lipid bilayer, the inner nanostructure of the cubosomes was preserved.

3.2. PF108-D, PF108 and fluorescent cubosomes. Physicochemical and photophysical characterization

Before analyzing the UV-visible light emission/absorption characteristics of the new cubosome formulation, the physicochemical and photophysical properties of the pure block copolymers (PF108 and PF108-D) in water were investigated. When dispersed in aqueous solution the PEO-PPO-PEO block copolymers essentially

Table 1

Space groups (in parenthesis), lattice parameters (a), and water channel radii (r_w) obtained from SAXS experiments, mean hydrodynamic diameters (d_H), polydispersity indexes from cumulant analysis (PDI), and ζ -potentials obtained from DLS experiments on the cubosome formulations prepared with a mixture of F108 and F108-D in the 65/35 ratio (wt%). An estimation of the sample stability is also reported. DLS and SAXS experiments were respectively performed on samples formulated with 3.6 and 7.2 wt% of the dispersed phase (see also the text). Errors are reported as $\pm$ estimated standard deviation (SD).

Formulation	a (Å)	r_w (Å)	d_H (nm)	PDI	ζ -Potential (mV)	Stability (weeks)
Cubosomes-D	104 ± 1 (Pn3 m) 146 ± 1 (Im3 m)	$24 \pm 128 \pm 1$	168 ± 1	0.15	-21 ± 1	>24
Cubosomes-D + QUE	102 ± 1 (Pn3 m) 143 ± 1 (Im3 m)	$23 \pm 127 \pm 1$	171 ± 1	0.14	-23 ± 1	>24

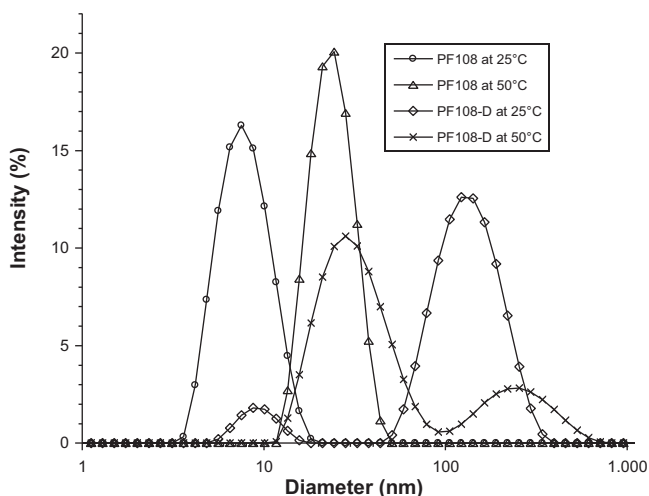


Fig. 3. Intensity-weighted size distributions (expressed in terms of apparent hydrodynamic diameter) obtained from DLS measurements on PF108 and PF108-D solutions with the concentrations of 3.8×10^{-4} and 2.9×10^{-4} M respectively at 25 and 50 °C.

behave like classical surfactants containing PEO. Therefore, above the critical micelle concentration (CMC), these block copolymers self-assemble forming variously shaped (spherical or rod-like depending on the PPO and PEO block length) micelles [38]. These copolymers also present a critical micelle temperature (CMT) due to the fact that PPO becomes hydrophobic at higher temperatures. The CMT can be determined using differential scanning calorimetry (DSC) as the DSC signal is related to the change in the hydration of PPO, a process with an endothermic transition enthalpy, which occurs when the micelles are formed [39]. Furthermore, in the same study, it was found that the CMT increases with a decreasing copolymer concentration. However, the description of spherical micelles with a core of dehydrated PPO blocks surrounded by hydrated PEO blocks constituting an outer corona, although useful for explaining most of the experimental data, is quite far from representing the real micellar organization, since water can penetrate the micellar core to some extent, and miscibility of the PEO and PPO blocks is also allowed [40].

Water solutions of PF108 and PF108-D with the concentrations of 3.8×10^{-4} M, and 2.9×10^{-4} M, respectively, were studied at 25 and 50 °C using dynamic light scattering. The intensity-weighted hydrodynamic diameter distributions obtained from the data analysis described above are reported in Fig. 3. The absence of micelles in both solutions at 25 °C was expected because of the low concentrations used, which were well below the CMC at this temperature [41]. In our measurements, the PF108 solution at 25 °C presents a monomodal size distribution with a mean hydrodynamic diameter of 8.1 nm, which can be attributed to the copolymer monomers. When the temperature is raised to 50 °C (above the critical micelle temperature for a PF108 concentration of 3.8×10^{-4} M), the monomodal size distribution shifts to a larger mean diameter of 24.3 nm. This mode can be assigned to micelles of PF108 and the size is in accordance with literature [41], differently from the unmodified copolymer solution, at 25 °C, the PF108-D solution shows a bimodal size distribution characterized by two peaks centered at 8.7 and 137.3 nm, respectively (Fig. 3). Bimodal size distributions are often found in DLS measurements on PEO–PPO–PEO block copolymer solutions with concentrations below CMC or at temperatures below CMT. The two modes are typically related to the simultaneous presence of unimers and clusters. The latter is an effect of the presence of low molecular weight (and more hydrophobic) copolymer fragments, the amount of which can differ between different commercial batches [42,43]. These

contaminants become incorporated into the micelles once they are formed, except the most hydrophilic components, that stay in solution [44,45]. Here, this scenario can be ruled out because of the absence of clusters in the PF108 solution (both block copolymers belong from the same batch). It is rather the hydrophobic interactions between the dansyl moieties that may play an important role in the formation of aggregates of PF108-D. This can justify the existence of a second slow mode in the distribution. The DLS results for the PF108-D solution at 50 °C also reveal two modes, one corresponding to a hydrodynamic mean diameter of 26.2 nm and one to 150.5 nm. The fast mode is attributed to PF108-D micelles, whereas the slower one could possibly be related to aggregates of some sort (however, not to unimer clusters as discussed above). It is worth noticing that they are very few in numbers, but could well be the effect of an ill-set base line in the calculations. Indeed, this peak was found strongly affected by a base line contribution (several measurements were performed). An evidence of their low number is given in Fig. S1 (Supplementary data), where the same size distribution presented in Fig. 3 is reported as number-averaged instead of intensity-averaged.

As a concluding remark, DLS results evidenced that conjugation of the dansyl moiety to the end of the PF108 arms significantly alter the self-assembling properties of this block copolymer.

Dansyl is a hydrophobic naphthalene derivative showing a fluorescence emission that shifts bathochromically as the polarity of the surrounding medium increases. Therefore, fluorescence spectroscopy studies can give important information about the environment experienced by this molecule. The absorption spectrum of PF108-D showed an absorption band at 247 nm ($\epsilon = 30,168 \text{ M}^{-1} \text{ cm}^{-1}$) and a less intense band at 344 nm ($\epsilon = 7980 \text{ M}^{-1} \text{ cm}^{-1}$) as shown in Fig. 4A. Upon excitation at 344 nm, an emission band centered at 532 nm ($\Phi = 4.25 \times 10^{-3}$) was observed, as shown in Fig. 4B. Previous studies on the dansyl molecule reported a fluorescence emission maximum, which shifts from 494 nm when dispersed in a 10% Pluronic L62 ($\text{EO}_6\text{PO}_{36}\text{EO}_6$) water solution (where the dye preferentially resides in the micelle hydrophobic core) to about 529 nm when the dispersion medium was a tetraethylene glycol/water mixture (used to mimic the micellar PEO corona) [46]. Moreover, Bright and co-workers [47], showed that in a dendrimeric family functionalized with a dansyl moiety increase in dendrimers generation (from the first to the third) caused a hypsochromic shift in the fluorophore emission (547 nm, 542 nm, and 535 nm, for the first, second, and third generation, respectively) as a result of a less polar microenvironment surrounding the dansyl group. These results are also in accordance with those reported in the literature for a dansyl group attached to dendrimers [47,48], silica nanoparticles [49], or cyclodextrines [50]. Consequently, the emission observed in the aqueous solution of the PF108-D can be considered a robust evidence that, rather than completely exposed to the water molecules, the fluorogenic fragments experience a less polar environment mainly constituted by the EO groups.

The absorption spectrum of the cubosomes formulation prepared using the mixture of PF108 and PF108-D as a stabilizer could not be recorded due to the intense scattering of the sample. However, when excited at 344 nm, an emission band at 531 nm was found. The similarity of this emission band with that observed in the PF108-D aqueous solution strongly suggests that the fluorogenic fragments are at least partially buried within the PEO corona.

3.3. Fluorescent cubosome internalization and cytotoxic features against HeLa cells

The fluorescent cubosome formulation was then investigated *in vitro* to test cellular internalization and cytotoxicity features in HeLa cell culture. Fig. 5A shows the optical images which highlight

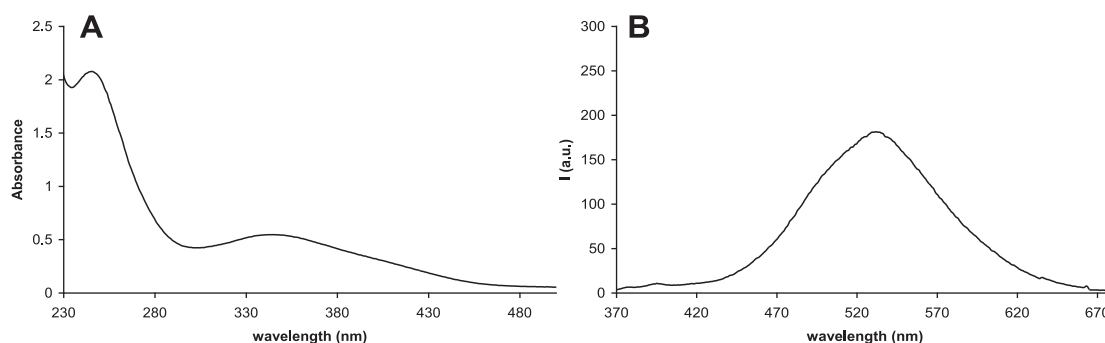


Fig. 4. Absorption (A) and emission (B) spectra ($\lambda_{\text{exc}} = 344 \text{ nm}$, $\lambda_{\text{em}} = 532 \text{ nm}$) of a $6.9 \times 10^{-5} \text{ M}$ water solution of PF108-D.

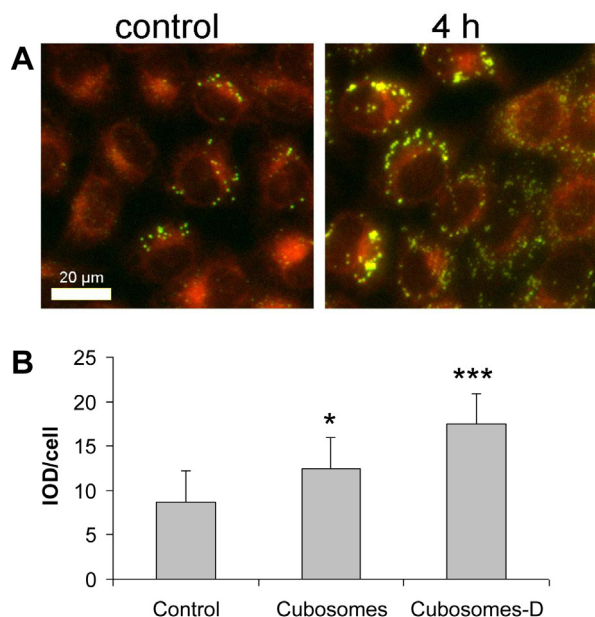


Fig. 5. Lipid droplet accumulations induced in HeLa cells by cubosome treatment. (A) Representative composite color images of HeLa cells exposed to monolein-based cubosomes (without quercetin loading) at 4 h incubation. Membranes (red) and lipid droplets (green) were stained with Nile Red. (B) IOD (integrated optical density) per cell related to lipid droplet accumulation in HeLa cells exposed to lipid-based cubosomes. Data are expressed as mean $\pm$ SD from at least two independent experiments. Lipid droplet quantification was performed with Image Pro Plus. * and *** represent $p < 0.05$ and $p < 0.001$ versus control, respectively.

the lipid droplet accumulation in HeLa cells as a result of cubosome treatment.

The lipid droplets (LDs), cytoplasmic organelles involved in the controlled synthesis and mobilization of fat stores, consist of a phospholipid monolayer surrounding a hydrophobic core of neutral lipids [51]. Although marked differences in terms of size, number, and lipid content, are observed from one cell type to another [52], their increase basically occurs because of exogenous lipids uptake. Indeed, cell internalization exposes cubosomes to endosomal/lysosomal degradation into the single components (MO and the block copolymer), with a consequent redistribution of the lipids between the cytoplasmic membranes and the lipid droplets. Therefore, the amount of LDs straightforwardly reflect the internalization of the cubosomes via endocytosis pathway, and LDs accumulation can be used, at least in principle, to validate the cellular uptake of all kinds of lipid-based nanoparticles. Accordingly, LDs accumulation following monolein-based nanoparticles (vesicles or cubosomes) administration was previously reported [20,53]. Here, the lipid droplet growth caused by the internalization of two cubosome formulations (not carrying the quercetin) prepared with PF108 or with

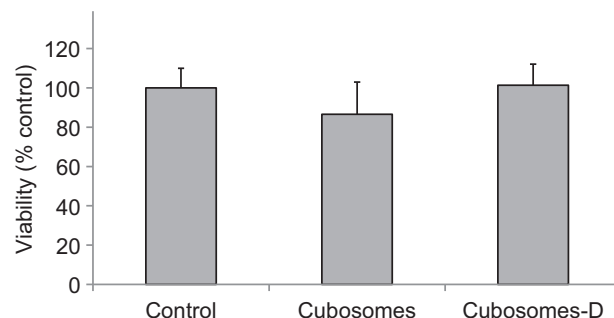


Fig. 6. Viability, expressed as % of the control, induced by incubation for 4 h with lipid-based cubosomes (1:500, 2 $\mu\text{l/ml}$) in HeLa cells culture. Three independent experiments were performed and data are presented as mean $\pm$ SD ($n = 12$).

the PF108/PF108-D mixture was confirmed and quantified via estimation of the IOD (integrated optical density) per cell. An increased production of lipid droplets was observed for both nanoparticles at 4 h of incubation time in comparison with untreated-control cells, as shown in Figs 5A and B. Results evidenced a statistically different IOD/cell between nanoparticle formulations, very likely caused by the dansyl moieties exposed on the surface of the cubosomes. Indeed, dansyl may to some extent, alter the molecular recognition characteristics of the cubosomes' surface, changing their interactions with the cells.

The two aforementioned nanoparticle formulations were also tested for cytotoxicity (MTT assay) in HeLa cells. Fig. 6 shows the viability, expressed as % of the control, induced in HeLa cells after 4 h incubation, and evidences that treatments with both nanoparticle formulations did not induce a relevant reduction in cell viability in comparison with control cells.

4. Conclusions

When dealing with soft matter based nanoparticles (such as cubosomes or hexosomes), one of the issues researchers working in the field of nanomedicine have to face is the preservation of their morphology and internal structure after cargos loading. To overcome this problem, we proposed here a strategy that considers the conjugation of a fluorescence probe, namely dansyl, to the end of the hydrophilic arms of the PEO–PPO–PEO block copolymer PF108, which is used to stabilize an aqueous dispersion of monolein-based cubosomes. In this way, both the morphology and topology were not significantly altered by the presence of the fluorescent molecules.

The physicochemical investigation presented here demonstrate that a mixture of this new fluorescently labeled block copolymer PF108-D and the original PF108 copolymer is effective in stabilizing in water the cubosome formulation also encapsulating the anti-cancer drug quercetin. The performed steady-state fluorescence

and UV–vis experiments evidenced good photophysical properties of such a formulation, while the microscopic observations on HeLa cell cultures proved their effective internalization and high biocompatibility (when not carrying the drug), when used at the same concentration suitable for imaging purposes.

Taken on the whole, the results presented in our study validate the potential use of this new cubosome formulation in theranostic nanomedicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2015.03.025>.

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IV

Docetaxel loaded fluorescent liquid crystalline nanoparticles for cancer theranostics.

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Docetaxel-Loaded Fluorescent Liquid-Crystalline Nanoparticles for Cancer Theranostics

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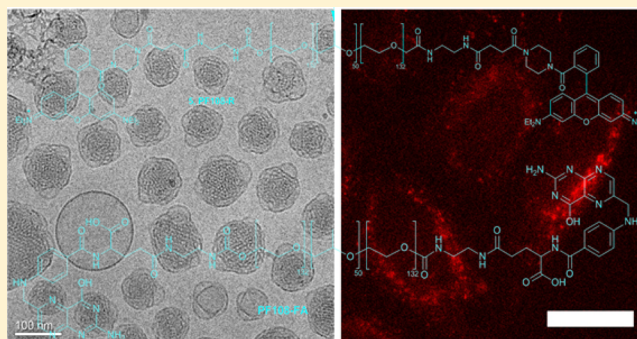
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ABSTRACT: Here, we describe a novel monoolein-based cubosome formulation engineered for possible theranostic applications in oncology. The Docetaxel-loaded nanoparticles were stabilized in water by a mixture of commercial Pluronic (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer) F108 (PF108) and rhodamine- and folate-conjugated PF108 so that the nanoparticles possess targeting, therapeutic, and imaging properties. Nanoparticles were investigated by DLS, cryo-TEM, and SAXS to confirm their structural features. The fluorescent emission characterization of the proposed formulation indicated that the rhodamine conjugated to the PF108 experiences an environment less polar than water (similar to chloroform), suggesting that the fluorescent fragment is buried within the poly(ethylene oxide) corona surrounding the nanoparticle. Furthermore, these nanoparticles were successfully used to image living HeLa cells and demonstrated a significant short-term (4 h incubation) cytotoxicity effect against these cancer cells. Furthermore, given their analogy as nanocarriers for molecules of pharmaceutical interest and to better stress the singularities of these bicontinuous cubic nanoparticles, we also quantitatively evaluated the differences between cubosomes and multilamellar liposomes in terms of surface area and hydrophobic volume.



1. INTRODUCTION

The amphiphilic characteristics of polar lipids drive their self-assembly in water, giving a number of liquid-crystalline phases.^{1,2} Among them, the most interesting are probably the reverse bicontinuous cubic phases.³ These are a family of three-dimensional cubic structures constituted by a curved, non-intersecting lipid bilayer folded to form two disconnected, continuous water channels with a crystal lattice that can be described by three different types of infinite periodic minimal surfaces: the double diamond (space group $Pn\bar{3}m$), the gyroid (space group $Ia\bar{3}d$), and the primitive (space group $Im\bar{3}m$). These phases have been extensively investigated in recent decades because they represent versatile nanostructures able to incorporate molecules of biological relevance.^{4–8} Interestingly, cubic phases can be formulated as aqueous nanoparticle dispersions, known as cubosomes.⁹ Although various emulsifiers have been suggested,^{10,11} in general, the stabilization in water of such liquid-crystalline nanoparticles is achieved using amphiphilic polymers having long poly(ethylene oxide) (PEO) chains, e.g., Pluronics (poly(ethylene oxide)-poly(propylene

oxide)-poly(ethylene oxide) triblock copolymers) or polysorbate 80.^{9,12}

Similar to the reverse liquid-crystalline cubic bulk phases, pharmaceutical applications are predicted for cubosomes,^{13–17} and their potential use as nanocarriers for MRI probes has been investigated.^{18,19} These nanoparticles have also been proposed for theranostic applications.^{20–25} Indeed, several peculiar characteristics make cubosomes appealing as theranostic tools in oncology. Among these, one of the most distinguishing is the higher hydrophobic volume with respect to liposomes, often considered to be the golden standard for lipid-based nanocarriers in medicine. However, a quantitative comparison of the hydrophobic portion of these two kinds of nanoparticles has never been reported in the literature. Other cubosome-specific features are the simultaneous possibility of host therapeutics and imaging agents as well as a size (typically in the range 100–

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200 nm) that, at least in principle, allows the exploitation of the enhanced permeation retention mechanism for the passive targeting of cancer tissues.^{9,26} In view of their application as nanomedicine, it is also worth mentioning that the clearance from the bloodstream via the mononuclear phagocytic system should be retarded since, by analogy to stealth liposomes, these nanoparticles are enclosed within a sort of a PEO corona that should prevent efficient recognition by opsonin, thus hindering phagocytosis.²⁷ Moreover, it was demonstrated that cubosomes (and hexosomes) can be suitably formulated by covering their surface with folate residues^{20,28} or anti-EGFR Fab²⁹ to actively target these nanocarriers toward cancer cells, while the effects on the subcellular level of pristine monoolein-based cubosomes were recently investigated for HeLa cells.³⁰ Remarkably, nanoparticles with reverse bicontinuous cubic internal structure endowed with long-time stability and thermoresponsive capabilities can be formulated using polymer–surfactant mixtures.³¹

To explore further the potential of lipid-based liquid-crystalline nanoparticles as theranostic nanomedicine in cancer treatments, we report here the loading of monoolein-based cubosomes with a potent anticancer drug (Docetaxel). Such a formulation was stabilized with a mixture of commercial Pluronic F108 (PF108), folate-conjugated PF108 (PF108-FA), and rhodamine-conjugated PF108 (PF108-R) to simultaneously confer targeting, therapeutic, and imaging abilities on these nanoparticles. It deserves notice that engineering stable, multifunctional cubosome formulations can be a hard task. While the hydrophobic effect represents the main force that drives the self-assembly of the lipids constituting the nanoparticles, their inner phase is basically determined by the geometrical features of the lipid hydrophobic tail and polar head. This is rationalized by the molecular effective packing parameter (P_{eff}) given by the ratio v/al , where v and l are, respectively, the hydrophobic chain volume and length (taken as 80% of the fully extended chain) and a is the headgroup area.¹ P_{eff} is strongly dependent on the surrounding environment; therefore, the type of phase that characterizes the nanoparticles (and their very existence) is barely predictable in the presence of additives, since encapsulation within the lipid bilayer of molecules useful for giving these nanoparticles theranostic skills may significantly alter P_{eff} , thus provoking the transition of the inner phase or the collapse of the formulation.⁹ In addition, the decoration of the cubosome surface with targeting and/or fluorescent moieties may disturb both the colloidal stability of the formulation and the cellular uptake of the nanoparticles. Throughout this paper we will demonstrate that the proposed cubosome formulation, although prepared with a high portion (40%) of conjugated PF108, is stable and successfully delivers its cargo to HeLa cells, taken as the standard for cancer cells.

Moreover, considering the analogies between liposomes and cubosomes, we also presented here a theoretical evaluation of the hydrophobic volume of these liquid-crystalline nanoparticles.

2. MATERIALS AND METHODS

2.1. Materials. Monoolein (MO, 1-monooleoylglycerol, RYLO MG 19 PHARMA, glycerol monooleate, 98.1 wt %) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. Pluronic F108 (PEO₁₃₂-PPO₅₀-PEO₁₃₂), Docetaxel ($\geq 97\%$), *N*-hydroxysuccinimide (NHS, 98%), *N,N'*-dicyclohexylcarbodiimide (DCC, $\geq 99.5\%$), triethylamine ($\geq 99.5\%$), and dimethyl sulfoxide ($\geq 99.7\%$)

were purchased from Sigma-Aldrich. Distilled water passed through a Milli-Q water purification system (Millipore) was used to prepare the samples.

2.2. Sample Preparation. Monoolein-based cubosomes were prepared and stabilized by dispersing the appropriate amount of MO in aqueous solutions of a 60/20/20 mixture of Pluronic F108 (PF108) and folate-conjugated and rhodamine-conjugated PF108 (PF108/PF108-FA/PF108-R) using an ultrasonic processor (UP100H by Dr. Hielscher, cycle 0.9, amplitude 90%) for 10 min. Docetaxel-doped cubosomes were obtained by dispersing the drug in the melted lipids (at 37 °C) with the help of an ultrasonic bath before mixing with the Pluronic solution. The sample volume was usually 4 mL with approximately 96.4 wt % water, 3.3 wt % MO, and 0.3 wt % Pluronic mixture.

2.3. Synthesis of Rhodamine-Conjugated PF108 (PF108-R). PF108-NH₂ (0.1976 g, 0.013 mmol), *N*-hydroxysuccinimide (NHS, 0.0125 g, 0.108 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC, 0.0188 g, 0.091 mmol), and *N*-(9-(2-(4-(3-carboxypropanoyl)piperazine-1-carbonyl)benzyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium (4; 0.024 g, 0.039 mmol) were dissolved in 8 mL of anhydrous DMSO in the presence of triethylamine (20 μ L). To precipitate DCU (dicyclohexylurea), 20 mL of deionized water was added, and the mixture was then centrifuged and filtered to obtain a red limpid solution. This was dialyzed (14 kDa MW cutoff membrane) for 3 days against ultrapure water, which was changed every 2–6 h. The resulting product was lyophilized for 1 day to remove all of the residual water. Yield 63% (130.3 mg, 0.008 mmol); mp 48–50 °C. ¹H NMR (400 MHz, DMSO-*d*₆, 298 K): δ H 1.045 (3H $\times$ 50, –CH₃ of PPO), 1.17–1.33 (m, 3H $\times$ 2, –N(CH₂–CH₃)₂), 2.66–2.69 (m, 4H $\times$ 2, aliphatic protons from rhodamine moiety), 3.29–3.55 (m, 3H $\times$ 50, 4H $\times$ 264, –CH₂–CH(CH₃)–O– of PPO and –CH₂–CH₂–O– of PEO), 3.60–3.73 (m, 2H $\times$ 2, –N(CH₂–CH₃)₂), 4.53–4.56 (m, 1H $\times$ 2, amidic –NH), 6.95–8.07 (m, 10H $\times$ 2, aromatic protons). IR (solid state, cm^{–1}) ν = 2876 (s), 1584, 1635, 1714 (w).

2.4. Photophysical Characterization. Cubosome dispersions were diluted with Milli-Q water (1:100) before performing the photophysical measurements. The emission and excitation spectra were recorded with a PerkinElmer LS 55 spectrofluorimeter. The fluorescence quantum yield on PF108-R was determined by using Rhodamine 6G dissolved in EtOH as the reference standard (Φ_{ref} = 0.94). The absorption spectra were recorded on a Thermo Nicolet Evolution 300 spectrophotometer.

2.5. Cryogenic Transmission Electron Microscopy (cryo-TEM). Vitrified specimens were prepared in a controlled environment vitrification system (CEVS) at 25 °C and 100% relative humidity. A drop (~ 3 μ L) of the sample was placed on a perforated carbon film-coated copper grid, blotted with filter paper, and plunged into liquid ethane at its freezing point. The vitrified specimens were transferred to a 626 Gatan cryo-holder and observed at 120 kV acceleration voltage in an FEI Tecnai T12 G² transmission electron microscope at about –175 °C in the low-dose imaging mode to minimize electron-beam radiation damage. Images were digitally recorded with a Gatan US1000 high-resolution CCD camera.

2.6. Small-Angle X-ray Scattering (SAXS). The characterization of the nanoparticle structure as a function of temperature was carried out by SAXS using a Ganesha 300XL (SAXSLAB ApS, Skovlunde, Denmark). This instrument is equipped with a 2D 300 K Pilatus detector (Dectris Ltd., Baden, Switzerland) and a Genix 3D X-ray source (Xenocs SA, Sassenage, France), generating X-rays at a wavelength, λ , of 1.54 Å. The scattering data was collected over a q range of $0.014 < q$ (Å^{–1}) < 0.753 , where the magnitude of the scattering vector, q , is defined as $q = (4\pi/\lambda) \sin(\theta/2)$, where θ is the scattering angle. The two-dimensional scattering pattern was radially averaged using SAXSGui software to obtain $I(q)$. Measurements were performed in 1.5 mm quartz capillaries (Hilgenberg GmbH, Malsfeld, Germany). The temperature was controlled by an external recirculating water bath within ± 0.3 °C. Samples were equilibrated for 1 h at the measurement temperature prior to performing the experiment.

Water channel radii of the reverse bicontinuous cubic phases were calculated using the relation $r_w = [(A_0/2\pi\chi)^{1/2}a] - L$, where L is the length of the lipid hydrophobic chain (17 Å, in the case of MO), a is the lattice parameter obtained from the SAXS analysis, and A_0 and χ are the surface area and the Euler characteristic (integral Gaussian curvature scaled by 2π) of the infinite periodic minimal surface geometries within the unit cell, explained further in the Appendix ($Pn3m$, $A_0 = 1.919$, $\chi = -2$; $Im3m$, $A_0 = 2.345$, $\chi = -4$). At least two Bragg peaks were used to estimate the errors associated with a and r_w , unless otherwise indicated.

2.7. Light Scattering. Dynamic light scattering (DLS) experiments were performed to obtain the size evolution of the nanoparticles as a function of temperature. For that we used a light-scattering goniometer instrument (3D LS spectrometer, LS Instruments, Fribourg, Switzerland), equipped with a 35 mW He–Ne laser light source (wavelength of 632.8 nm). The instrument implements the so-called cross-correlation scheme to suppress contributions from multiple scattering^{32–34} together with a modulation unit.³⁵ The samples were placed in 10 mm diameter cylindrical borosilicate disposable culture tubes (Fisherbrand, Thermo Fisher Scientific Inc., Waltham, MA, USA) and kept at the measurement temperature in a temperature-controlled index-matching bath. Samples were equilibrated at the measurement temperature for 10 min before the experiment. Measurements were performed at a range of scattering angles between 40 and 100° (in triplicate at each scattering angle). The initial decay rate, Γ , was derived from the second-order cumulant analysis of the normalized field autocorrelation function, which was used to calculate the apparent collective diffusion coefficient, D_0 , of the nanoparticles

$$\Gamma = D_0 q^2 \quad (1)$$

where q is the module of the scattering vector, $q = (4\pi n/\lambda)\sin(\theta/2)$, with n being the refractive index of the solvent, λ being the wavelength of the incoming laser light, and θ being the scattering angle. The hydrodynamic radius, R_h , was obtained by the Stokes–Einstein relation ($D_0 = k_B T / 6\pi\eta R_h$), where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent at temperature T .

2.8. Cell Culture. HeLa cells (ATCC collection) were grown in phenol-red-free Dulbecco's modified Eagle's medium (DMEM, Molecular Probes, USA) with a high glucose concentration, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL^{−1}), and streptomycin (100 μg mL^{−1}) (Invitrogen) in a 5% CO₂ incubator at 37 °C. Cells were seeded in 35 mm dishes; experiments were carried out 2 days after seeding, when cells reached 90% confluency. Liquid-crystalline nanoparticles were added to the cells at a concentration of 1:500 (2 μL in 1 mL of fresh medium) and incubated at 37 °C for 4 h. For live cell imaging, fresh serum-free medium was used to remove the extracellular particle suspension before the imaging session.

2.9. Fluorescence Microscopy. Light microscopy observations were made using a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with 10×, 20×, and 40×/0.75 NA water immersion objectives and an HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome-cooled CCD camera (QICAM, Qimaging, Canada). For the observation of dye fluorescence, filters were ex 546 ± 6 nm and em 620 ± 60 nm. Digital images were obtained with Image Pro Plus software (Media Cybernetics, Silver Springs, MD, USA).

2.10. Cytotoxic Activity (MTT Assay). The cytotoxic effect of cubosome formulations was evaluated in HeLa cells by the MTT assay. HeLa cells were seeded in 24-well plates at a density of 3×10^4 cells/well in 500 μL of serum-containing media. Experiments were carried out 2 days after seeding when cells had reached 90% confluency. The different formulations were added to the cells at a concentration of 1:500 (2 μL in 1 mL of serum-free medium) and incubated at 37 °C for 4 h. The effect of Docetaxel 10 μM (specifically, 1 μL of a 10 mM solution of Docetaxel in DMSO was added to 1 mL of serum-free

medium) on HeLa cell viability was also tested for comparison at the same incubation time. A 50 μL portion of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL in H₂O) was then added and left for 2 h at 37 °C. The medium was aspirated, 500 μL of DMSO was added to the wells, and color development was measured at 570 nm with an Infinite 200 automicroplate reader (Infinite 200, Tecan, Austria). The absorbance is proportional to the number of viable cells. All measurements were performed in quadruplicate and repeated at least three times. Results are shown as the percentage of cell viability in comparison with nontreated control cells. Data were expressed as a mean ± standard deviation (SD) of three independent experiments involving quadruplicate analyses for each sample.

2.11. Determination of MO and Docetaxel Content in Cubosomes. After loading with Docetaxel, the cubosome dispersion was purified from the nonencapsulated drug by dialysis: 2 mL was loaded into a dialysis tubing cellulose membrane (14 kDa MW cutoff) and dialyzed against water (1000 mL) for 2 h (by replacing the water after 1 h) at 25 °C. It should be noticed that this purification step did not influence the concentration of the dye in the formulation since the fluorophore-conjugated PF108 has a MW higher than the membrane cutoff (about 16 kDa). Therefore, in comparison to traditional methods that require enclosing the dye within the lipid bilayer, this new procedure allows a huge saving of the (typically very expensive) dye. Aliquots (10 μL) of the nanoparticle preparations were dissolved in 300 μL of CH₃CN with 0.14% CH₃COOH (v/v). The samples were vortex mixed to obtain a clear solution, and aliquots (20 μL) were injected into the Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD). Analyses of MO (detected at 200 nm) and Docetaxel (at 230 nm) in nanoparticle dispersions were carried out with a XDB–C₁₈ Eclipse (150 × 4.6 mm², 3.5 mm particle size) (Agilent Technologies) equipped with a Zorbax XDB–C₁₈ Eclipse (12.5 × 4.6 mm², 5 mm particle size) guard column (Agilent Technologies), with a mobile phase of CH₃CN/H₂O/CH₃COOH (75/25/0.12 v/v/v) at a flow rate of 2.3 mL/min. The temperature of the column was maintained at 37 °C. Recording and integration of the chromatogram data was carried out through an Agilent OpenLAB chromatography data system. The identification of lipid components and drug was made using standard compounds and conventional UV spectra. Calibration curves of all of the compounds were constructed using standards and were found to be linear, with correlation coefficients >0.995.

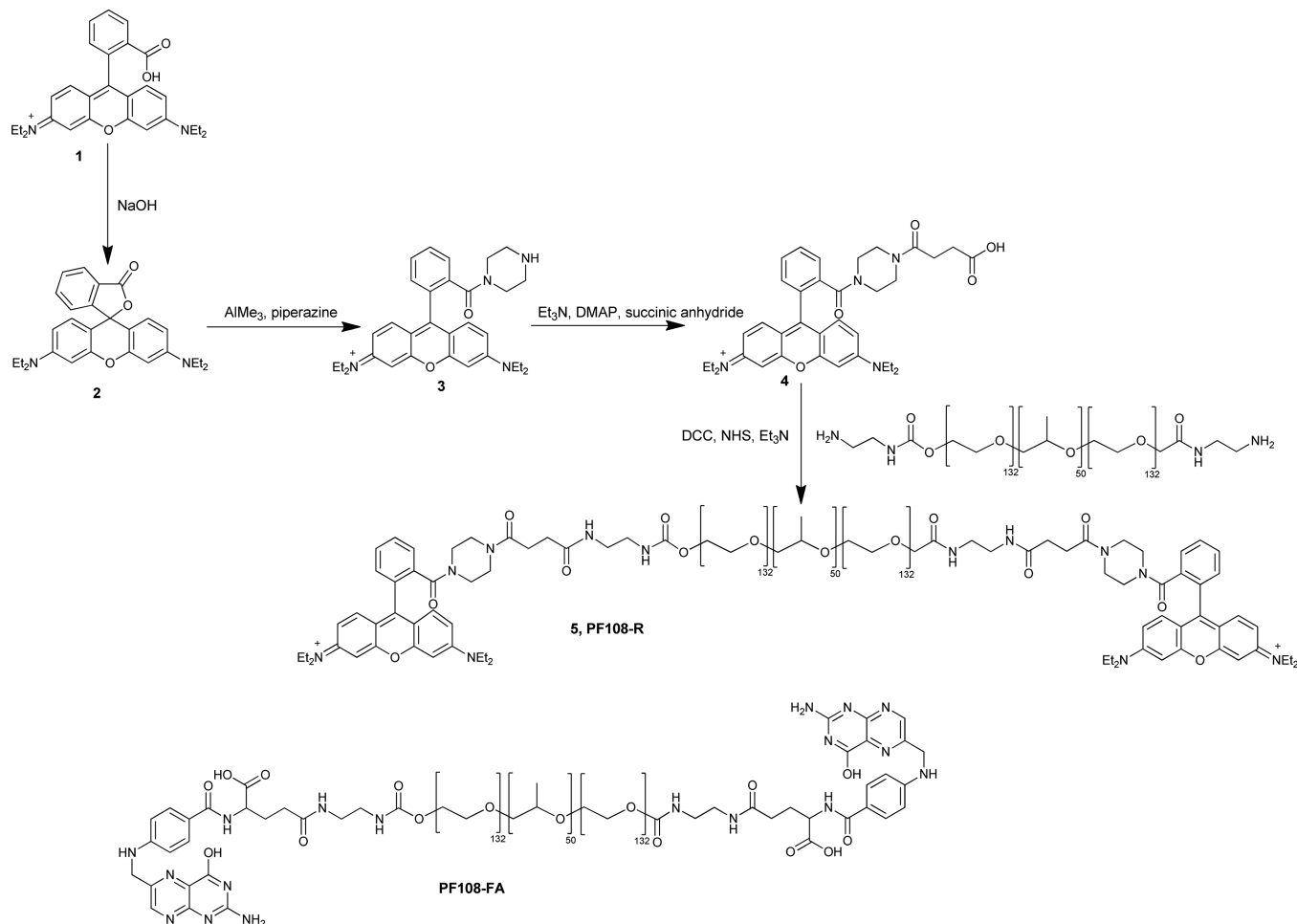
The drug encapsulation efficacy (EE%) was calculated by the following equation:³⁶ EE% = (weight of the drug in nanoparticles)/(weight of the feeding drug) × 100%. Data were expressed as a mean ± standard deviation (SD) of three independent experiments involving duplicate analyses for each sample.

2.12. Statistical Analysis. An evaluation of the statistical significance of differences was performed using Graph Pad INSTAT software (GraphPad software, San Diego, CA, USA). Comparison between groups was assessed by one-way analysis of variance (one-way ANOVA) followed by the Bonferroni multiple comparisons test.

3. RESULTS

3.1. Synthesis of Rhodamine-Conjugated PF108 and Liquid-Crystalline Nanoparticle Characterization. When designing a nanoparticle for diagnostic or theranostic applications, two different approaches could be adopted: one is doping the nanoparticle with the imaging agent (a fluorescent probe, for instance); the other is functionalizing the nanoparticle with the imaging agent. This approach is quite straightforward in the case of silica-based nanoparticles, which can be easily functionalized by well-established siloxane chemistry.³⁷ The functionalization of nanoparticles as the cubosomes reported here can efficiently be achieved by conjugating the amphiphilic polymers used to stabilize the formulation (Pluronic F108, PF108, in our case) with a suitably

Scheme 1. Synthesis Procedure Adopted for the Preparation of PF108-R



chemically modified imaging agent.³⁸ Here, fluorogenic molecule rhodamine B was conjugated to PF108 via a multisteps approach (Scheme 1). First, the fluorophore was synthetically modified as reported in the literature³⁹ to obtain its piperazine amide derivative (4) that was subsequently conjugated to the aminated derivative of PF108.⁴⁰ The reaction was carried out in DMSO in the presence of DCC, NHS, and Et₃N. Then, the product was dialyzed against deionized water and lyophilized.

Since the folate receptor is overexpressed on the cancer cell membrane, PF108 was also conjugated with folic acid as previously reported²⁰ to actively target the nanoparticles toward cancer tissues.

A mixture of commercial PF108, folate-conjugated PF108 (PF108-FA), and rhodamine-conjugated PF108 (PF108-R) was used to formulate cubosome dispersions in water also loaded with anticancer drug Docetaxel (DTX). According to preliminary tests, it was found that a mixture having the ratio PF108/PF108-FA/PF108-R = 60/20/20 (wt %) suitably stabilizes the dispersion for more than two months. This formulation was then investigated for its morphological and structural aspects by cryo-TEM, SAXS, and DLS measurements.

Figure 1 shows a typical cryo-TEM image of the cubosome formulation we proposed. A close inspection of the image in Figure 1 reveals a number of spherical cubosomes of different sizes and an inner structure characterized by a dark matrix and

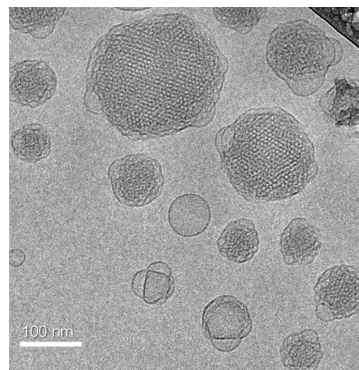


Figure 1. Cryo-TEM image of the cubosome formulation stabilized by a 60/20/20 mixture of commercial PF108, folate-conjugated PF108, and rhodamine-conjugated PF108.

alternate bright spots, respectively, representing the lipid bilayer and the water channels. As often occurs, some vesicular materials can be seen to be attached to the liquid-crystalline nanoparticles while, differently from other cubosome formulations, only a few vesicles are noticed. Indeed, unilamellar or oligolamellar vesicles typically form during the process of cubosome production, very likely because of the presence of the Pluronic used as a dispersant (MO in water does not produce small unilamellar or oligolamellar vesicles).⁴¹ Actually, depending on the method of preparation, vesicles may exceed

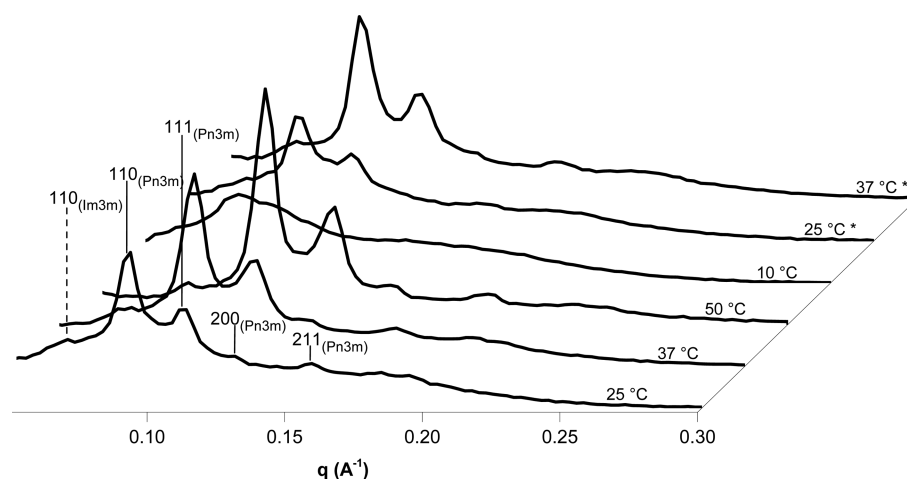


Figure 2. $I(q)$ vs q data obtained by SAXS recorded at different temperatures of the cubosome formulation stabilized by the same Pluronic mixture as in Figure 1. The Miller indices are reported on top of the corresponding Bragg peaks with the indication of the space group in the case of the bicontinuous cubic phases.

cubosomes in terms of number density.⁴² Therefore, the absence of vesicular material observed here could be ascribed to the use of the conjugated Pluronics as a stabilizer for cubosome formulation.

Taking into account the possible use of the system under study as a theranostic nanocarrier, the variation of the size and inner structure upon temperature changes was monitored through DLS and SAXS measurements in the range of 10–50 °C. In particular, the temperatures were chosen to include two extreme storage temperatures (10 and 50 °C) as well as room and physiological temperatures. Moreover, to find out if the system nanostructure after temperature cycling is reestablished, SAXS measurements were performed in the following order: 25, 37, 50, 10, 25, and 37 °C. Results are reported in Figure 2 and Table 1.

Table 1. Cubosome Lattice Parameters (a) and Radius of the Water Channels (r_w)^a

T (°C)	$Pn3m$		$Im3m$ ^b	
	a (Å)	r_w (Å)	a (Å)	r_w (Å)
25	97.0 ± 0.5	20.9 ± 0.2	129.6	22.6
37	89.2 ± 0.2	17.9 ± 0.1	118.7	19.3
50	80.7 ± 0.2	14.5 ± 0.1	109.6	16.5
25	97.1 ± 0.6	21.2 ± 0.3	123.1	20.6
37	90.5 ± 0.7	18.4 ± 0.3	118.7	19.3

^aExperiments were performed at different temperatures in the order reported in the table. ^bStandard deviations on a and r_w pertaining to $Im3m$ phase were not reported since these parameters were obtained from the single Bragg peak discernible in the diffraction pattern.

SAXS results confirmed the bicontinuous cubic inner structure of the formulation. Actually, the diffraction pattern of the cubosome formulation at 25 °C (Figure 2) is dominated by four Bragg peaks placed in a q ratio of $2^{1/2}:3^{1/2}:2:6^{1/2}$ that clearly identify the double diamond ($Pn3m$) bicontinuous cubic phase. However, a weak reflection at low q reveals the simultaneous presence of the primitive ($Im3m$) bicontinuous cubic phase. The coexistence of the $Pn3m$ and the $Im3m$ phases in cubosome formulations is widely reported in the literature; it is attributed to a $Pn3m$ to $Im3m$ intercubic phase transition

caused by the interaction of the hydrophobic moiety of the Pluronic with the surface of the cubosome nanoparticles.⁴³

The lattice parameter, a , obtained from the SAXS experiments performed at different temperatures along with the calculated radius of the water channels r_w (section 2.3) are reported in Table 1. As usually detected in liquid-crystalline systems, although the nanostructure is retained, the lattice parameter (and consequently the radius of the water channels) of the cubosomes decreases while the temperature increases in the sequence 25, 37, 50 °C. This phenomenon is related to the enhanced disorder of the lipid chain at higher temperatures that, in turn, induces higher negative curvature at the lipid/water interface.⁴³ The disappearance of the weaker Bragg peaks detected in the diffraction patterns obtained at 10 °C (Figure 2) indicates that the inner structure is in some way altered, although not completely lost. When the temperature is raised again to 25 or 37 °C, the nanostructure is recovered. However, some significant differences can be noticed in the intensity of the Bragg peaks and the general appearance of the diffractograms after the temperature cycle. Therefore, it can be concluded that while the nanostructure is reestablished, the system evolution upon temperature changes is characterized by a certain degree of hysteresis.

The overall trend described by the DLS measurements of the nanoparticles' hydrodynamic radius can be extrapolated from the results reported in Table 2. Essentially, the nanoparticle average size does not vary significantly with temperature changes.

3.2. Rhodamine-Conjugated Liquid-Crystalline Nanoparticle Photophysical Characterization and HeLa Cell Imaging. The photophysical properties of PF108-R and those of cubosomes were investigated. Rhodamine dyes belong to the

Table 2. Average Hydrodynamic Radius (R_h) and Polydispersity Index (PDI) of the Cubosome Formulation at Different Temperatures

T (°C)	R_h (nm)	PDI
10	85	0.13
25	90	0.16
37	88	0.14
50	87	0.21

class of xanthene dyes, characterized by an intense red fluorescence, high brightness, excellent photostability, and the ability to modulate the properties of the dye through substitution.⁴⁴ A key property of these dyes is the equilibrium between an open, colored, fluorescent quinoid form or a closed, colorless, nonfluorescent lactone, which can be controlled by appropriate substitution.⁴⁵ In particular, rhodamine B (bearing two diethyl amino substituents) is one of the most common derivatives.

The emission/absorption properties of PF108-R were investigated in water and chloroform to test its photophysical features in media of different polarities. In both solvents PF108-R has a strong absorption band at 560 nm ($\epsilon = 30\,660\text{ M}^{-1}\text{ cm}^{-1}$, $31\,770\text{ M}^{-1}\text{ cm}^{-1}$ in water and chloroform, respectively) and a shoulder at 522 nm ($\epsilon = 13\,100\text{ M}^{-1}\text{ cm}^{-1}$, $9950\text{ M}^{-1}\text{ cm}^{-1}$ in water and chloroform, respectively). Upon excitation at 522 nm in water, an emission band at 594 nm is generated. Conversely, the excitation at the same wavelength in chloroform generates a strong emission band at 584 nm. The quantum yields measured were 0.16 and 0.58, respectively, in water and chloroform. As expected, water has a quenching effect on the rhodamine fluorescence. The shift of the emission to higher energy could be explained by considering the solvatochromic properties of the fluorophore.

The emission spectrum of the cubosome formulation stabilized with the PF108/PF108-FA/PF108-R = 60/20/20 (wt %) mixture was also recorded after 1:100 dilution in water. Excitation at 522 nm resulted in emission bands centered at 581 nm (Figure 3). The position of the maximum emission

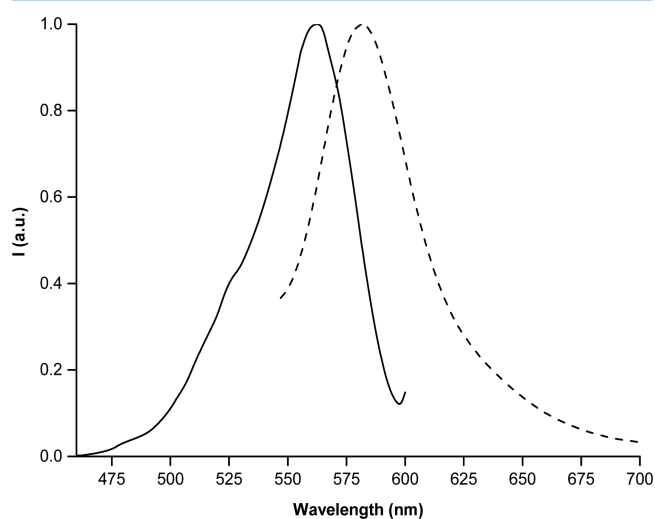


Figure 3. Normalized emission ($\lambda_{\text{exc}} = 522\text{ nm}$) and excitation ($\lambda_{\text{em}} = 581\text{ nm}$) spectra of the aqueous cubosome formulation stabilized using the same Pluronic mixture as in Figure 1.

wavelength is similar to that observed for PF108-R in chloroform. These results suggest that the rhodamine fragment conjugated to the Pluronic F108, rather than protruding toward the bulk water, is hidden within the poly(ethylene oxide) corona surrounding the nanoparticle, thus experiencing a less polar environment. The excitation spectrum of cubosomes is in accordance with PF108-R absorption in both water and chloroform. Due to the turbidity of the solution the determination of the quantum yield of the dye in the nanoparticles was not possible.

The uptake of cubosome nanoparticles was investigated by fluorescence microscopy. After 4 h of incubation, faint diffuse fluorescence was distributed within the cytoplasm of HeLa cells, whereas in untreated control cells no fluorescence was detected (Figure 4). However, it was not possible to determine whether the dye molecules were localized in specific cell compartments.

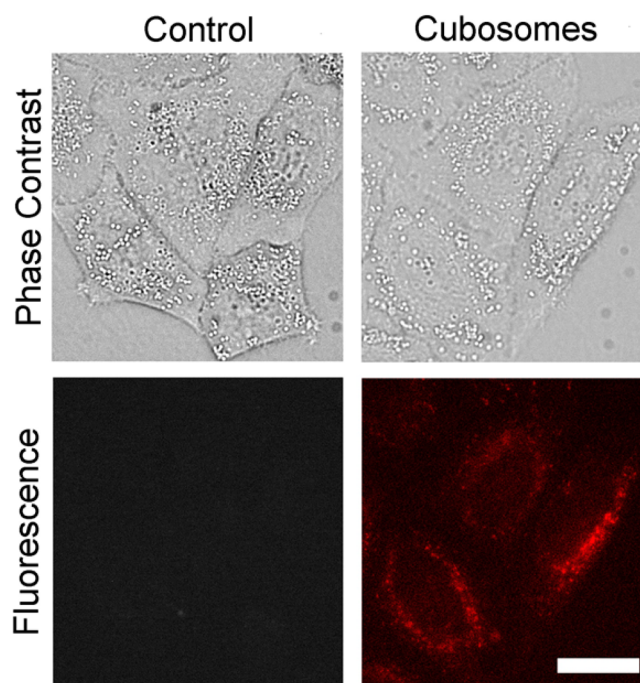


Figure 4. Fluorescence microscopy of living HeLa cells treated with cubosomes not carrying the drug (incubation for 4 h) showing a diffuse cytoplasmic fluorescence in treated cells indicating nanoparticle uptake. Scale bar = 20 μm .

3.3. Monoolein and Docetaxel Levels in Cubosome Formulations and Cytotoxic Activity (MTT Assay). The amount of MO (expressed as mg/mL of dispersion) and Docetaxel (DTX, $\mu\text{g/mL}$) in different cubosome formulations was monitored by HPLC. The formulations analyzed were the traditional cubosome formulation stabilized with PF108 (CUB), the cubosome formulation stabilized with the PF108/PF108-FA/PF108-R = 60/20/20 (wt %) mixture (CUB-R), and the latter formulation also loaded with DTX (CUB-R/DTX).

Monoolein contents in CUB, CUB-R, and CUB-R/DTX formulations were 33 ± 3 , 35 ± 2 , and $29 \pm 2\text{ mg/mL}$, respectively. The CUB-R/DTX formulation was loaded with $328 \pm 25\text{ }\mu\text{g/mL}$ of drug, with a DTX/MO molecular ratio of 1/200. The encapsulation efficacy (EE%) was $103 \pm 13\%$.

These formulations were tested for cytotoxicity (MTT assay) in HeLa cells. Figure 5 shows the viability, expressed as the % of the control, induced in HeLa cancer cells in the presence of the nanoparticle formulations at a concentration of 1:500 (2 $\mu\text{L/mL}$ of medium) after 4 h of incubation. The cytotoxic effect of 10 μM DTX (section 2.10) was also determined after 4 h of incubation for comparison. Treatment with CUB and CUB-R formulations did not induce a significant reduction in cell viability, in comparison with control cells (Ctrl). Differently, the incubation with CUB-R/DTX particles induced a statistically significant decrease in HeLa cell viability (36% reduction)

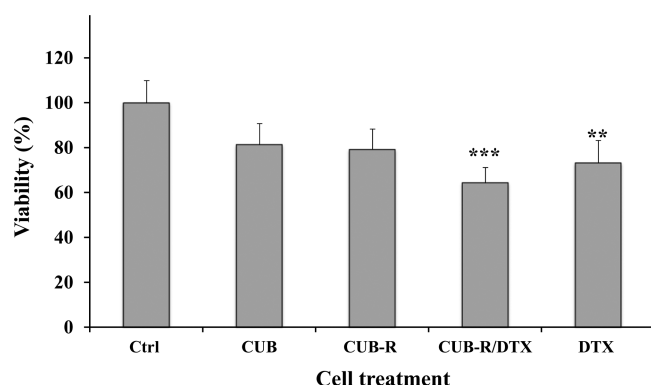


Figure 5. Viability, expressed as % of the control, induced in HeLa cells by incubation for 4 h in the presence of different types of cubosome formulations (CUB, CUB-R, and CUB-R/DTX, see the text) and Docetaxel (DTX). The drug concentration in the CUB-R/DTX formulation ($0.8 \mu\text{M}$) is 12.5-fold lower than in the DTX molecular dispersion ($10 \mu\text{M}$). Results are expressed as a mean $\pm$ standard deviation (SD) of four independent experiments involving quadruplicate analyses for each sample ($n = 4$). *** = $P < 0.001$; ** = $P < 0.01$ versus Ctrl, and data were analyzed using one-way ANOVA.

with respect to the control as a result of the presence of the anticancer drug. A significant cytotoxic effect (27% reduction in cell viability) was also observed in cells treated with the anticancer drug (DTX) directly added to the cell culture. Remarkably, the HeLa cell treatment with DTX-loaded cubosomes (DTX dose of approximately $0.8 \mu\text{M}$) induced a superior cytotoxic effect compared to the molecularly dispersed anticancer drug tested at much higher (12.5-fold) concentration.

DTX is one of the most effective anticancer agents and has activity against a wide range of cancers. The growth inhibitory effect of DTX was examined in several cancer cell lines, and the IC_{50} (50% inhibitory concentration) values of this taxoid were in the nanomolar range at long-time incubation (72 h)^{46,47} The 24 and 48 h IC_{50} values of the free DTX in HeLa cells were previously found to be 5.01 and $2.62 \mu\text{g/mL}$, respectively.⁴⁸ Here, the higher cytotoxicity after 4 h of incubation of DTX administered via the nanoparticles ($0.66 \mu\text{g/mL}$) with respect to free drug molecules ($8.08 \mu\text{g/mL}$) can be explained by calling into play different uptake mechanisms. Indeed, cubosomes can deliver DTX into cells via endocytosis while free DTX can enter cells via passive diffusion with inherent lipophilicity, and various ABC efflux transporters, expressed at the cellular membrane, can minimize its effective penetration by efficiently eliminating the drug from the cell.⁴⁹

3.4. Cubosomes versus Multilamellar Liposome Hydrophobic Volume. One important feature that distinguishes cubosomes (reverse bicontinuous cubic liquid-crystalline nanoparticles) from liposomes (their lamellar counterpart) is the higher hydrophobic volume of the former. Since cubosomes are mainly proposed as nanocarriers for hydrophobic molecules of pharmaceutical interest as an alternative to liposomes, this fact may be extremely relevant in view of their theranostic applications. Given the very convoluted volumes occupied by lipid chains in bicontinuous morphologies compared to the shells of liposomes, it is tempting to assume that the former contains a far larger hydrophobic volume (suitably) normalized than the latter. Surprisingly, the quantification of the hydrophobic volumes of bicontinuous

cubosomes with respect to multilamellar liposomes has never been reported.

Assume for simplicity that we have two nanoparticles composed of monoolein in water whose outer diameter is 100 nm. Assume that the liposome is composed of N concentric spherical monoolein bilayers, composed of pairs of monolayers, each with a hydrophobic thickness of $17 \text{ \AA}$, corresponding to the likely chain length in molten monoolein assemblies. Furthermore, assume that adjacent bilayers are separated by polar films that are $16 \text{ \AA}$ thick, so that each monolayer has an associated polar layer of thickness of $8 \text{ \AA}$ (corresponding to dimensions in the fully hydrated lamellar mesophase).⁵⁰ Each repeat unit of chains, monoolein head-groups, and water is therefore $50 \text{ \AA}$ thick. The hydrophobic volume is then calculated as the chain contribution to the total volume within N concentric nested shells whose outermost radius is scaled such that the outermost monolayer and associated polar layer has a diameter of 100 nm. These dimensions imply that the hydrophobic volume fractions within the liposome (whose total volume is $5.24 \times 10^5 \text{ nm}^3$) are approximately 0.18, 0.33, 0.445, 0.53, and 0.59 nm^3 for $N = 1$ –5 bilayers, respectively. The exposed outermost monolayer has an area of $2.83 \times 10^4 \text{ nm}^2$.

The structural dimensions of a typical cubosome can be estimated as follows. We assume the cubosome to be a single-crystalline domain of the $\text{Im}\bar{3}m$ phase, also contained within a spherical shell of diameter 100 nm (cubosome nanoparticles, although characterized by a cubic inner nanostructure, can be spherical in shape, such as those shown in Figure 1). We choose a typical lattice dimension of the $\text{Im}\bar{3}m$ unit cell, namely, $130 \text{ \AA}$, and fix the chain length to be the same as that in the liposome, $17 \text{ \AA}$ (the polar dimensions are then fixed by those parameters). The resulting total exposed area is then $1.68 \times 10^5 \text{ nm}^2$ (equal to the summed area of each monolayer displaced $17 \text{ \AA}$ from the bilayer midsurface, whose geometry is assumed to lie on the P surface, the cubic periodic $\text{Im}\bar{3}m$ minimal surface). Surprisingly, the membrane area exposed to water in the liposome is substantially larger than the total area in the cubosome. The corresponding hydrophobic volume fraction within the 100 nm cubosomal sphere is 0.59 nm^3 , which is greater than that of the liposome with up to five bilayers (Table 3).

Table 3. Cubosome-to-Liposome Hydrophobic Volume Ratios (V_h) as a Function of the Number of Concentric Bilayers in the Liposome^a

number of bilayers	1	2	3	4	5
V_h ratios	3.22	1.79	1.33	1.12	1.00

^aSee also the text.

4. CONCLUSIONS

In this paper we demonstrated that cubosomes loaded with an anticancer drug, namely, Docetaxel, can be formulated by decorating their nanoparticle surface with both a cancer-cell-targeting ligand and an imaging probe. It is worth noticing that this is the first time that Docetaxel is successfully loaded within cubosomes and that the cubosome surface is simultaneously decorated with targeting and imaging moieties. To this aim, the commercial block copolymer Pluronic F108 (PF108) and its folate- and rhodamine-conjugated counterparts were used in a 60/20/20 (wt %) mixture to stabilize the lipid liquid-crystalline

dispersion. SAXS, DLS, and cryo-TEM investigation, over the temperatures range explored, confirmed the cubic bicontinuous inner structure of the nanoparticles and showed the physicochemical aspects (morphology and size) akin to those of traditionally prepared cubosome dispersions. Photophysical measurements suggested that the fluorogenic fragment, although conjugated to the terminal ethylenoxide moiety of PF108, was very likely exposed to the less polar environment constituted by the poly(ethylene oxide) corona surrounding the nanoparticles, rather than protruding toward the bulk water. This formulation, not loaded with the drug, was successfully used to image living HeLa cells. Besides, viability tests revealed a significant cytotoxic effect (more than 1 order of magnitude larger than the molecularly dispersed drug) of the Docetaxel-loaded nanoparticles against HeLa cells. These results evidenced that the loading of cubosomes with a Docetaxel dose able to induce cytotoxic effects on HeLa cells and the decoration of their surface with 40% conjugated PF108 did not compromise the intrinsic nature of these peculiar nanoparticles and their cellular uptake. We have also evaluated the differences between cubosomes and multilamellar liposomes in terms of surface area and hydrophobic volume. Under the imposed constraints (same molecular building block and nanoparticle volume, cubosome nanostructure characterized by the $Im\bar{3}m$ space group with a lattice parameter of 130 Å, and multilamellar liposome constituted of adjacent bilayers), these calculations demonstrated that the hydrophobic volume of cubosomes is more than 3 times larger than that of single-bilayer liposomes, but the cubosome-to-liposome hydrophobic volume ratio rapidly converges to unity as the number of the bilayers in the liposome increases. It is worth noting that this ratio will be even larger if the liposome contains nonadjacent bilayers. Remarkably, the cubosome surface exposed to water is about 60% with respect to that of the liposome.

Taken together, these findings demonstrate the good performance of cubosomes as peculiar nanocarriers for hydrophobic molecules with therapeutic/diagnostic relevance and encourage the investigation of a cubic bicontinuous liquid-crystalline dispersion for possible applications as theranostic tools.

■ APPENDIX

Geometrical Calculations

Denote the chain length by d , the thickness of the polar layer (containing water plus headgroups) by t , and the lattice parameter in the cubic mesophase by a . Assume that the outer shape of the liposome and the cubosome is a sphere of outer diameter L .

We assume that all interfaces, lining the monolayers at the chain–water interface and the free chain ends, are parallel. We then use the formalism described elsewhere¹ to deduce dimensions. All dimensions are calculated from the geometry of the imaginary surface S that runs between the chain ends in opposed monolayers at the midsection of the hydrophobic shell for each bilayer. The “outer” surfaces, exposed to water, are parallel to S and displaced by d from S . We have two formulae derived from the geometry of parallel surfaces, both related to the mean and Gaussian curvatures of S , denoted H_S and K_S , respectively. One is for the area of the surface Sx , parallel to S , displaced by a distance x

$$\text{area}(Sx) = \text{area}(S)(1 + 2Hx + Kx^2)$$

(where the sign of x depends on whether it is on the outside or the inside of the surface). The second describes the volume of the film lying in between S and Sx :

$$\text{volume}(Sx) = \text{area}(S)x \left(1 + Hx + \frac{Kx^2}{3} \right)$$

In the liposome, those surfaces are parallel spheres, and if it is made of N bilayers, there are N such spheres, each one with radius

$$r(i) = \frac{L}{2} - (2i - 1)(d + t)$$

These dimensions impose mean and Gaussian curvatures on bilayer i equal to

$$H(i) = r(i)^{-1}$$

$$K(i) = r(i)^{-2}$$

The exposed area of the liposome is that of the outermost spherical monolayer (displaced a distance t inwards from the 100 nm external shell), namely, $4\pi r(1)^2$. The total volume is $V_{\text{total}} = (4/3)\pi(L/2)^3$. The hydrophobic (chain) volume in the liposome is determined by summing over all $2N$ monolayers, N displaced x and N displaced $-x$:

$$\begin{aligned} \text{volume}(N) = 4\pi d \sum_{i=1}^N r(i)^2 & \left\{ \left(1 + H(i)d + \frac{d^2}{3K(i)} \right) \right. \\ & \left. + \left(1 - H(i)d + \frac{d^2}{3K(i)} \right) \right\} \end{aligned} \quad (1)$$

The corresponding calculation for the cubosome follows directly from the normalized surface to volume ratio or “homogeneity index” characteristic of a particular folded surface geometry

$$\mathcal{H} = \frac{\text{area}_Q^{3/2}}{(-2\pi\chi)^{1/2} \text{volume}}$$

where χ is the Euler characteristic of the hyperbolic bilayer within the particle. If the cubosome is contained within a spherical shell of diameter L and the membrane within folds onto a periodic minimal surface to form a cubic lattice of edge a whose topology per unit cell is χ_0 , then $\chi = \chi_0(V_{\text{total}}/\alpha^3)$. The radius of curvature of the minimal surface is given by the expression

$$r_Q = \left\{ \frac{\mathcal{H}}{-2\pi\chi} \right\}^{1/3}$$

which corresponds to minimal surface curvatures $H_Q = 0$ and $K_Q = -r_Q^{-2}$.⁵⁰ It follows that the minimal surface area in the cubosome is

$$\text{area}_Q = \{\mathcal{H}V_{\text{total}}(-2\pi\chi)^{1/2}\}^{2/3}$$

so that the exposed area (at the hydrophobic/hydrophilic interface) is

$$\text{area}_{\text{exposed}} = 2\text{area}_Q(1 - Kd^2)$$

and the hydrophobic volume is

$$\text{volume}_Q = 2\text{area}_{\text{exposed}} \left(1 + \frac{d^3}{3K_Q} \right) \quad (2)$$

If the internal bilayer geometry within the cubosome corresponds to the $Im\bar{3}m$ bicontinuous mesophase, then the relevant data are $\mathcal{H} = 0.7163$ and $\chi_0 = -4$.⁵¹ The ratios of hydrophobic volumes are calculated from the results of eqs 1 and 2 above.

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Notes

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Solvatochromic fluorescent BODIPY derivative as imaging agent in camptothecin loaded hexosomes for possible theranostic applications.

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Solvatochromic fluorescent BODIPY derivative as imaging agent in camptothecin loaded hexosomes for possible theranostic applications†

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We here discuss the potential theranostic nanomedicine application of an innovative formulation consisting of monoolein-based nanoparticles with a two-dimensional hexagonal inner structure stabilized in water using a mixture of PEO₁₃₂–PPO₅₀–PEO₁₃₂ block copolymers with and without conjugated folate for targeting. The proposed tumor-cell targeted formulation was shown to be able to simultaneously host the model anticancer drug camptothecin and a pyrene-modified BODIPY fluorophore, based on dynamic light scattering, small-angle X-ray scattering, and cryogenic transmission electron microscopy. The photophysical properties of the fluorophore were studied in solution in various solvents. A marked fluorescent solvatochromism, whose origin was explained by time-dependent density functional theory theoretical calculations, was observed. Fluorescence microscopy showed that HeLa cells readily internalize these nanoparticles, and that the fluorophore localizes within the lipid droplets. In addition, cytotoxicity test revealed that these nanoparticles are not toxic at the concentration used for the imaging analysis.

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1. Introduction

Polar lipids such as phospholipids or monoglycerides are amphiphilic in nature, therefore they self-assemble in aqueous solution to form a number of different liquid crystalline phases. Among others, monoolein (MO, glycerol monooleate) received much attention in the last three decades because of its rich structural polymorphism.^{1–3} Besides a reverse micellar phase found at very low water content, the MO binary phase diagram at 25 °C is characterized by a lamellar (L_α) and two cubic bicontinuous liquid crystalline phases, namely, the gyroid (C_G, space group *Ia3d*) and the double diamond (C_D, space group *Pn3m*). The last two are constituted by a curved, triply periodic, and non-intersecting MO bilayer folded to form two disjoint continuous water channels. Although the role of the C_G and the C_D phases in the cell machinery is not fully clarified, they were

virtually found ubiquitous in nature,⁴ and several papers were devoted to shed some light on the biological function of this amazing nanostructures.^{5–7} Remarkably, the entrapment of lipophilic molecules into these cubic bicontinuous phases may alter the MO effective packing parameter, causing the evolution towards a reverse hexagonal phase⁸ (*i.e.*, a bulk phase organized in water cylinders surrounded by a MO monolayer, and packed in a two-dimensional hexagonal array), while the local order and dynamics of the MO alkyl chain remains unaltered.^{9,10} The polymorphism of MO in water can be extended by adding small amounts of lauroylcholine chloride, which causes the formation of a very stable solution of small unilamellar vesicles.^{11,12} When increasing the dispersed phase content, they organize into a vesicle gel.¹³

As for lamellar phases that can be dispersed into vesicles, the cubic and hexagonal phases can be formulated into stable colloidal dispersion in water, known as cubosomes and hexosomes, respectively. In order to obtain mono-disperse formulations, a dispersion agent like the nonionic poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) tri-block copolymers, known as Pluronic, can be used.^{14,15} These copolymers anchor their hydrophobic moiety in the nanoparticle, while protruding their hydrophilic segments into the solvent. This provides the particles with a PEO corona, which ensures a strong steric stabilization of the nanoparticles. Recently, such dispersions were proposed as platforms in theranostic nanomedicine.^{16–20} With the purpose of discovering and treating

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† Electronic supplementary information (ESI) available: Material and methods; synthesis of Py-BODIPY; details of DFT calculations. See DOI: 10.1039/c5ra01025j

diseases at the earliest stage and with limited side effects, a new strategy to engineer different kinds of nanocarriers for the sustained, controlled, and targeted delivery of both therapeutic and diagnostic agents at the same time has emerged.²¹ Indeed, our group has demonstrated that, as with other kinds of nanoparticles, cubosomes can be simultaneously loaded with optical imaging agents and anticancer drugs.^{16,19} Cubosome and hexosome formulations possess a number of properties that can be exploited in nanomedicine but, most of all, (i) they have the right size for theranostic applications, and (ii) they are biodegradable. Moreover, these nanoparticles can be considered to be stealth particles, because their PEO corona is expected to prevent the adsorption of macromolecules on the nanoparticle surface. This prevents the formation of the biomolecular corona, thus reducing the clearance from the bloodstream *via* the mononuclear phagocytic system (MPS).²² When considering cubosomes and hexosomes for cancer diagnostic and therapy (as in the case discussed in this paper), it deserves also noticing that their size is within the range required for the tumor tissues passive targeting through the enhanced permeation mechanism (EPR).²³ We also proved that decoration of their surface with targeting moieties capable of addressing them at cancer cells is feasible, making these lipid-based nanoparticles even more appealing for theranostic nanomedicine applications in oncology.¹⁶

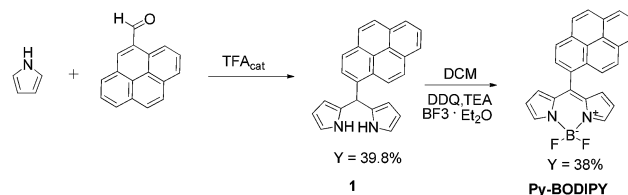
Over the past decade increasing efforts have been devoted to the synthesis of new organic chromophores or nanoparticles^{24,25} with strong absorption and emission bands for imaging applications, both *in vitro* and *in vivo*. In particular, considerable attention has been addressed towards the development of derivatives of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) because of their high molar extinction coefficients and quantum yields, excellent photostability and high solubility in commonly used organic solvents. BODIPY derivatives have been included into different type of nanoparticles,^{26–33} belonging both to the hard and the soft matter realm.

Here, we report the formulation of MO-based hexosomes loaded with both the model chemotherapeutic drug camptothecin and a pyrene-modified BODIPY fluorophore (Py-BODIPY) also stabilized by a mixture of Pluronic F108 (PF108) and folate-conjugated PF108 (PF108-FA), and investigate their physico-chemical and photophysical properties. Theoretical calculations were used to elucidate the origin of the peculiar fluorescent properties of Py-BODIPY and the localization of the dye inside the nanoparticles.

2. Results and discussion

2.1. Pyrene-modified BODIPY (Py-BODIPY) synthesis and solvatochromic properties

The typical emission of unsubstituted BODIPY chromophore falls in the region 470–530 nm, but the position of the maximum of luminescence can be modulated by substitution at different positions of the BODIPY skeleton, and in particular of the *meso*-carbon atom. The strategy of functionalizing BODIPYs on the *meso*-carbon with polyaromatic rings such as naphthalene,³⁴ anthracene,³⁴ and phenanthrene,³⁵ in order to improve



Scheme 1 Adopted synthetic procedure for the preparation of Py-BODIPY.

the photophysical properties of the chromophore have been widely adopted.

In particular, the BODIPY derivative containing a pyrene group (Py-BODIPY) in the *meso* position was reported for the first time by Peña-Cabrera and co-workers³⁶ using the Liebeskind–Srögl cross-coupling reaction between 8-thiomethylBODIPY and pyrene boronic acid, catalyzed by copper(i) thiophene-2-carboxylate (CuTC). Herein, we succeeded in synthesizing Py-BODIPY using the classic Lindsey method,³⁷ *i.e.*, acid-catalyzed condensation of 1-pyrenecarboxaldehyde with pyrrole, followed by oxidation with DDQ and exposure to triethylamine and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (Scheme 1 and ESI† for synthetic details).

Although the method reported by Peña-Cabrera avoids the synthesis of dipyrromethanes precursors which might

Table 1 Quantum yield (Φ), excitation and emission wavelength (λ_{exc} and λ_{emis} , respectively) of Py-BODIPY with different solvents

Solvent	λ_{exc} (nm)	λ_{emis} (nm)	Φ
Hexane	504	523	0.25
Toluene	507	582	0.14
DCM	505	670	2.0×10^{-2}
THF	504	630	4.0×10^{-2}
EtOAc	502	627	4.3×10^{-3}
Acetone	502	662	8.9×10^{-3}
DMF	505	670	6.1×10^{-3}
MeCN	499	708	4.9×10^{-3}
DMSO	507	709	9.5×10^{-3}
EtOH	502	651	6.8×10^{-3}
MeOH	501	662	5.6×10^{-3}

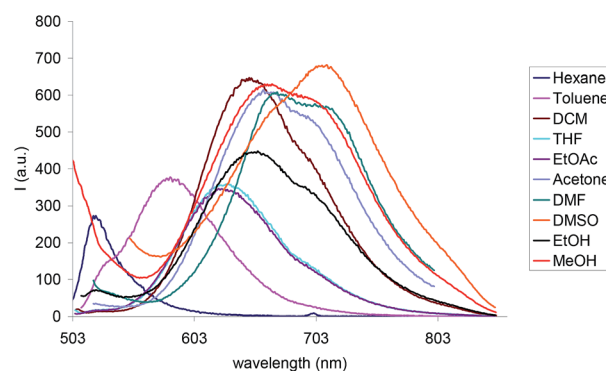


Fig. 1 Fluorescence spectra of Py-BODIPY in different solvents. [Py-BODIPY] = 2.5×10^{-5} M; λ_{exc} between 499 and 507 nm (see Table 1).

be difficult to purify, it requires the synthesis of both 8-thio-methylBODIPY and the catalyst. The Lindsey method is more straightforward to prepare Py-BODIPY as it requires two steps, being the 1-pyrenecarboxaldehyde commercially available.

Here, we studied the photophysical properties of Py-BODIPY in different solvents covering a variety of polarities larger than that previously reported,³⁸ and ranging from apolar hexane to polar DMSO (Table 1). While the absorption spectrum of Py-BODIPY showed negligible changes in the different solvents (ESI, Fig. S1†), its emission properties were strongly dependent on the polarity of the solvent, with the maximum of the fluorescence emission ranging from 523 nm in hexane to 709 nm in DMSO (Fig. 1).

2.2. Nanoparticles physicochemical and photophysical characterization

The hexosome nanoparticles investigated are engineered for theranostic applications in oncology. Therefore, to confer on them targeting properties towards tumor tissues, the stabilization of the nanoparticles was achieved using a 80/20 mixture of commercial PF108 and the folate-conjugate PF108 (PF108-FA). The synthesis of PF108-FA and its use for the formulation of targeted theranostic cubosomes were previously described in ref. 16. Since tumor cells often overexpress folate receptors, the folate molecules conjugated to the terminal PEO moieties of PF108 on the particle surface will actively favor the enrichment of nanoparticles at cancer cells compared to healthy cells. Hexosomes were also simultaneously loaded with Py-BODIPY, for optical imaging purposes, and camptothecin, an anti-cancer drug that, along with its analogues, was demonstrated to be effective in the treatment of a broad spectrum of tumors. The loading efficiency of the dye and the drug ($E\%$, expressed as percentage of the amount of the drug and the dye present in the formulation before dialysis, see ESI†) were 97% and 54%, respectively. The physicochemical characterization of the hexosome formulation containing both the diagnostic and the therapeutic agent (HexCBFA) was performed by DLS, SAXS, and cryo-TEM. For the sake of comparison the other targeting hexosome formulations, empty (HexFA) as well as those containing only the drug (HexCFA) or the dye (HexBFA), were also investigated by DLS (see ESI† for Experimental details).

In general, DLS results reported in Table 2 confirm that the 80/20 mixture of PF108/PF108-FA was able to stabilize the formulations as a nanometer-size particle dispersion with quite a low polydispersity and highly negative ζ -potential. Moreover, encapsulation of the fluorophore and the drug, even when they are formulated simultaneously, did not cause appreciable alterations in the size, the PDI, and the ζ -potential of the nanoparticles (Table 2).

The inner symmetry and the stability as a function of the temperature of the HexCBFA formulation were determined by synchrotron SAXS experiments. The diffraction patterns recorded at 25, 37, and 50 °C are reported in Fig. 2. All of them are dominated by a strong reflection peak followed, at higher q values, by two weaker reflections. The positions of these three Bragg peaks, in the ratio $1 : \sqrt{3} : 2$, are always consistent with

Table 2 Average nanoparticle diameter (D_{av}), polydispersity index (PDI), and ζ -potential of various hexosome formulations. HexFA, indicate the hexosome formulation stabilized by the folate-conjugated PF108, while the capital letters B and C in the acronyms indicate formulations loaded with Py-BODIPY and/or camptothecin, respectively

Formulation	D_{av} (nm)	PDI	ζ -potential (mV)
HexFA	168 ± 1	0.20	-33 ± 1
HexCFA	170 ± 1	0.20	-36 ± 1
HexBFA	177 ± 1	0.22	-32 ± 1
HexCBFA	172 ± 1	0.20	-35 ± 1

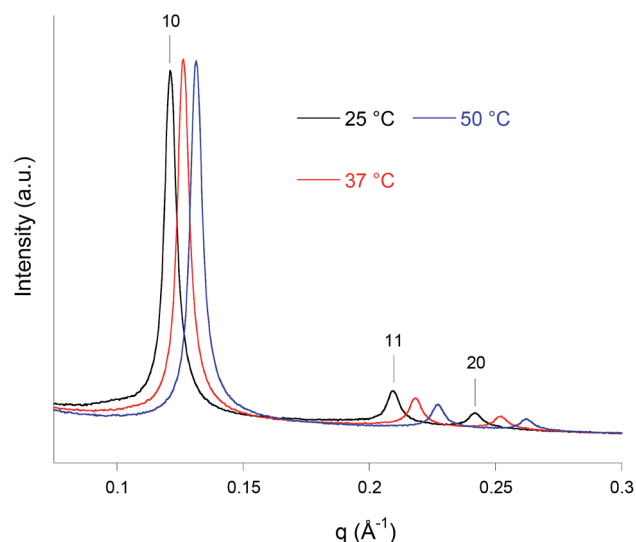


Fig. 2 Synchrotron SAXS diffraction patterns recorded at different temperatures of the hexosome formulation stabilized by the mixture PF108/folate-conjugated PF108 and loaded with Py-BODIPY and camptothecin (HexCBFA). The Miller indices (hk) are reported on top of the corresponding Bragg reflections.

that expected for a reverse hexagonal phase. With temperature increasing, the lattice parameters, a , were found to be $59(\pm 1)$, $57(\pm 1)$, and $55(\pm 1)$ Å, respectively, while the corresponding radius of the water channels r_w were $31(\pm 1)$, $30(\pm 1)$, and $29(\pm 1)$ Å, respectively. This is as generally observed for liquid crystalline systems.³⁹ Nonetheless, the nanoparticles retain their internal reverse hexagonal symmetry even at the highest temperature (50 °C).

Cryo-TEM observations of the same sample (Fig. 3) showed the presence of quasi-spherical nanoparticles characterized by curved striations and the absence of the small unilamellar vesicles usually found in cubosomes formulations, either attached to the nanoparticles or dispersed as single entities. Curved striations are a characterizing feature of the hexosomes,^{40,41} and are related to the peculiar configuration adopted by the close (hexagonally) packed and deformable water tubes that constitutes the internal nanostructure.⁴²

From a photophysical point of view, when the HexCBFA formulation (dilution 1 : 30) was excited at 330 nm an emission band with the maximum centered at 532 nm (Fig. 4) was

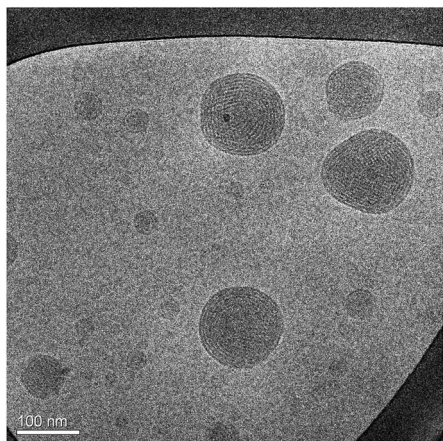


Fig. 3 Cryo-TEM image of the same sample investigated in Fig. 2.

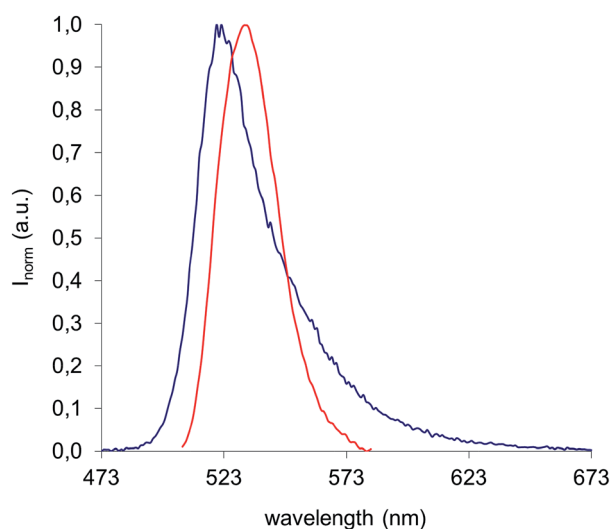


Fig. 4 Emission spectra of Py-BODIPY in hexane (blue curve) ($\lambda_{\text{exc}} = 350$ nm) and of HexCBFA in water (dilution 1 : 30), $\lambda_{\text{exc}} = 330$.

observed, a position quite similar to that observed for the fluorophore dissolved in hexane ($\lambda_{\text{emiss}} = 523$ nm).

The photostability of the HexCBFA formulation was tested by irradiating the sample under a UV-lamp ($\lambda_{\text{exc}} = 365$ nm) for 24 h. No changes were observed in the emission properties of the formulation in these conditions.

2.3. Internalization, viability, and Py-BODIPY localization against HeLa cells

The uptake of the HexBFA nanoparticles was investigated by fluorescence microscopy (see ESI† for Experimental details). The image in Fig. 5 clearly shows that cytoplasmic fluorescent signals were observed in the cytoplasm of living cells after HexBFA nanoparticle incubation, whereas no fluorescence was detected in HexFA-treated cells. Remarkably, rather than a diffuse fluorescence the microscopic observations revealed intense fluorescent spots dispersed throughout the cytoplasm.

Given the shape and the size of these spots and the apolar nature of the dye, it could be inferred that the Py-BODIPY

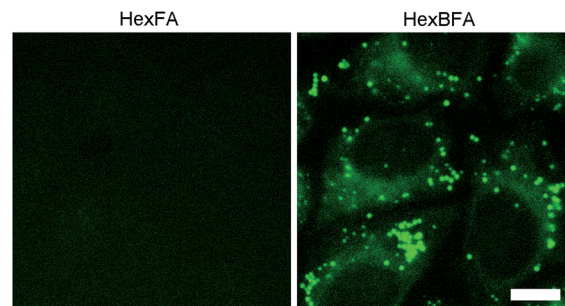


Fig. 5 Fluorescence microscopy of living HeLa cells treated with HexFA and HexBFA nanoparticles (incubated for 4 h) showing cytoplasmic fluorescence spots only inside the HexBFA-treated cells. Scale bar = 10 μm .

localizes in the lipid droplets. The latter are dynamic storage organelles that represent an intracellular reservoir of neutral lipids, such as triacylglycerols and cholesteryl esters, part of the lipid metabolism and used for membrane lipid synthesis. We recently demonstrated that monoolein-based nanoparticles (cubosomes) treatment induces intracellular accumulation of neutral lipids in the cytoplasmic lipid droplets.¹⁶ To investigate whether the Py-BODIPY fluorescence is localized in the lipid droplets, HexBFA-treated cells, grown on coverslips, were fixed and labeled with LipidTOX, a red neutral lipid stain with an extremely high affinity for neutral lipid droplets. This treatment resulted in the intracellular appearance of a colocalized Py-BODIPY (green) and LipidTOX (red) fluorescence, as shown

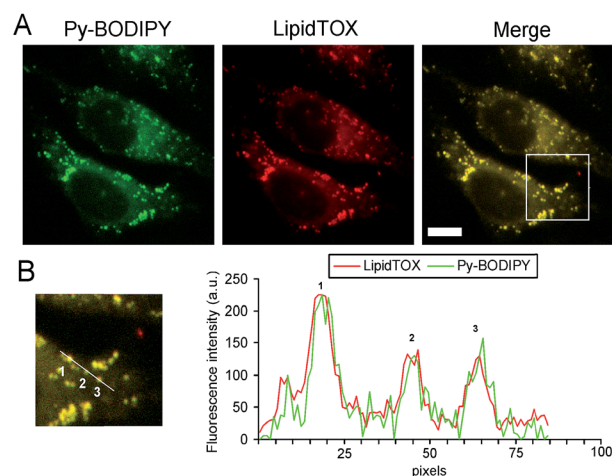


Fig. 6 Colocalization of Py-BODIPY with LipidTOX Red Neutral Lipid Stain. HeLa cells were treated with HexBFA nanoparticles (incubated for 4 h), fixed with 4% PFA and stained with LipidTOX. (A) Cells are displayed in separate images showing the fluorescence of Py-BODIPY (green), LipidTOX (red) and in a composite image (colocalization in yellow). Scale bar = 10 μm . (B) Magnification of the square in the merged image as shown in (A) and fluorescent intensity profiles of Py-BODIPY and LipidTOX. The linear plot (white line), sampled crossing over the cell cytoplasm and three lipid droplets, shows the fluorescence intensity of Py-BODIPY-green and LipidTOX-red fluorescence. The Py-BODIPY profile colocalizes perfectly with the LipidTOX profile thus indicating that Py-BODIPY stains neutral lipids in the lipid droplets.

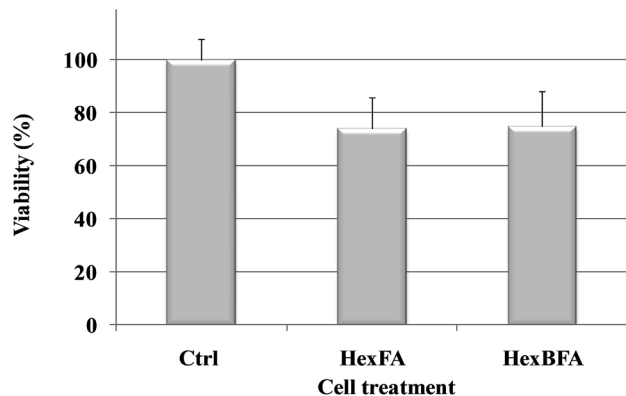


Fig. 7 Viability, expressed as % of the control, induced by incubation for 4 h with HexFA and HexBFA nanoparticles ($1 : 500$, $2 \mu\text{L mL}^{-1}$) in HeLa cells culture. Results were expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving quadruplicate analyses for each sample.

by yellow fluorescence in the merged image and by intensity fluorescence profiles (Fig. 6).

The two nanoparticle formulations, HexFA and HexBFA, were also tested for cytotoxicity (MTT assay) in HeLa cells. Fig. 7 shows the viability, expressed as % of the control, induced in HeLa cells after 4 h incubation. Evidently, treatments with both nanoparticle formulations did not induce a relevant reduction in cell viability as compared with control cells (see ESI† for Experimental details).

2.4. Theoretical calculations

In order to correlate the photophysical properties of Py-BODIPY and the environment experienced by the dye in the formulation, theoretical calculations were carried out at Density Functional Theory (DFT) level. The effect of implicit solvation on the optimised metric parameters (Fig. 8; ESI, Table S1†) was evaluated at IEF-PCM level by considering the same solvents investigated in paragraph 2.1 (see also Table 1). In all cases, the optimised metric parameters were only marginally affected (see ESI† for Experimental details).

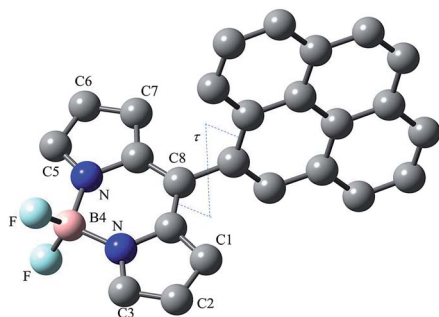


Fig. 8 Ball and stick drawing of the structure of Py-BODIPY optimised at DFT level in the gas phase. The numbering scheme for the BODIPY core is also reported. Hydrogen atoms have not been depicted for clarity.

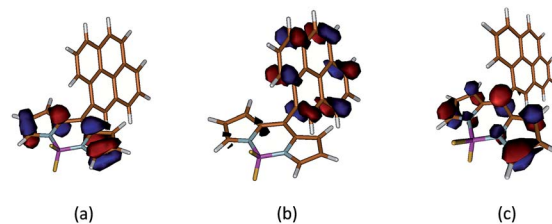


Fig. 9 Molecular orbital isosurfaces of HOMO-1 (a), HOMO (b), and LUMO (c) calculated for Py-BODIPY. Cutoff value $0.05 e$.

In agreement with what was reported previously,³⁸ the HOMO-1 and the LUMO are π -orbitals located on the BODIPY moiety, while the HOMO is centred on the pyrenyl substituent (Fig. 9), both in the gas phase and in solution.

Time-Dependent (TD) DFT calculations show that the singlet electron transition at the lowest energy $S_0 \rightarrow S_1$ (about 2.2 eV , Table S1 and Fig. S3, ESI†) calculated for Py-BODIPY arises entirely from a HOMO $\rightarrow$ LUMO mono-electronic excitation, corresponding to a charge transfer (CT) process between the pyrene and the indacene systems. Due to the π -nature of the molecular orbitals (Fig. 9), in order for the overlap integral between the wavefunctions of HOMO and LUMO to be different from zero, the deviation of τ (the rotation of the pyrenyl ring, see ESI† for Experimental details) from orthogonality is required. Accordingly, this transition was calculated to be forbidden when the pyrene and the indacene planes were forced to be perpendicular. As a consequence of the CT nature of the transition, the $S_0 \rightarrow S_1$ transition, which shows a very low oscillator strength at the optimised geometry, is calculated to be moderately solvatochromic in character (with a variation $\Delta\lambda$ in the calculated absorption wavelength of about 25 nm on passing from toluene to DMF).

The $S_0 \rightarrow S_2$ transition (falling at about 3.0 eV , see Table S1 and Fig. S3, ESI†) is instead assigned to a HOMO-1 $\rightarrow$ LUMO excitation. Due to its non-polar character, this transition is independent of the solvent ($\Delta\lambda = 5 \text{ nm}$). Although difference in the excitation energies between the two transitions (about 0.8 eV , see Table S1, ESI†) might be overestimated by TDDFT calculations because of their difference in polarity, the structured experimental absorption band at about 500 nm can be attributed to the convolution of the transitions leading to the S_1 and S_2 excited states. TDDFT calculations allow accounting for the fluorescence of Py-BODIPY. Indeed, it is conceivable that the UV excitation from S_0 to a S_k excited state is followed by an internal conversion to the S_2 excited state. The possibility of the pyrene group to rotate (the libration vibration is calculated at about 20 cm^{-1} for the ground state), shown by the PES analysis (see ESI and Fig. S2†), could be responsible for the vibrational internal conversion $S_2 \rightarrow S_1$. Hence, fluorescence emission would involve the $S_1 \rightarrow S_0$ radiative relaxation. From a qualitative point of view, the polar nature of S_1 nicely accounts for the remarkable solvatochromism of the emission discussed above. Indeed, in polar solvents (such as DMF, alcohols, acetonitrile) the S_1 singlet excited state is stabilised ($E < 2.2 \text{ eV}$, Table S1 and Fig. S3, ESI†) by up to 0.1 eV with respect to nonpolar solvents (hexane, toluene), so that emission is

expected to occur at lower energies, as found experimentally (Fig. 1). Accordingly, the fluorescent emission observed in the hexosomes, similar to that detected in hexane, reflects the non-polar nature of the environment experienced by the imaging agent (the hydrophobic MO chains), strongly supporting the localization of the Py-BODIPY within the bilayer.

3. Conclusions

In this paper we showed that nanoparticles with a 2D hexagonal nanostructure can be loaded with a pyrene-modified BODIPY fluorophore and the antineoplastic drug camptothecin and, at the same time, stabilized using a mixture of PF108 and folate-conjugated PF108, so that they possess targeting properties toward tumor cells. The striking solvatochromism of the modified BODIPY, emitting at energies directly dependent on the polarity of the chemical environment, and the QM calculations performed, confirm the localization of the imaging agent within the bilayer. Moreover, we demonstrated that these nanoparticles are easily internalized by HeLa cells and that the fluorophore localizes within the lipid droplets. The cytotoxicity experiments, performed using the HexBFA formulation at a concentration of $75 \mu\text{g mL}^{-1}$ of the dispersed phase (MO + block copolymers, or $68 \mu\text{g mL}^{-1}$ of MO), the same as used for the fluorescence microscopy experiments, proved that the proposed formulation can be safely used for cellular imaging purposes. Remarkably, these results are in good agreement with our previous cytotoxicity tests performed on HeLa cells using cubosomes formulations, and those obtained by other groups working on similar nanoparticles and different cellular lines.^{43,44}

Integration of therapeutics and imaging agents within a single theranostic nanoparticle allows monitoring drug delivery, release, and efficacy, contributing to realize tailor-made therapies that, besides the study of genetic variations and biomarkers, exploit the development of imaging methods for predicting and evaluating therapeutic responses.⁴⁵ Therefore, results here presented encourage the use of these nanoparticles in theranostic nanomedicine for personalized diagnosis and treatment of tumors.

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VI

Effects of monoolein-based cubosome formulations on lipid droplets and mitochondria of HeLa cells.

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Effects of monoolein-based cubosome formulations on lipid droplets and mitochondria of HeLa cells

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Despite the remarkable development of nanoparticles for different purposes, relatively little is known about their interaction with biological systems and individual cells. Here the effects of two monoolein-based cubosome formulations stabilized by Pluronic F108 and F127 were investigated against HeLa cells. Microscopy analysis on living cells loaded with organelle-specific fluorescent probes was performed to assess the formation of cytoplasmic lipid droplets after nanoparticle treatment. Mitochondrial membrane potential and mitochondrial ROS generation were also investigated in relation to the capability of the accumulated lipids to affect mitochondrial functions. Values of the main cellular unsaturated fatty acids were also measured to assess cell lipid profile modulation. Results from this study show that the uptake of both cubosome formulations induced modification of the cell lipid profile, lipid droplet accumulation, mitochondrial hyperpolarization and mitochondrial ROS generation. These results shed some light on the influence exerted by monoolein-based cubosome formulations on subcellular organelles and their possible adverse effects on cell functions.

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Introduction

Monoolein (MO, glycerol monooleate) self-assembles in water, forming a number of nanostructures, including two bicontinuous cubic phases characterized by a different space group: the *Ia3d*, and the *Pn3m*.¹ These amazing liquid crystalline nanostructures are composed of a curved bilayer whose three-dimensional folding originates two disconnected, continuous water channels.^{2,3} They were ubiquitously found in Nature, and several studies were dedicated to shed some light on their role in the cell machinery.^{4–6} Since MO-based bicontinuous cubic phases are nanostructures that can suitably host hydrophobic molecules with biological relevance, they were extensively investigated in the past for their possible applications in the pharmaceutical field.^{7–9} Remarkably, cubic phases can be formulated as nanoparticle dispersions, known as cubosomes, typically stabilized in water using amphiphilic polymers having long polyethylene oxide (PEO) chains, such as Pluronics or polysorbate 80.^{10–12} These liquid crystalline nanoparticles are often proposed to be suitable drug delivery nanocarriers

and, recently, their possible application as theranostic nanomedicine was discussed in several papers.^{13–15} However, little information about *in vitro* cellular interactions and toxicity of monoolein-based cubosomes has been reported so far, and the lack of shared protocols precludes straightforward comparisons between these investigations. For example, focusing on monoolein-based formulations exposed to adherent cells, to the best of our knowledge only six papers (two from our group, not including the present) have discussed the cytotoxicity of pristine cubosomes (*i.e.*, not decorated with targeting agents or loaded with drugs/imaging agents).^{13,16–20} In all these cases, experiments were performed using different cell lines, at different incubation times, and with formulations having different compositions (basically, they were prepared using a different monoolein/Pluronic ratio, sometimes using different types of Pluronic). It is worth noting that several papers have reported on the haemolytic properties of monoolein-based cubosome formulations tested on mouse blood,^{18,21} indicating 20 $\mu\text{g mL}^{-1}$ of monoolein as the maximum tolerable concentration.²⁰ However, another recent investigation conducted on human and porcine plasma has disputed these results, attributing to the same formulation a very low haemolytic activity.²² We have previously shown no toxic effects of monoolein-based nanoparticles (liposomes and cubosomes) at short times against HeLa cells and 3T3 fibroblasts^{16,23} based on an 80% viability threshold.

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All cell types have been found to readily take up the lipids present in culture media and sequester them into lipid droplets (LDs). LDs are cytoplasmic organelles that consist of a hydrophobic core of neutral lipids (such as triglycerides and cholesterol esters) encased by a phospholipid monolayer harboring a set of enzymes and regulatory proteins that catalyze the highly metabolically controlled synthesis and mobilization of fat stores.²⁴ LDs, found in almost all cells under physiological or pathological conditions, are heterogeneous not only in size but also in the lipid content and the composition of their protein coat.²⁵ The most widely accepted model for LD formation hypothesizes that they arise in the endoplasmic reticulum (ER) through the accumulation of neutral lipid at specific sites between the leaflets of the phospholipid bilayer (ER budding model).²⁴ Dynamical interactions between LDs and organelles other than ER, including mitochondria and peroxisomes, have been suggested to facilitate the exchange of proteins and lipids in cells, while interactions between the ER and mitochondria are crucial for processes such as lipid synthesis, storage and transport as well as mitochondrial functions (calcium homeostasis and apoptosis).^{26,27}

In the present study, we used organelle-specific dyes to explore the changes occurring in lipid droplets and mitochondria of living HeLa cells when exposed to treatment with monoolein-based cubosomes stabilized by Pluronic F108 or Pluronic F127. Analyses were carried out to examine lipid droplet content, mitochondria membrane potential, reactive oxygen species (ROS), and cellular lipid profile at 4 and 24 h after nanoparticle treatment. Our results demonstrate that the uptake of both cubosome formulations induces a modification in the cellular lipid profile, accumulation of lipid droplets associated with a significant dysfunction of mitochondria,

including mitochondrial hyperpolarization and mitochondrial ROS generation.

Results and discussion

Cubosome characterization

Colloidal dispersions of a bicontinuous cubic phase (cubosomes) were prepared by dispersing *via* sonication melted monoolein (MO) either in a Pluronic F108 (PF108) or in a Pluronic F127 (PF127) aqueous solution. These nanoparticle formulations were then characterized for particle size and morphology, as well as for their inner structure. The cryo-TEM images of MO-based PF127 and PF108 stabilized cubosomes (MO/PF127 and MO/PF108) are shown in Fig. 1A and B. These images show various nanoparticles revealing the regular alternation of bright spots (water channels) and dark matrix (lipid bilayer) classically observed in the presence of a cubic arrangement of the interface, along with the vesicular material that typically co-exists with the cubosomes in these kinds of formulations.¹⁶ On the basis of repeated observations, the samples under investigation *via* cryo-TEM were found to be a dispersion of cubosomes having diameter in the range of 100–200 nm, in striking agreement with DLS analysis (Table 1). SAXS definitely confirmed the inner cubic structure of both cubosome formulations. As reported in Table 1, the diffraction patterns showed the Bragg peaks of the bicontinuous cubic double diamond and primitive phases (with space groups *Pn3m* and *Im3m*, respectively, see also Fig. 1C and D). It deserves noticing that the simultaneous presence of these two phases has already been described for cubosome formulations having composition similar to those investigated here,²⁸ and their occur-

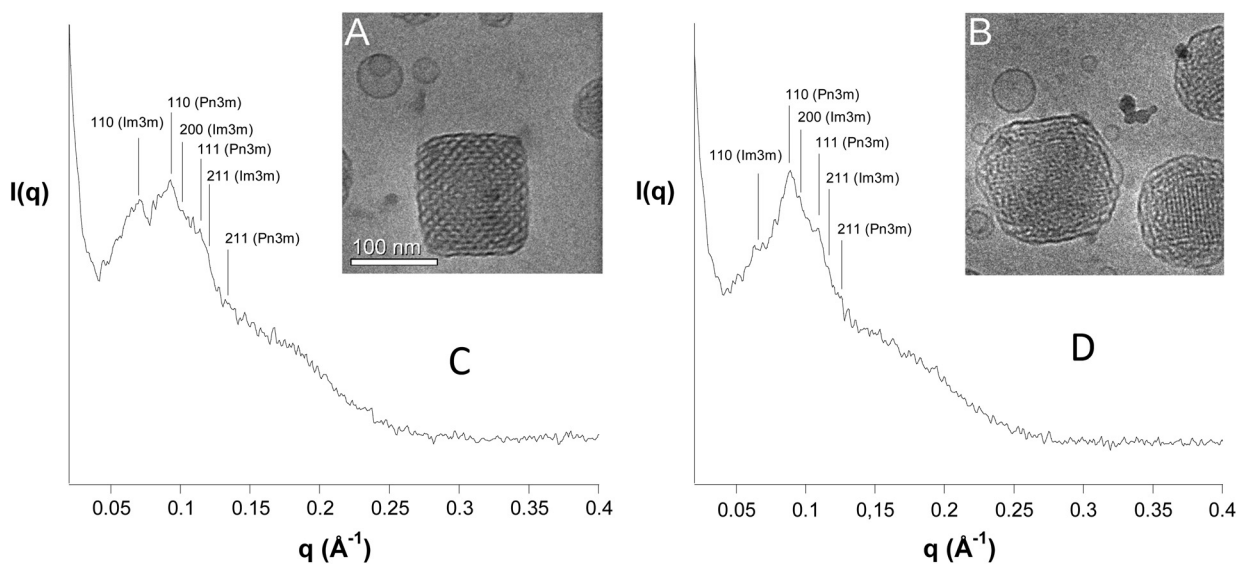


Fig. 1 Cryo-TEM images of MO-based cubosomes stabilized by PF127 (A) and PF108 (B). $I(q)$ vs. q data obtained by SAXS of the cubosome formulation stabilized by PF127 (C) and PF108 (D). The Miller indices are reported on top of the corresponding Bragg peaks along with the indication of the space group.

Table 1 Space group, lattice parameter (a), mean diameter (D_{av}), poly-dispersion index (PDI), and ζ -potential of the cubosome nanoparticles stabilized with either PF108 or PF127

Cubosome stabilizers	Space group	a (Å)	D_{av} (nm)	PDI	ζ -Potential (mV)
PF108	<i>Pn3m</i>	101 ± 4	133 ± 1	0.11	−28 ± 2
	<i>Im3m</i>	137 ± 4			
PF127	<i>Pn3m</i>	98 ± 3	136 ± 2	0.14	−19 ± 1
	<i>Im3m</i>	136 ± 6			

rence is explained with the non-uniform partition of the Pluronic. Indeed, during the dispersion process the dispersant preferentially localizes at the cubosome surface, originating Pluronic-depleted regions characterized by double diamond symmetry.²⁹

Accumulation of lipids in the lipid droplets

Several studies have shown LD accumulation after treatment with free fatty acids³⁰ or associated with oxidative stress induced by nanoparticle treatment.^{31,32} Indeed, after the internalization in the cells, unsaturated fatty acids such as oleic acid (OA) can be either esterified and stored as neutral lipids inside LDs or become part of the cellular membranes or, after activation to fatty acyl-CoAs, oxidized in mitochondria for energy production.³⁰ Here, to investigate LD accumulation, HeLa cells were treated with cubosome formulations (5 μ L in 1 mL of medium) at 4 and 24 h and, after nanoparticle wash-out, co-loaded with Nile Red (NR) and Hoechst probes, to stain LDs and the nucleus, respectively.

In untreated control cells the number of small LDs, detected as punctuated green fluorescent cytoplasmic structures, was very different from cell to cell. Upon short exposure to cubosomes, the number of lipid droplets dispersed throughout the cytoplasm and their size increased in a remarkable way and droplets appeared larger and more numerous in all cells (Fig. 2A), suggesting that LD accumulation results from the synthesis of neutral lipids from nanoparticle-derived monoolein.

When compared to untreated control cells, an increase in the IOD (Integrated Optical Density) per cell of 7.9 ($p < 0.001$)- and 3.1 ($p < 0.001$)-fold was estimated for, respectively, MO/PF127 and MO/PF108, indicating a statistically significantly increased production of LDs (Fig. 2B). Subsequently, HeLa cells were grown in the presence of MO dissolved in DMSO and Pluronic aqueous solutions (administered at the same concentrations as MO and Pluronics present in the cubosome formulations) and OA (18:1 n-9) (used as the positive control at the non-cytotoxic concentration of 100 μ M in DMSO). At short times, MO and OA treatments caused 1.3 ($p < 0.05$)- and 2.8 ($p < 0.001$)-fold increase, respectively, in IOD per cell in comparison with untreated cells, whereas Pluronic solutions showed the same IOD per cell as the control (Fig. 2B). At longer exposure time (24 h), differently from MO- and OA-treated cells where the IOD did not change, the IOD of

MO/PF127 and MO/PF108-treated cells increased by 25.6 ($p < 0.001$)- and 31.5-fold ($p < 0.001$), respectively. Because of the huge cubosome uptake, cells appeared to be full of lipids inside the cytoplasm with many large droplets aggregated in several clusters resembling grapes (Fig. 2A and B).

While the LD formation induced by long-chain unsaturated fatty acids such as OA is a well-known phenomenon, data reported here proved that LD formation also occurs upon MO and MO-based cubosome treatment. Compared with MO-treated cells, statistically significantly increased production of lipid droplets was detected after cubosome exposure, thus suggesting a strong internalization ability of both MO-based cubosome formulations. Since the amount of MO administered was exactly the same, the differences could be ascribed to different uptake mechanisms. Indeed, while nanoparticles can be transported into the cells *via* endocytosis, the fatty acid uptake across the lipid bilayer of the plasma membrane was proposed to proceed by diffusion or through membrane-associated proteins (acting as fatty acid transporters).^{33,34} Remarkably, statistically significant differences ($p < 0.001$) between the two cubosome formulations were observed in the IOD only at short time treatments.

Modification of the lipid cell profile

The same MO/PF108 and MO/PF127 cubosome formulations and MO, OA, and Pluronic solutions previously described were then tested to assess their effects on the lipid composition of HeLa cells. After 4 and 24 h of incubation, the cell lipid fraction was extracted and the variations in the levels of unsaturated fatty acids and cholesterol were analyzed with respect to untreated control cells. The unsaturated fatty acid composition (expressed as μ g per plate) and the chromatographic profile of HeLa control cells obtained by HPLC are shown in Fig. 3. The cell content of the most abundant unsaturated fatty acids was detected as follows: 78.19, 19.87, 6.38, 4.89, 3.11, and 3.05 μ g per plate for 18:1 isomers (OA and 18:1 n-7), 16:1 n-7, arachidonic acid (20:4 n-6), docosahexaenoic acid (DHA, 22:6 n-3), eicosapentaenoic acid (EPA 20:5 n-3), and 18:2 n-6, respectively; minor amounts were measured for 18:3 n-3, 20:3, and 22:4 n-6. The incubation of HeLa cells with PF108 or PF127 solution did not induce changes in unsaturated fatty acid levels, with treated cells showing a profile similar to that of control cells at both incubation times (data at 4 h are shown in Fig. 3A).

Fig. 4 shows the values of the most abundant unsaturated fatty acids 16:1 n-7, 20:4 n-6, DHA, OA, and cholesterol (expressed as % of the control) measured in HeLa cells after 4 (Fig. 4A) and 24 h (Fig. 4B) of incubation in the presence of cubosomes, MO, and OA. The incubation of cells at 4 h treatment with the compounds induced a significant increase in the cell level of OA, which reached values approximately 3, 3, 2, and 2 times higher than that of the control cells in MO/PF108, MO/PF127, MO and OA treated cells, respectively. Treatments were not associated with changes in the levels of all the other unsaturated fatty acids. Experiments performed at 24 h of incubation time revealed a further increase of the OA levels

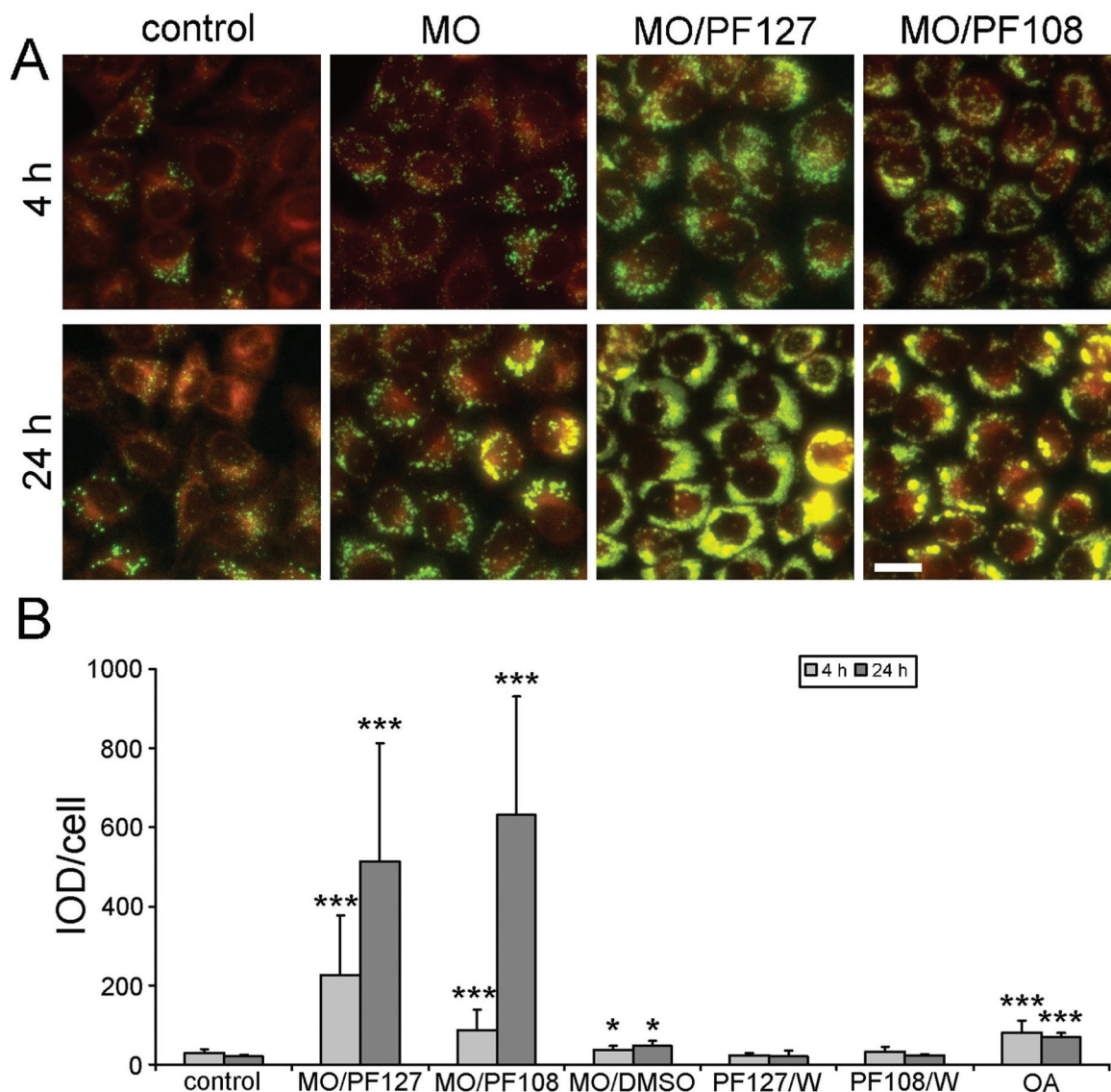


Fig. 2 Lipid droplet accumulation induced in HeLa cells by cubosome treatment. (A) Representative composite color images of HeLa cells exposed to the monoolein (MO), MO/PF127 and MO/PF108 cubosomes at 4 and 24 h of incubation time. Membranes (red) and lipid droplets (green) were stained with Nile Red (colocalization in yellow). Scale bar = 20 μ m. (B) Results of the lipid droplet formation at 4 and 24 h of incubation time. Oleic acid (OA) was used as the positive control at the non-cytotoxic concentration of 100 μ M. Data are expressed as mean $\pm$ SD from four independent experiments. Statistically significant differences are indicated by * ($p < 0.05$), *** ($p < 0.001$) by *t*-test vs. untreated-control cells.

(5 times higher than the control) in cells treated with cubosome formulations, while levels of all the other unsaturated fatty acids remained substantially unaltered with respect to the control. Remarkably, the levels of OA recorded in MO and OA treated cells were very similar to those observed at 4 h-treated HeLa cells. This result parallels the finding discussed in the previous paragraph concerning the lipid droplet accumulation and, similarly, can be reconciled taking into account the different uptake mechanisms. Using HPLC, the total cholesterol level was measured in control cells as the mean content of 72.52 ± 0.55 μ g per plate. Fig. 4 also shows the values of cholesterol measured in the control and 4 and 24 h-treated

HeLa cells. The different treatments did not seem to affect the level of cholesterol.

Mitochondrial membrane potential

Mitochondria play a central role in normal cell functions such as energy supply and in cell death by inducing apoptosis. Changes in the mitochondrial membrane potential (mitoMP) represent one of the adaptation processes to a variety of environmental factors that regulate the increased energy demands, cell growth and differentiation, or cellular stress. In mitochondria, mitoMP is produced by the electron transport chain as it pumps H^+ ions into the membrane space,

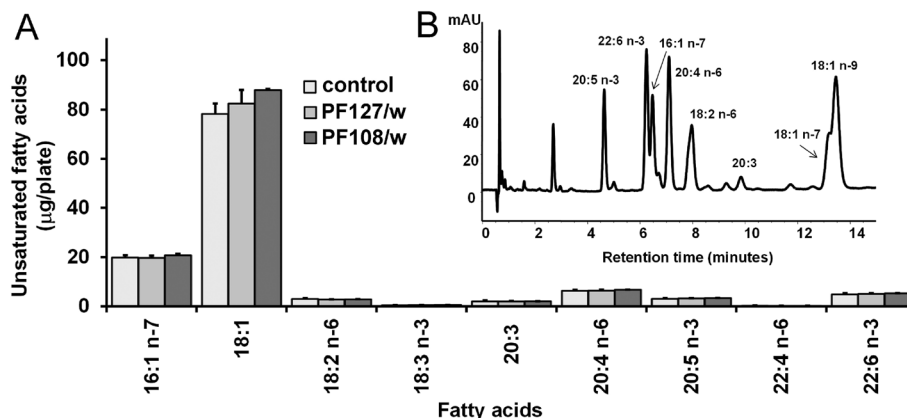


Fig. 3 Fatty acid composition and chromatographic profile of HeLa cells. Values of the unsaturated fatty acids (16:1 n-7, 18:1, 18:2 n-6, 18:3 n-3, 20:3, 20:4 n-6, 20:5 n-3, 22:4 n-6, 22:6 n-3, expressed as μg per plate) measured in control cells and after 4 h of incubation in the presence of PF127 and PF108 solutions (A) and the corresponding HPLC chromatographic profile of the control HeLa cells (B). Data are expressed as mean $\pm$ SD from four independent experiments (involving duplicate analyses for each sample).

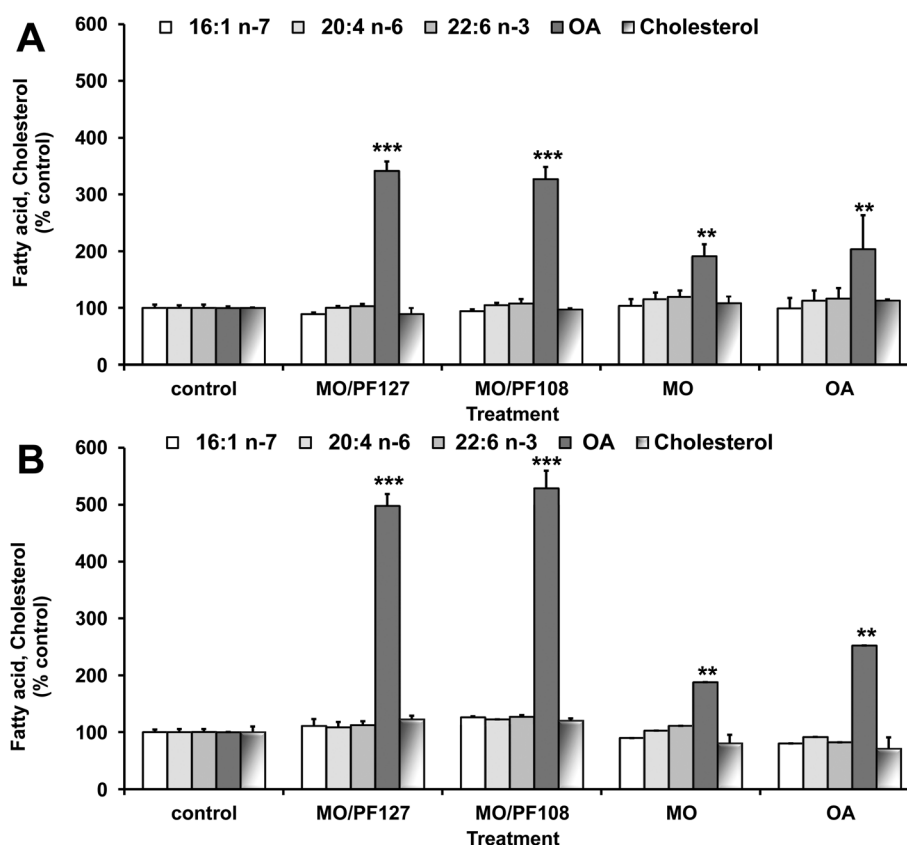


Fig. 4 Lipid profile modulation in cubosome-treated cells. The values of the main unsaturated fatty acids, 16:1 n-7, 18:1 (OA), 20:4 n-6, 22:6 n-3, and cholesterol (expressed as % of the control) measured in HeLa control cells and after 4 (A) and 24 h (B) of incubation in the presence of MO/PF127 and MO/PF108 cubosomes, MO, and OA (used as the positive control). Data are expressed as mean $\pm$ SD from four independent experiments (involving duplicate analyses for each sample). Statistically significant differences, using one-way analysis of variation (one-way ANOVA) and the Bonferroni post test (GraphPad INSTAT software, San Diego, CA), are indicated by ** ($p < 0.01$), *** ($p < 0.001$) vs. untreated-control cells.

thus creating an electrochemical proton gradient which drives ATP synthesis. Variations of mitoMP are usually measured in living cells with the cationic fluorescent dye TMRM,

which accumulates in the mitochondrial matrix driven by the voltage gradient across the inner mitochondrial membrane. Using the TMRM probe, control cells showed morphologically

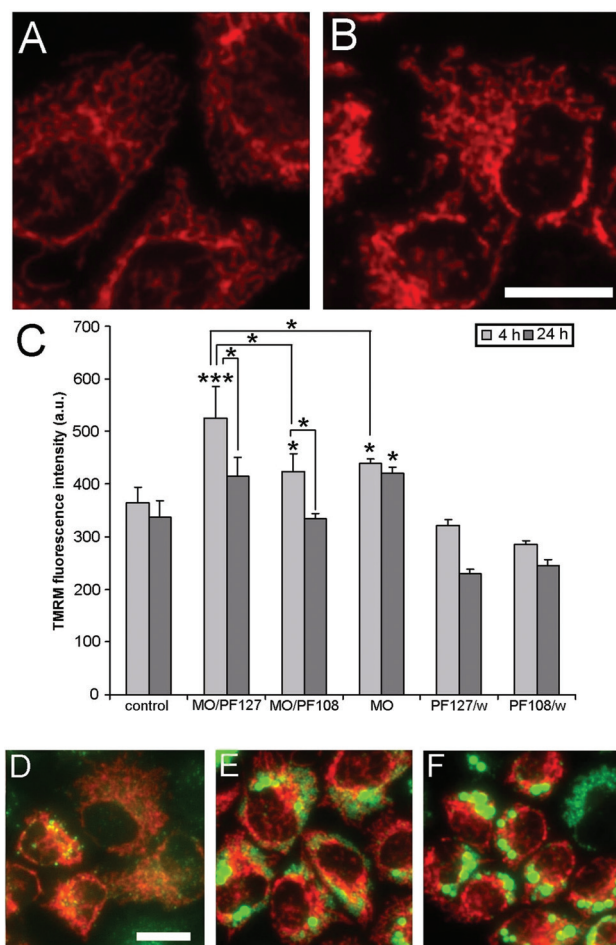


Fig. 5 Effect of cubosome treatment on mitochondrial membrane potential and mitochondrial network appearance. (A, B) Representative images of the control (A) and short-term cubosome-treated (B) cells stained with the mitochondria-specific dye TMRM. Scale bar = 20 μ m. (C) Analysis of mitochondrial membrane potential (mitoMP) evaluated by TMRM fluorescence intensity (arbitrary units) in the control and treated cells. Data are expressed as the mean $\pm$ SD from four independent experiments. Statistically significant differences are indicated by * ($p < 0.05$), *** ($p < 0.001$) by *t*-test vs. untreated-control cells and among differently treated cells. (D–F) Representative composite color images of HeLa cells, co-loaded with TMRM (red) and Bodipy 493/503 (green), after 24 h of incubation time with MO (D), MO/PF127 (E) and MO/PF108 (F) cubosomes. Scale bar = 10 μ m.

smooth and variously stained mitochondrial filaments (Fig. 5A).

Following short term incubation of HeLa cells with both cubosome formulations and MO, a significant hyperpolarization of the mitochondrial membrane potential was observed as compared with untreated-control cells (Fig. 5B and C). Moreover, MO/PF127-treated cells showed a larger mitoMP ($p < 0.05$) than MO/PF108- and MO-treated cells. In contrast, analysis of mitoMP in cells treated with Pluronic solutions showed the same mitoMP as the control. At long time treatment, differently from MO-treated cells, a significant decrease of mitoMP ($p < 0.05$) was detected in both cubosome-treated cells.

On co-loading cells with TMRM and Bodipy 493/503, it was observed that, in cubosome-treated cells, the cytoplasmic distribution of mitochondria (the so-called mitochondrial networking) was considerably modified, and unlike MO-treated cells where the mitochondrial network appeared to be regular (Fig. 5D), clusters of mitochondria were confined in various areas of the cytoplasm and excluded by the large droplets (Fig. 5E and F). Therefore, the loss of mitoMP associated with altered mitochondrial morphology could suggest that, at long time treatment, mitochondrial dysfunction arises from lipid accumulation.³⁵

Mitochondrial ROS generation

The mitochondrion is the major free radical-producing compartment of the cell, representing, at the same time, an important target for the reactive oxygen species (ROS) action. Mitochondrial ROS production usually occurs during electron transport along the respiratory chain complexes, and same pathophysiological conditions can increase mitochondrial ROS generation such as an enhancement of the mitochondrial membrane potential.³⁶ To specifically investigate the effect of nanoparticles on mitochondrial ROS generation, control and treated cells were loaded with the mitochondrial superoxide indicator MitoROS Red in combination with the mitochondria-specific probe MitoTracker Green with the aim of verifying the mitochondrial origin of ROS and correlating mtROS with changes in the mitochondrial morphology. A significant and progressively increased ROS production was detected in cells treated with both types of cubosomes as compared with the control and MO-treated cells (Fig. 6A–C). ROS production increased by 1.3 ($p < 0.01$)- and 1.9-fold ($p < 0.001$) at short term treatment and by 2.3- and 2.4-fold ($p < 0.001$) at long time exposure to, respectively, MO/PF108 and MO/PF127 cubosomes. The mitochondrial origin of ROS was confirmed by their co-localization with MitoTracker Green. It is worth mentioning that, in cells with high mtROS levels, mitochondria appeared fragmented (Fig. 6D), suggesting involvement of the mitochondrial permeability transition pore (mPTP) and the inner membrane anion channel (IMAC) in maintaining healthy mitochondria homeostasis.³⁷

Analysis of the mtROS level in HeLa cells treated with Pluronic solutions showed different results (Fig. 6A). Whereas in PF108 solution-treated cells, mtROS production was not dissimilar from the control and MO-treated cells, cells treated with a PF127 solution showed a significant mtROS increase at both 4 h and 24 h treatment. This effect is in agreement with that reported in a previous study where Pluronic P85 co-localized with mitochondria and caused mitochondria impairment accompanied by ROS production.³⁸

Nuclear staining and externalization of phosphatidylserine

LD and mitoMP evaluation was performed on cubosome-treated cells with no signs of morphological damage and/or chromatin condensation (Fig. 7A). Chromatin condensation was determined by nuclear staining with Hoechst 33258. After 4 h cubosome treatment along with healthy cells, we detected

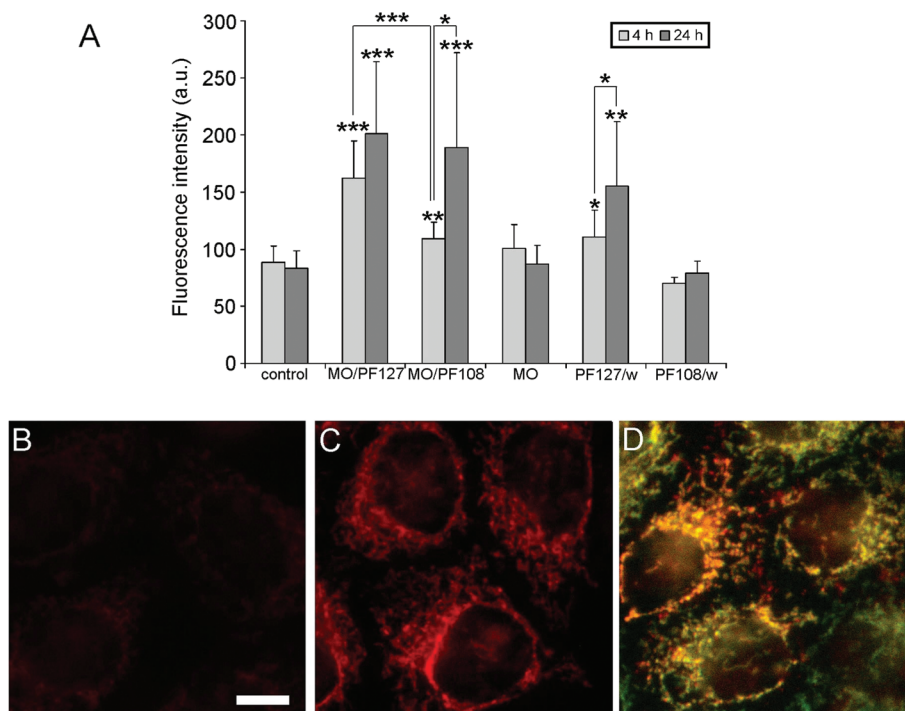


Fig. 6 Mitochondrial ROS generation. (A) Analysis of mitochondrial ROS evaluated by MitoSOX fluorescence intensity (arbitrary units) in untreated-control and treated cells. Data are expressed as mean $\pm$ SD from four independent experiments. Statistically significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) by t-test vs. untreated-control cells and among differently treated cells. (B, C) Representative images of MitoSOX Red fluorescence in untreated (B) and short-term cubosome-treated (C) cells. (D) Composite color image of cubosome-treated cells co-loaded with MitoSOX Red (red) and MitoTracker Green (green), co-localization in yellow-orange. Scale bar = 10 μ m.

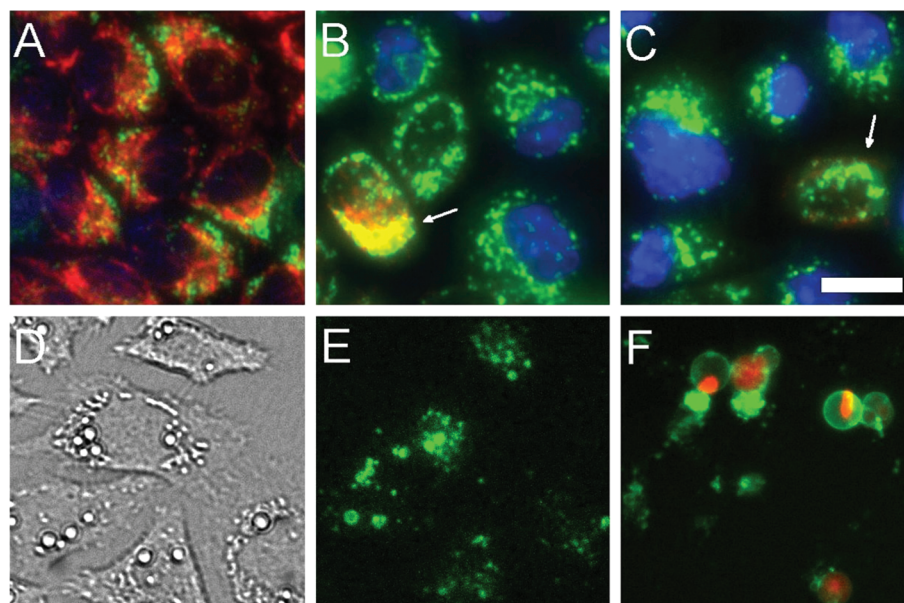


Fig. 7 Cells undergoing apoptosis with chromatin condensation and externalized PS. (A–C) Merged color images of cubosome-treated cells co-loaded with TMRM (red), Bodipy 493/503 (green) and Hoechst 33258 (HOE, blue) probes. (A) Healthy cells showing polarized mitochondria, lipid droplet accumulation and no signs of chromatin condensation. (B, C) Round cells, full of cytoplasmic lipids, showing chromatin condensation (HOE-positive) and depolarized mitochondria (TMRM-negative). Arrows indicate some round cells showing still polarized mitochondria. (D–F) Phase contrast (D) and relative fluorescence image (E) of cells showing distinct round domains positive for FITC-Annexin V with nuclei negative for propidium iodide (PI). (F) Apoptotic cells positive for FITC-Annexin V and PI. Scale bars = 10 μ m.

the presence of clusters of round cells (still attached at the dish) with the cytoplasm full of lipids, showing condensed nuclei and low or completely missing TMRM fluorescence, indicating mitochondrial depolarization (Fig. 7B and C). To verify whether these round cells were undergoing apoptosis, untreated control and cubosome-treated cells were co-stained with FITC-conjugated Annexin V and propidium iodide (PI). As untreated-control cells were largely negative for externalized phosphatidylserine (PS) and nuclear condensation, in cells treated with cubosomes we were able to observe and distinguish differently labeled cells: (a) healthy cells (~80%), negative for both Annexin-FITC and PI (Fig. 7A); (b) cells undergoing apoptosis (~15%), with exposed PS Annexin positive and PI negative (Fig. 7D and E); (c) apoptotic cells (~5%), positive for both Annexin-FITC and PI (Fig. 7F). This result showed the slight impact of the cubosomes on the cells and, at the same time, confirmed that lipid loading might induce apoptosis in cells.

MTT assay

The effects of cubosome formulations, MO, PF127 and PF108 solutions were evaluated against HeLa cells at 4 and 24 h according to the MTT assay, which is sensitive to mitochondrial activity and is used to evaluate cell apoptosis and cell division inhibition. The MTT value of non-treated control cells was set to 100% and the values of treated cells were expressed as a percent of the control. As shown in Fig. 8, a slight decrease of MTT reduction was observed with both cubosome formulations (but also with MO and Pluronic solutions) in the first 4 h of incubation time. Remarkably, at long term exposure (24 h), the absorbance of cubosome-treated cells increased and was not different from control cells, whereas treatment with MO and both Pluronic solutions showed the same decrease as MTT reduction at short time. This unexpected

result may be due to variation in the redox status of the cells and/or mitochondrial membrane hyperpolarization and/or interference of the MTT reaction with the large amount of LDs in the cell cytoplasm. Indeed various lines of evidence suggest that MTT formazan deposits accumulate in the lipid droplets.^{39,40} According to MTT results further supported by fluorescence analysis, HeLa cells appeared healthy beside mitochondrial dysfunction and lipid droplet accumulation, suggesting no relevant toxic effect of cubosome treatment at these incubation times and this concentration.

Conclusion

Here, the effects caused by two different cubosome formulations on HeLa cells were compared. The formulations were prepared using the same building block (monoolein), but were stabilized by two different Pluronics. By employing multiple labeling with organelle-specific dyes, we provide a helpful procedure to investigate nanoparticle–cell interactions. Results evidenced that both cubosome formulations influenced the cell behavior and, particularly, organelles such as lipid droplets and mitochondria. In long time experiments the differences between the two cubosome formulations were levelled out, but significant differences were observed at short time treatments.

Physicochemical investigations demonstrated that, from a morphological point of view, two almost indistinguishable formulations were compared. Moreover, the lipid profile confirmed that the same amount of formulation was taken up by the cells. Therefore, the detected differences can be entirely ascribed to the different Pluronic used to stabilize the cubosome formulations, and could be explained in terms of the different hydrophobicity of these block copolymers, as exemplified by their Hydrophilic Lipophilic Balance values (HLB equal to 22 and 27 for PF127 and PF108, respectively).⁴¹ Results concerning the Pluronic solutions evidenced different mitochondrial ROS production depending on the dispersant used, reinforcing this hypothesis. The MTT test proved the low toxicity of these formulations against HeLa cells at the concentration used in this investigation ($165 \mu\text{g mL}^{-1}$ of monoolein). This finding agrees with others previously reported on the same cellular line.¹⁷

Taking into consideration the close advent on the market of various nanotechnologically based products, we strongly believe that the presented results may be useful to understand the impact of cubosome formulations in biological tissues and their possible hazardous nature.

Experimental

Cubosome preparation

Cubosomes were prepared by dispersing the appropriate amount of melted monoolein (MO) in an aqueous solution of Pluronic (F108 or F127) using an ultrasonic processor UP100H

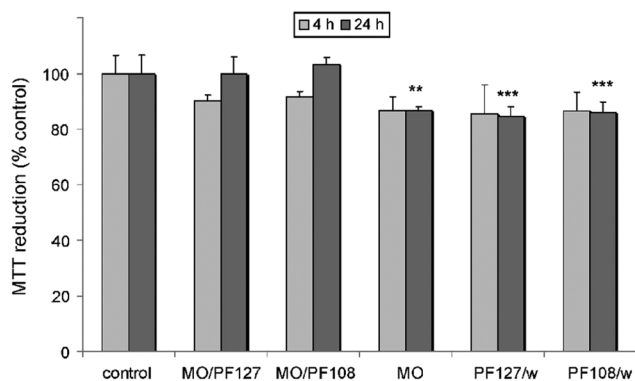


Fig. 8 Results of the MTT assay of HeLa cells exposed to treatment with cubosomes, monoolein and Pluronic solutions. The MTT value of non-treated control cells was set to 100% and the values of treated cells were expressed as a percent of the control. Data are expressed as the mean $\pm$ SD from three independent experiments (involving triplicate analyses for each sample). Statistically significant differences are indicated by **($p < 0.01$) and ***($p < 0.001$) vs. untreated-control cells by *t*-test.

by Dr Hielscher, cycle 0.9, amplitude 90%, for 10 minutes. The sample volume was 4 mL with 96.4 wt% of water, 3.3 wt% of MO and 0.3 wt% of Pluronic (F108 or F127). MO (RYLO MG 19 PHARMA, glycerol monooleate, 98.1 wt%) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. Pluronic F108 (PEO₁₃₂-PPO₅₀-PEO₁₃₂) and Pluronic F127 (PEO₁₀₀-PPO₆₅-PEO₁₀₀) were purchased from Sigma-Aldrich. Distilled water passed through a Milli-Q water purification system (Millipore) was used to prepare the samples.

Cryogenic-transmission electron microscopy (cryo-TEM)

Vitrified specimens were prepared in a controlled environment vitrification system (CEVS) at 25 °C and 100% relative humidity. A drop of the sample was placed on a perforated carbon film-coated copper grid, blotted with filter paper, and plunged into liquid ethane at its freezing point. The vitrified specimens were transferred to a 626 Gatan cryo-holder, and observed at 120 kV acceleration voltage in a FEI Tecnai T12 G2 transmission electron microscope at about -175 °C in the low-dose imaging mode to minimize electron-beam radiation-damage. Images were digitally recorded with a Gatan US1000 high-resolution CCD camera.

Small-angle X-ray scattering (SAXS) experiments

Small-angle X-ray scattering was recorded with an S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). Cu K α radiation of wavelength 1.542 Å was provided by a GeniX X-ray generator, operating at 50 kV and 1 mA. A 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels of width 54.0 μ m was used for the detection of scattered X-rays in the small-angle region. The working q -range ($\text{\AA}^{-1}$) was $0.003 \leq q \leq 0.6$, where $q = 4\pi \sin(\theta)\lambda^{-1}$ is the scattering wave vector. Thin-walled 2 mm glass capillaries were used. The lattice parameter a of the cubic phase was determined using the relation $a = d(h^2 + k^2 + l^2)^{1/2}$ from linear fits of the plots of $1/d$ versus $(h^2 + k^2 + l^2)^{1/2}$, where $d = 2\pi/q$ and h , k , and l are the Miller indices.

Dynamic light scattering (DLS)

Particle size and ζ -potential determinations of the nanoparticles were performed with a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK) at a temperature of 25 ± 0.1 °C. Samples were backscattered by a 4 mW He-Ne laser (operating at a wavelength of 633 nm) at an angle of 173°. Diluted samples (1 : 50) were housed in disposable polystyrene cuvettes of 1 cm optical path length with water as the solvent. At least 2 independent samples were taken, each of which was measured 3–5 times. The width of the DLS hydrodynamic diameter distribution is indicated by PDI (polydispersion index).

Cell culture and treatments

The human cervical carcinoma cell line HeLa (ATCC collection), chosen as model cancer cells, was grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL⁻¹) and streptomycin (100

μ g mL⁻¹) (Invitrogen) in a 5% CO₂ incubator at 37 °C. Cells were seeded in 35 mm dishes and experiments were carried out two days after seeding when cells had reached 90% confluency. MO-based cubosomes were added to the cells at a concentration of 1 : 200 (5 μ L in 1 mL of fresh medium) and incubated at 37 °C for 4 and 24 h. Treatments were also performed with oleic acid (OA) in DMSO (100 μ M), MO in DMSO (463 μ M) and Pluronic F108 and F127 aqueous solutions (205 μ M and 238 μ M, respectively) equimolar respectively with the lipid and surfactant present in the cubosome formulations. OA and MO stock solutions in DMSO were 1000-fold concentrated to not exceed the 0.1% concentration of vehicle in the medium. The same concentration of vehicle was added to control cells when required by experimental design. For live cell imaging, fresh serum-free medium was used to remove the extracellular particle suspension. Then, cells were loaded with fluorescent probes and, after the incubation time, the latter were washed before the imaging session.

Fluorescence microscopy

Cells were stained with the following probes: 300 nM Nile Red (NR) (9-diethylamino-5*H*-benzo[*a*]phenoxazine-5-one) for 15 min; 25 nM tetramethylrhodamine methyl ester perchlorate (TMRM) for 30 min; 5 μ M MitoSox Red for 10 min; 100 nM MitoTracker Green FM (MitoTracker) for 30 min; 38 μ M Bodipy 493/503 for 30 min; 650 nM Hoechst 33258 for 30 min. NR was from Fluka (Buchs, SG, Switzerland); TMRM, MitoTracker Green, MitoSox Red and Bodipy 493/503 were from Molecular Probes (Eugene, OR, USA); Hoechst from Sigma-Aldrich (St. Louis, MO, USA). Microscopy observations were made using a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with 10 $\times$, 20 $\times$ and 40 $\times$ /0.75 NA water immersion objectives and a HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome cooled CCD camera (QICAM, Qimaging, Canada) with variable exposure. For the observation of TMRM and MitoSOX, filters were: ex 546 ± 6 nm, em 620 ± 60 nm. For MitoTracker Green, Bodipy 493/503 and Annexin V-FITC, filters were: ex 470 ± 20 nm, em 535 ± 40 nm. For Hoechst 33258, filters were: ex 360 ± 20 nm, em 460 ± 25 nm. For Nile Red, filters were: ex 470 ± 20 nm, em 535 ± 40 nm for nonpolar lipids; ex 546 ± 6 nm, em 620 ± 60 nm for total lipids.

Lipid droplet quantification

Fluorescent-based detection of the lipid droplet number, volume and cellular distribution is usually achieved in live cells with NR and Bodipy 493/503. NR is an ideal probe for the detection of lipids, as it exhibits high affinity, specificity and sensitivity to the degree of hydrophobicity of lipids. The latter feature results in a shift of the emission spectrum from red to green in the presence of polar and non-polar lipids respectively.⁴² For this reason, on staining living cells with NR, the cytoplasmic membranes are stained in red whereas neutral lipids of LDs are stained in green. Fluorescence images of NR-stained cells were acquired at 10 $\times$ under a fluorescence micro-

scope. Measurements concerned the amount of LD content that was evaluated by the fluorescence intensity of NR-green emission. IOD (Integrated Optical Density) per cell was calculated using Image ProPlus software. Alternatively, LDs were stained with Bodipy 493/503. This nonpolar dye for neutral lipid staining was used when cells were co-loaded with TMRM or MitoSOX Red for simultaneous observations of LDs with mitochondria or mitochondrial ROS, respectively.

Detection of mitochondrial membrane potential and mitochondria-derived ROS

TMRM is a red potential-sensitive probe that is concentrated inside mitochondria by their negative membrane potential. MitoTracker Green FM is a green selective dye that localizes to the lipid environment of the mitochondria. MitoSOX Red is a live-cell permeant fluorogenic probe that is selectively targeted to the mitochondria of live cells. Once in the mitochondria, MitoSOX Red is oxidized by superoxide anions and exhibits red fluorescence. In order to analyze the mitochondrial membrane potential (mitoMP) and the mitochondrial production of superoxide anions (mtROS), cells were incubated with TMRM or MitoSOX and, after removal of the probes from the medium, images of living cells were captured under a fluorescence microscope. The fluorescence intensities of TMRM and MitoSOX were assumed to be a conventional estimate of mitoMP and mtROS concentrations, respectively. Using Image ProPlus software, the fluorescence intensity of TMRM and MitoSOX was measured around the perinuclear area where mitochondria are densely packed. Mitochondrial fragmentation was morphologically assessed.

Imaging

Fluorescence intensity measurements, made on a single cell basis, were performed with Image ProPlus software (Media Cybernetics, Silver Springs, MD) in the following way: cells were seeded in 35 mm dishes and, after nanoparticle treatment, loaded with fluorescent probes. Ten randomly selected fields were acquired from each dish using a 10 $\times$ objective. Data of the fluorescence intensity of NR, TMRM or MitoSOX were expressed as the mean $\pm$ standard deviation (SD) of four independent experiments. Statistical evaluations were done by Student's *t*-test. The differences were considered to be significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

Nuclear and Annexin V-FITC staining

Cells were co-loaded with the DNA fluorochrome Hoechst 33258 together with all dyes. This probe is a water-soluble nuclear dye commonly used to distinguish apoptotic nuclei (characterized by nuclear condensation of chromatin and/or nuclear fragmentation) from healthy ones. To visualize the externalized phosphatidylserine (PS) as an early apoptosis marker, treated cells were incubated with FITC-conjugated Annexin V and propidium iodide (PI) for 10 min at room temperature according to the Instruction Manual for the kit (Annexin V-FITC Apoptosis detection kit, Sigma-Aldrich).⁴³ FITC-conjugated Annexin V and PI were detected as green and

red fluorescence, respectively. Fluorescence images were acquired at 10 $\times$ and viable and non-viable cells were counted in each field (5 fields per well) using Image ProPlus. Results are expressed as the percent ratio of damaged *versus* total number of cells.

Extraction and analyses of cell lipid components

Total lipids were extracted from HeLa cell pellets using the Folch procedure.⁴⁴ The CHCl₃ fraction, containing the lipids, from each cell sample was dried down and dissolved in EtOH. Separation of cholesterol and unsaturated fatty acids was obtained by mild saponification.⁴⁵ The hexane phase containing the unsaponifiable fraction (cholesterol) was collected and the solvent was evaporated. A portion of the dried residue, dissolved in MeOH, was injected into the high-performance liquid chromatography (HPLC) system. The hexane phase (saponifiable fraction) with fatty acids, dissolved in CH₃CN with 0.14% CH₃COOH (v/v), was injected into the HPLC system. The recovery of fatty acids and cholesterol during the saponification was calculated using an external standard mixture prepared by dissolving 1 mg of triolein, trilinolein, and cholesterol in EtOH and processed as samples. All solvent evaporation was performed under vacuum. Analyses of cholesterol and unsaturated fatty acids were carried out with an Agilent Technologies 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector as previously described.⁴⁵ Identification of lipid components was made using standard compounds and the conventional UV spectra, generated using the Agilent OpenLAB CDS Chemstation C.01.04. Calibration curves of all of the compounds were constructed using standards and were found to be linear, with correlation coefficients >0.995 . Cholesterol, triolein, trilinolein, standards of fatty acids, high-purity solvents, and Desferal were purchased from Sigma-Aldrich. Data are expressed as mean $\pm$ SD from four independent experiments (involving duplicate analyses for each sample). Statistically significant differences, using one-way analysis of variation (one-way ANOVA) and the Bonferroni post test (GraphPad INSTANT software, San Diego, CA), are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

MTT assay

HeLa cells were seeded in 24-well plates (3 $\times$ 10⁴ cells per well), cultured overnight and then treated with the cubosomes, MO and Pluronic solutions for 4 and 24 h at the same concentrations as those described in the "Cell culture and treatments" section. After removal of the extracellular particle suspension with fresh serum-free medium, cells were incubated with MTT (3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (0.5 mg mL⁻¹) for 2 h at 37 $^{\circ}$ C, and then lysed with DMSO. Absorbance was measured at 570 nm using an Infinite auto 200 microplate reader (Infinite 200, Tecan, Austria). Results are shown as percent of cell viability in comparison with non-treated control cells. Data are expressed as mean $\pm$ SD from three independent experiments (involving triplicate analyses for each sample). Statistical evaluations

were done by Student's *t*-test. The differences were considered to be significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Conflict of interest

The authors declare that they have no conflict of interest.

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VII

Monoolein-based cubosomes affect lipid profile in HeLa cells.

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Monoolein-based cubosomes affect lipid profile in HeLa cells



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ABSTRACT

Monoolein-based cubosomes are promising drug delivery nanocarriers for theranostic purposes. Nevertheless, a small amount of research has been undertaken to investigate the impact of these biocompatible nanoparticles on cell lipid profile. The purpose of the present investigation was to explore changes in lipid components occurring in human carcinoma HeLa cells when exposed to short-term treatments (2 and 4 h) with monoolein-based cubosomes stabilized by Pluronic F108 (MO/PF108). A combination of TLC and reversed-phase HPLC with DAD and ELSD detection was performed to analyze cell total fatty acid profile and levels of phospholipids, free cholesterol, triacylglycerols, and cholesteryl esters. The treatments with MO/PF108 cubosomes, at non-cytotoxic concentration (83 µg/mL of MO), affected HeLa fatty acid profile, and a significant increase in the level of oleic acid 18:1 *n*-9 was observed in treated cells after lipid component saponification. Nanoparticle uptake modulated HeLa cell lipid composition, inducing a remarkable incorporation of oleic acid in the phospholipid and triacylglycerol fractions, whereas no changes were observed in the cellular levels of free cholesterol and cholesteryl oleate. Moreover, cell-based fluorescent measurements of intracellular membranes and lipid droplet content were assessed on cubosome-treated cells with an alternative technique using Nile red staining. A significant increase in the amount of the intracellular membranes and mostly in the cytoplasmic lipid droplets was detected, confirming that monoolein-based cubosome treatment influences the synthesis of intracellular membranes and accumulation of lipid droplets.

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1. Introduction

Nanoparticles (NPs) are a class of functional materials characterized by size-dependent properties, generally defined as engineered structures with at least one dimension less than 100 nm (Kroll et al., 2009), for which applications in medicine as therapeutic drug delivery and/or medical imaging systems have been predicted (Faraji and Wipf, 2009; Kroll et al., 2009; Panariti et al., 2012). Relevant examples include micelles, liposomes, solid lipid, polymeric, silicon-based, gold, or iron oxide NPs, as well as dendrimers, and quantum dots (Faraji and Wipf, 2009; Panariti et al., 2012). Lipid-based reverse cubic bicontinuous liquid crystalline phases possess a three-dimensional structure consisting of non-intersecting bilayers folded on an infinite periodic minimal surface characterized by a cubic symmetry and organized to form two disconnected, continuous water channels (Hyde, 1989). They were broadly investigated for pharmaceutical purposes in the past as their nanostructure can incorporate molecules of biological

relevance (Caboi et al., 2001; Murgia et al., 2001). Remarkably, these peculiar cubic phases can be dispersed in water originating nanoparticles known as cubosomes (Larsson, 1983; Larsson and Tiberg, 2005), often pictured as the non-lamellar counterpart of liposomes. Cubosomes can be easily prepared sterically stabilizing a dispersion of monoolein (MO, Fig. 1A) in water by Pluronic series. By virtue of peculiar characteristics such as high mechanical rigidity, high hydrophobic volume, and the possibility of being biodegraded in vivo through enzyme-catalyzed reactions (carboxylesterases and phosphatases) (Hinton et al., 2014; Mulet et al., 2013), monoolein-based cubosomes were recently proposed for application in theranostic nanomedicine (Caltagirone et al., 2014; Murgia et al., 2013).

Several investigations were conducted on the in vitro cytotoxicity of MO-based nanoparticles, and results were found related to cell line, incubation time, dose, and formulation type (Falchi et al., 2015; Hinton et al., 2014; Murgia et al., 2010, 2015; Tran et al., 2015). Previous studies also provided evidence that, as result of their internalization, MO-based cubosomes induce accumulation of lipids in treated cells, causing the increase (both in size and number) of the cytoplasmic lipid droplets (LDs) (Caltagirone et al., 2014; Falchi et al., 2015; Murgia et al., 2015). LDs are

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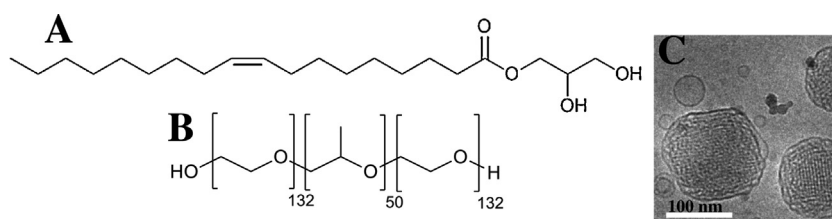


Fig. 1. Chemical structures of monoolein (MO) (A) and pluronic PF108 (B). Cryo-TEM images of MO/PF108 cubosomes (Falchi et al., 2015) (C).

macromolecular lipid assemblies consisting of neutral lipids, such as triacylglycerols, diacylglycerols, cholesterol esters, and cholesterol, surrounded by a monolayer of phospholipids and associated proteins (Bartz et al., 2007; Khatchadourian and Maysinger, 2009; Przybytkowski et al., 2009; Suzuki et al., 2012). LDs are now regarded as metabolically active organelles, with a particular structure and organization, engaged in a wide range of activities and formed, under physiological conditions, when free (unesterified) fatty acids from exogenous or endogenous sources are available inside the cells (Khatchadourian and Maysinger, 2009; Przybytkowski et al., 2009; Suzuki et al., 2012). Their main functions include lipid storing and supplying for various cellular needs (β -oxidation, membrane biogenesis, and lipoprotein synthesis) (Suzuki et al., 2012).

Remarkably, although numerous articles were devoted to validate cubosome relevance in nanomedicine, only a small amount of research was undertaken to investigate the impact of these nanoparticles on the cell lipid profile (Falchi et al., 2015). The present investigation aimed to fill this gap by exploring the changes in lipid components occurring in HeLa cells when exposed to short-term treatments with MO-based cubosomes stabilized by Pluronic F108 (MO/PF108). The effect of nanoparticles (at 2 and 4 h of incubation) on HeLa cell viability was preliminarily evaluated by the MTT assay. MO/PF108 cubosomes, at non-cytotoxic concentration, were then tested to evaluate their effect on lipid component profile, with particular regard to triacylglycerols (TAG), phospholipids (PL), free cholesterol (FC), cholesteryl esters (CE), and total fatty acid composition. In situ fluorescent quantification of cytoplasmic membranes and LDs after cubosome treatment was also assessed in living cells loaded with Nile red, a fluorescent hydrophobic probe for the detection of polar and non-polar lipids (Greenspan et al., 1985).

2. Materials and methods

2.1. Materials

Monoolein (RYLO MG 19 PHARMA, glycerol monooleate, 98.1 wt %; MO) was kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark. Pluronic F108 (PEO₁₃₂-PPO₅₀-PEO₁₃₂) (Fig. 1B), cholesterol, cholesteryl oleate (CO), cholesteryl arachidonate, standards of fatty acids, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC 18:1/18:1), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:1), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 18:1/16:0), 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:2), 2-arachidonoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/20:4), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (PC 18:2/18:2), 1,2-dieicosapentaenoyl-*sn*-glycero-3-phosphocholine (PC 20:5/20:5), trilinolein (LLL), triolein (OOO), 1,3-dioleoyl-2-palmitoyl-glycerol (OOP), trilinolenin (LnLnLn), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1,2-dilinoleoyl-3-oleoyl-rac-glycerol (LLO), 1,2-dioleoyl-3-linoleoyl-rac-

glycerol (OOL), and Desferal (deferoxamine mesylate salt), were purchased from Sigma-Aldrich (Milan, Italy). The code letters used for the fatty acid in TAG are: L, linoleic; Ln, linolenic; O, oleic; P, palmitic. All solvents used, of the highest available purity, were also from Sigma-Aldrich. All of the other chemicals used in this study were of analytical grade. Cell culture materials were purchased from Invitrogen (Milan, Italy).

2.2. Cubosome preparation and characterization

Cubosomes were prepared by dispersing the appropriate amount of melted MO in a solution of Pluronic F108 using an ultrasonic processor UP100H by Dr. Hielscher, cycle 0.9, amplitude 90%, for 10 min. The sample volume was 4 mL with 96.4 wt% of water, 3.3 wt% of MO and 0.3 wt% of Pluronic F108 (Falchi et al., 2015). Distilled water passed through a Milli-Q water purification system (Millipore) was used to prepare the samples. MO/PF108 cubosomes (Fig. 1C) were characterized for particle size, morphology, and inner structure as previously reported (Falchi et al., 2015).

2.3. Cell Culture

Human carcinoma HeLa cell line (ATCC collection) was grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL⁻¹), and streptomycin (100 μ g mL⁻¹) (Invitrogen) in a 5% CO₂ incubator at 37 °C.

2.4. Cytotoxic activity (MTT assay)

The cytotoxic effect of the nanoparticle formulation MO/PF108 was evaluated in HeLa cells by the MTT assay (Rosa et al., 2013; Schiller et al., 1992). HeLa cells were seeded in 24-well plates at density of 3×10^4 cells/well in 500 μ L of serum-containing media. Experiments were carried out two days after seeding when cells had reached 90% confluence. Cubosomes were added to the cells at concentrations of 1:400 and 1:200 (2.5 μ L and 5 μ L in 1 mL of serum-free medium, respectively) and incubated at 37 °C for 2 h and 4 h. A 50 μ L portion of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL in H₂O) was then added and left for 2 h at 37 °C. The medium was aspirated, 500 μ L of DMSO was added to the wells, and colour development was measured at 570 nm with an Infinite 200 auto microplate reader (Infinite 200, Tecan, Austria). The absorbance is proportional to the number of viable cells. All measurements were performed in quadruplicate and repeated at least three times. Results are shown as percent of cell viability in comparison with non-treated control cells.

2.5. Lipid profile modulation in HeLa cells

HeLa cells were plated in Petri dishes at a density of about 10^6 cells/10 mL of complete medium and were used for fatty acid profile modulation experiments at 2 days post-seeding when cells

had reached 90% confluence. Cubosomes were added to the cells at a concentration of 1:400 (25 μ L in 10 mL of fresh medium) and incubated at 37 °C for 2 h and 4 h. Cells were also incubated for 4 h at 37 °C with a water solution of Pluronic F108 equimolar with the surfactant present in the cubosome formulation. After treatment, the cells were scraped and centrifuged at 1200 g at 4 °C for 5 min. After centrifugation, pellets were separated from supernatants and used for lipid extraction and analyses (Rosa et al., 2013).

2.6. Extraction and preparation of lipid components

2.6.1. Determination of MO in cubosomes

Aliquots (10 μ L) of the MO/PF108 nanoparticle preparation were dissolved in 300 μ L of CH₃CN with 0.14% CH₃COOH (v/v), and aliquots of the samples were injected into the HPLC system for the quantification of MO.

2.6.2. Extraction of HeLa cell lipids

Total lipids were extracted from HeLa cell pellets using the Folch et al. procedure (Folch et al., 1957) by the addition of 12 mL of the mixture CHCl₃/MeOH 2:1. After the addition of 4 mL of H₂O, samples were left in the dark at room temperature for 1 h, then were centrifuged for 1 h at 900 g. The CHCl₃ fraction, containing the lipid compounds, was separated from the MeOH/H₂O mixture, and stored at –80 °C before further analysis. Aliquots of the lipid extracts were subjected to mild saponification and TLC analysis. Another aliquot of the CHCl₃ fraction, from each cell sample, was dried down, dissolved in 300 μ L of MeOH, and injected into the HPLC system for the analysis of lipid components: MO, phospholipids (PL), free cholesterol (FC), triacylglycerols (TAG) and cholesteryl esters (CE).

2.6.3. Saponification of HeLa cell lipids

An aliquot of the CHCl₃ fraction from each cell sample was collected, dried down, and dissolved in 5 mL of EtOH. Separation of fatty acids was obtained by mild saponification (Rosa et al., 2013) as follows: 100 μ L of Desferal solution (25 mg/mL of H₂O), 1 mL of a water solution of ascorbic acid (25% w/v), and 0.5 mL of 10 N KOH were added to lipids in EtOH solution. The mixtures were left in the dark at room temperature for 14 h. After the addition of n-hexane and of H₂O to the mixtures, samples were acidified with 37% HCl to pH 3–4 and then centrifuged for 1 h at 900 $\times$ g. The hexane phase with fatty acids was collected and the solvent was evaporated. A portion of the dried residue was dissolved in 300 μ L of CH₃CN with 0.14% CH₃COOH (v/v), and aliquots of the samples were injected into the HPLC system. The recovery of fatty acids during the saponification was calculated using an external standard mixture prepared dissolving 1 mg of triolein and trilinolein, in 5 mL of EtOH and processed as samples. All solvent evaporation was performed under vacuum.

2.6.4. Qualitative profile of HeLa cell lipids by TLC

An aliquot of the CHCl₃ fraction obtained from control and treated cells (2 h) was subjected to TLC analysis for a preliminary evaluation of the HeLa cell lipid classes. The lipids were separated on TLC plates in hexane:diethyl ether:acetic acid (80:20:1, v/v) for 40 min. Lipid classes were visualized by iodine vapor. Neutral lipid classes were identified according to TLC standards.

2.7. HPLC analysis of lipid compounds

Analyses of lipid compounds extracted from HeLa cells and MO in cubosomes were carried out with an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD) and with an Agilent Technologies 1260 Infinity ELSD. Analyses of unsaturated (detected

at 200 nm with DAD) and saturated (ELSD detection) free fatty acids, obtained from cell lipid saponification, and MO (at 200 nm) in cubosome dispersion, were carried out with a XDB–C₁₈ Eclipse (150 $\times$ 4.6 mm, 3.5 μ m particle size) (Agilent Technologies) equipped with a Zorbax XDB–C₁₈ Eclipse (12.5 $\times$ 4.6 mm, 5 μ m particle size) guard column (Agilent Technologies), with a mobile phase of CH₃CN/H₂O/CH₃COOH (75/25/0.12, v/v/v), at a flow rate of 2.3 mL/min (Rosa et al., 2013). The temperature of the column was maintained at 37 °C. Analyses of MO, PL, FC, TAG, and cholesteryl oleate (CO) in the lipid extracts obtained from cell pellets were carried out with 1100 HPLC–ELSD. Separation was performed with an Inertsil ODS–2 column, 150 $\times$ 4.6 mm, 5 μ m particle size (Superchrom), and MeOH as the mobile phase, at a flow rate of 0.7 mL/min for MO, PL, and FC, and 2 mL/min for TAG and CO. The column temperature was set at 37 °C. ELSD detector settings were: evaporator temperature 40 °C, nebulization temperature 40 °C, with nitrogen as the nebulizing gas at a flow of 1 L/min. Recording and integration of the chromatogram data was carried out through an Agilent OpenLAB Chromatography data system. The identification of cell lipid components was made using standard compounds and conventional UV spectra. Calibration curves of all of the compounds were constructed using standards and were found to be linear (DAD) and exponential (ELSD), with correlation coefficients >0.995.

2.8. Fluorescent quantification of cytoplasmic membranes and LDs in HeLa cells

2.8.1. Fluorescence microscopy

Cells were seeded in 35 mm dishes and experiments were carried out two days after seeding when cells had reached 90% confluence. MO-based cubosomes were added to the cells at a concentration of 1:400 (2.5 μ L in 1 mL of fresh medium) and incubated at 37 °C for 2 and 4 h. Cells were also incubated for 4 h at 37 °C with a water solution of Pluronic F108 equimolar with the surfactant present in the cubosome formulation. For live cell imaging, cells were stained with 300 nM Nile red (NR) (9-diethylamino-5H-benzo[a]phenoxazine-5-one) for 30 min and, after incubation time, the probe was washed out before imaging session. Microscopy observations were made using a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with 10 $\times$, 20 $\times$ and 40 $\times$ /0.75 NA water immersion objectives and a HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome cooled CCD camera (QICAM, Qimaging, Canada) with variable exposure. For observation of NR, filters were: ex 470 $\pm$ 20, em 535 $\pm$ 40 for non-polar/neutral lipids; ex 546 $\pm$ 6, em 620 $\pm$ 60 for polar lipids.

2.8.2. Measurements

NR is a fluorescent hydrophobic probe for the detection of neutral lipids or mixtures of lipids and proteins that create a hydrophobic environment (Greenspan et al., 1985). It is characterized by a shift of emission from red to green in the presence of polar and non-polar lipids respectively. Accordingly, cytoplasmic membranes, typically composed of PL, are stained in red whereas neutral lipids such as CE or TAG are stained in green. Images of NR-loaded cells were acquired with 10 $\times$ objective and color composite images were generated after background subtraction. Mean values of red and green fluorescence intensity were recorded from Histogram data. Measurements, made on single cell basis, concerned the amount of cytoplasmic membranes and neutral lipids, evaluated by the fluorescent intensity of the NR-red and NR-green emission, respectively. Measurements were performed with Image Pro Plus software (Media Cybernetics, Silver Springs, MD).

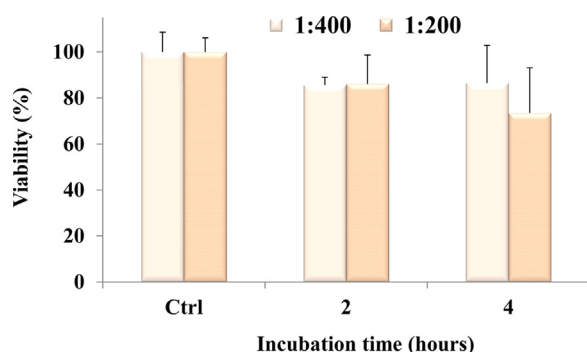


Fig. 2. Viability, expressed as percentage of the control, induced in HeLa cells by incubation for 2 h and 4 h in the presence of MO/PF108 cubosomes (concentrations 1:400 and 1:200, corresponding to 83 and 166 $\mu\text{g/mL}$ of MO, respectively). Data are expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving quadruplicate analyses for each sample.

2.9. Statistical analysis

Evaluation of statistical significance of differences was performed using Graph Pad INSTAT software (GraphPad software, San Diego, CA, USA). Comparison between groups was assessed by one-way analysis of variance (one-way ANOVA) followed by the Bonferroni Multiple Comparisons Test. Data were expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving duplicate/quadruplicate analyses for each sample.

3. Results

3.1. Determination of MO level in cubosome formulation

Before any biological evaluation was performed, the amount of MO in cubosome formulation was monitored by HPLC determination. A MO level of $33.14 \pm 2.56 \mu\text{g}/\mu\text{L}$ was determined in cubosome dispersion. Experiments in HeLa cells were performed using two concentrations of MO/PF108 cubosomes, 1:400 and 1:200, corresponding to 82.85 ± 6.41 and $165.69 \pm 12.82 \mu\text{g/mL}$ of MO, respectively.

3.2. Cytotoxic activity (MTT assay)

MO-based cubosomes prepared with PF108 were tested for cytotoxicity (MTT assay) in HeLa cells. Fig. 2 shows the viability,

expressed as percentage of the control, induced in HeLa cells after 2 and 4 h of incubation in the presence of two concentrations of the nanoparticle formulation MO/PF108 (1:400 and 1:200). Treatment with nanoparticle formulation at dose 1:400 did not induce a relevant reduction in cell viability in comparison with control cells at both incubation times. Cubosomes at dose 1:200 was not toxic at 2 h-incubation time, nevertheless a slight decrease in HeLa cell viability was observed at 4 h-incubation time as previously reported (Falchi et al., 2015).

3.3. Effect of MO/PF108 cubosomes on total fatty acid profile of HeLa cells

Freshly prepared MO/PF108 cubosomes were then tested in HeLa cells at the concentration 1:400 (83 $\mu\text{g/mL}$ of MO) to assess their effects on cell lipid composition. Also PF108 solution was tested to evaluate its effect on the HeLa cell lipids. After 2 and 4 h incubation, lipid fraction was extracted from cell pellets and an aliquot was subjected to mild saponification, in order to observe the effect of MO-based cubosomes on the total fatty acid profile. Fig. 3A shows the fatty acid chromatographic profile of HeLa control cells obtained by HPLC analysis with DAD (at 200 nm for unsaturated fatty acid determination) and ELSD (for saturated fatty acids) detection. The content of the most abundant fatty acids in HeLa control cells, detected by HPLC analysis, is reported in Table 1. HeLa control cells showed a lipid composition characterized by a high level of 18:1 isomers ($104.99 \pm 8.23 \mu\text{g/plate}$; mainly oleic acid 18:1 *n*-9, OA), while 16:0 ($29.59 \pm 2.58 \mu\text{g/plate}$), and 22:6 *n*-3 ($11.01 \pm 0.16 \mu\text{g/plate}$) represented the most abundant fatty acids among saturated and polyunsaturated, respectively. High amounts of 18:2 *n*-6 and 20:4 *n*-6 were also measured. Values of the fatty acids measured in HeLa cells after 4 h of incubation in the presence of PF108 solution are also reported in Table 1. The incubation (4 h) of HeLa cells with PF108 solution did not induce changes in fatty acid levels, with treated cells showing a profile similar to that of control cells, as previously observed (Falchi et al., 2015). Fig. 3B–C show the chromatographic profile, obtained by DAD and ELSD detection, of HeLa cells after 2 h-incubation with MO/PF108 cubosomes with respect to untreated-control cells. The values of the main unsaturated fatty acids 16:1 *n*-7, 20:4 *n*-6, 20:5 *n*-3, 22:6 *n*-3, 16:0, and OA (expressed as $\mu\text{g/plate}$) measured in HeLa cells after 2 and 4 h of incubation in the presence of MO/PF108 cubosomes are reported in Table 1. The incubation of HeLa cells with the

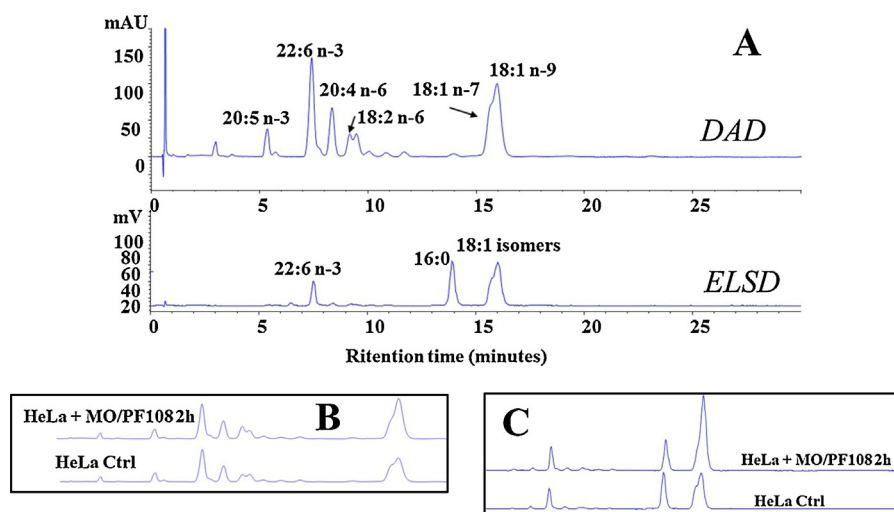


Fig. 3. Fatty acid chromatographic profile of control HeLa cells obtained by HPLC analysis with DAD (at 200 nm) and ELSD detection (A). Effect of 2 h-treatment with MO/PF108 cubosomes on HeLa fatty acid profile measured by HPLC analysis with DAD (200 nm) (B) and ELSD (C) detection.

Table 1

Fatty acid composition (expressed as $\mu\text{g}/\text{plate}$), obtained by HPLC analysis, of control HeLa cells (Ctrl), cells treated with Pluronic F108 (PF108) (4 h of incubation) and MO/PF108 cubosomes (2 and 4 h of incubation).

Fatty acid	Ctrl cells	PF108 treated cells (4 h)	Cubosome treated cells (2 h)	Cubosome treated cells (4 h)
16:0	29.79 $\pm$ 2.58	30.24 $\pm$ 3.40	30.88 $\pm$ 1.84	28.83 $\pm$ 2.59
16:1 <i>n</i> -7	2.48 $\pm$ 0.12	2.59 $\pm$ 0.07	2.99 $\pm$ 0.70	2.79 $\pm$ 0.72
18:1 <i>n</i> -7 + 18:1 <i>n</i> -9	104.99 $\pm$ 8.23	118.08 $\pm$ 0.60	163.63 $\pm$ 40.10**	180.85 $\pm$ 23.50***
18:2 <i>n</i> -6	5.53 $\pm$ 0.01	5.21 $\pm$ 0.17	6.06 $\pm$ 0.18	6.19 $\pm$ 0.32
18:3 <i>n</i> -3	0.68 $\pm$ 0.01	0.82 $\pm$ 0.03	0.63 $\pm$ 0.17	0.71 $\pm$ 0.14
20:3 <i>n</i> -3 + 20:3 <i>n</i> -6	1.57 $\pm$ 0.11	1.61 $\pm$ 0.01	1.64 $\pm$ 0.20	1.65 $\pm$ 0.40
20:3 <i>n</i> -9	1.55 $\pm$ 0.33	2.02 $\pm$ 0.14	1.57 $\pm$ 0.39	1.71 $\pm$ 0.47
20:4 <i>n</i> -6	6.50 $\pm$ 0.02	6.91 $\pm$ 0.04	6.72 $\pm$ 1.17	6.56 $\pm$ 0.52
20:5 <i>n</i> -3	2.92 $\pm$ 0.01	3.17 $\pm$ 0.07	2.97 $\pm$ 0.60	2.88 $\pm$ 0.27
22:4 <i>n</i> -6	Trace	Trace	Trace	Trace
22:5 <i>n</i> -3	2.31 $\pm$ 0.07	2.09 $\pm$ 0.05	2.24 $\pm$ 0.25	2.03 $\pm$ 0.06
22:6 <i>n</i> -3	11.01 $\pm$ 0.16	12.95 $\pm$ 0.01	10.75 $\pm$ 1.72	10.91 $\pm$ 1.71

Results are expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving duplicate analyses for each sample. ** $P < 0.01$; * $P < 0.05$ versus Ctrl.

MO/PF108 nanoparticles induced a significant increase in the cell level of OA, which reached values approximately 1.5 and 1.7 times higher than that of control cells at 2 and 4 h of incubation, respectively. The treatment did not seem to affect the levels of the other saturated and unsaturated fatty acids, with respect to control cells.

3.4. Effect of MO/PF108 cubosomes on HeLa cell polar lipids

Another aliquot of the lipid fraction extracted from cell pellets was subjected to a simple chromatographic method using a reversed-phase HPLC analysis combined with evaporative light scattering detection (HPLC–ELSD) for the analyses of the polar

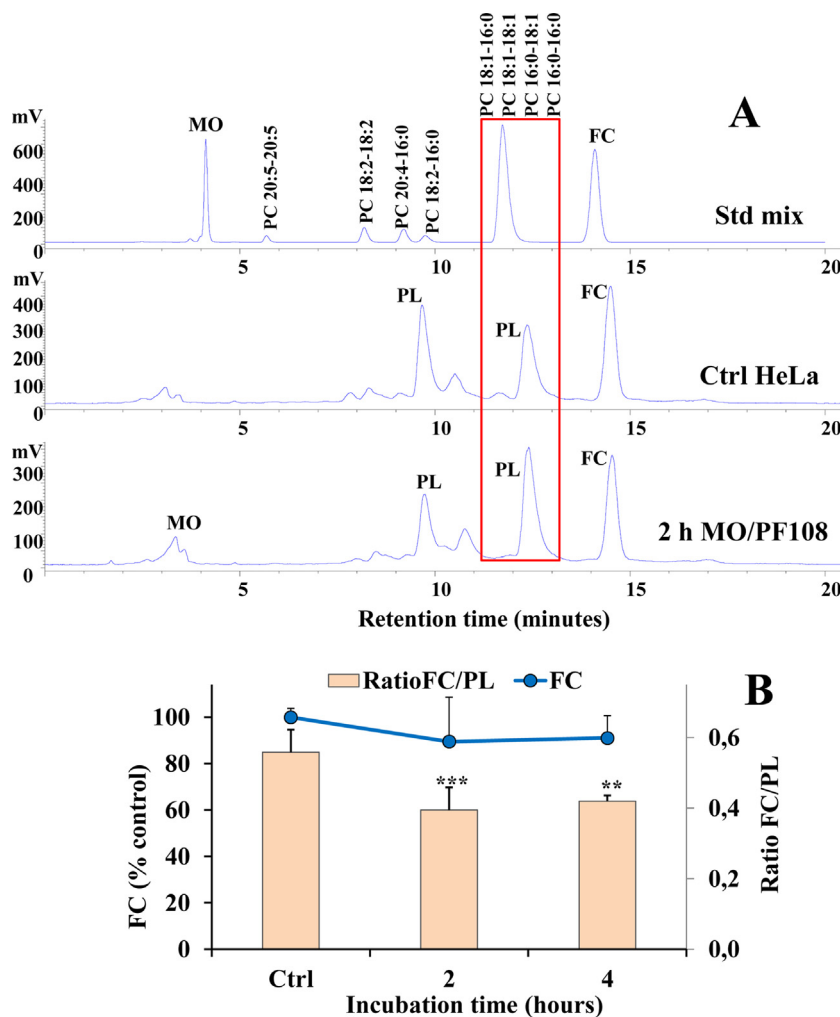


Fig. 4. Chromatographic profile, obtained by HPLC–ELSD analysis, of polar lipids monoolein (MO), phospholipids (PL) and free cholesterol (FC) measured in control HeLa cells and cells treated for 2 h with MO/PF108 cubosomes; a standard mix composed by MO, FC, and a mixture of phosphatidylcholines (PC 16:0/16:0, PC 18:1/18:1, PC 16:0/18:1, PC 18:1/16:0, ECN 32; PC 16:0/18:2, ECN 30; PC 16:0/20:4, PC 18:2/18:2, ECN 28; PC 20:5/20:5, ECN 20), is reported for comparison (A). FC values (expressed as percentage of the control) and values of FC/PL ratio measured in HeLa control cells (Ctrl) and after 2 h and 4 h of incubation in the presence of MO/PF108 cubosomes (B). Results were expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving duplicate analyses for each sample. *** $P < 0.001$; ** $P < 0.01$ versus Ctrl.

lipids MO, PL, and FC. The reversed phase mode used for chromatographic separation allowed to separate phospholipids on the basis of equivalent carbon number (ECN) ($=CN-2n$, where CN is the number of acyl group carbons and n the number of double bonds) (Walczak et al., 2015) and lipids containing fatty acids with the same ECN co-eluted. Phosphatidylcholines (PC) represent the predominant polar lipid class (53%) in HeLa cell membranes (Li and Yuan, 2011; Yu et al., 2014), therefore the identification of PL was made on the basis of the retention time of standard PC. Fig. 4A shows the chromatographic profile of polar lipids of HeLa control cells and cells treated for 2 h with MO/PF108 cubosomes obtained by HPLC-ELSD. The chromatographic profile of a standard mix composed by MO, cholesterol, and a mixture of unsaturated and saturated PC, is also reported as reference in Fig. 4A. In our experimental conditions, control HeLa cells showed a lipid profile characterized by two main peaks of PL at 9.8 and 12.5 min (corresponding to ECN 28/30 and 32, respectively), and the peak of FC was detected at 14.5 min. The 2 h-treatment with MO/PF108 cubosomes induced in HeLa cells a change in the profile of polar lipids, in particular it was observed a marked increase in the area of the peak corresponding to PL containing OA (PC 18:1-18:1, PC 18:1-16:0, and PC 16:0-18:1) (Fig. 4A) with respect to the other peaks, and a similar trend was observed at 4 h-incubation. Fig. 4B shows the levels of FC (expressed as percentage of the control) and the values of the ratio FC/PL containing OA measured in HeLa control cells and after 2 h and 4 h of incubation in the presence of MO/PF108 cubosomes. The determination of the ratio FC/PL represented a useful approach to bypass eventual differences in cell number and nanoparticle uptake rate among experiments. The FC level was measured in control cells as mean content of $67.87 \pm 8.18 \mu\text{g}/\text{plate}$, and a value of $122.56 \pm 12.73 \mu\text{g}/\text{plate}$ was determined for PL containing OA (quantified as PC 18:1-18:1). Both treatments with MO-based nanoparticles did not significantly affect FC level in HeLa cells, while a significant decrease of the FC/PL ratio value was observed at both the incubation times, due to a synthesis of phospholipids containing OA, which confirmed the significant increase of the OA amount measured in treated cells after lipid saponification. HeLa cells treated with PF108 solution showed the same FC level and FC/PL ratio of control cells (data not shown). The cell uptake of MO/PF108 cubosomes was also evaluated by monitoring the intracellular amount of MO. MO values of 5.95 ± 3.11 and $9.84 \pm 1.22 \mu\text{g}/\text{plate}$ were determined in treated HeLa cell pellets after 2 and 4 h of incubation, respectively.

3.5. Effect of MO/PF108 cubosomes on neutral lipids of HeLa cell

Initially, we set out to characterize the neutral lipid composition of HeLa cells in the absence or in the presence of MO/PF108 nanoparticles by TLC. Polar phospholipids remained at the origin in this solvent system and represented the main lipid classes in control and treated cells (Fig. 5). Control HeLa cells showed the bands of FC and CE, the latter migrated in the correspondence of cholesteryl oleate (CO), while the band of TG was not detectable in these experimental conditions, as previously observed (Galli et al., 1999). The treatment with MO/PF108 nanoparticles induced an evident increase in the level of TAG with respect to controls, as shown in Fig. 5 for cells at 2 h-incubation. Then, we used HPLC-ELSD to identify and quantify the dominant CE and TAG species. Fig. 6A shows the chromatographic profile of lipids extracted from control and treated HeLa cells (2 and 4 h) obtained by HPLC-ELSD analysis at 2 mL/min with MeOH as eluent. This chromatographic condition allowed to separate cell polar lipids (PL and FC) from neutral lipids (CE and TAG) that eluted at higher retention times. Peak identification and improvement of separation was based on standards-individually and as part of several mixtures prepared specifically to confirm

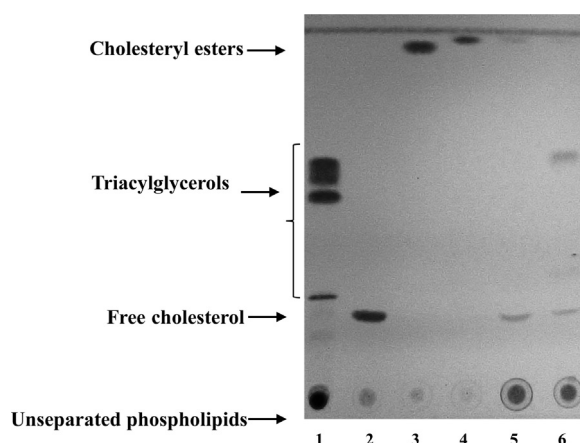


Fig. 5. Identification of HeLa cell lipid classes, separated on silica gel thin-layer chromatography using hexane-diethyl ether-acetic acid (80:20:1) as a solvent. Lane 1, standards. From top to bottom: mix of triacylglycerols (TAG) and phosphatidylcholines (PC). Lane 2, standard of free cholesterol (FC). Lane 3, standard of cholesteryl arachidonate. Lane 4, standard of cholesteryl oleate (CO). Lane 5, control HeLa cells. Lane 6, HeLa cells treated for 2 h with MO/PF108 cubosomes. Polar phospholipids remained at the origin in this solvent system. Lipids were visualized with iodine vapors.

retention times. The reversed phase mode allowed also to separate TAG on the basis of ECN (Lisa and Holcapek, 2008). Two main peaks were detected: peak 1 at 45 min identified as CO (Fig. 6B), and peak 2 at 43 min, identified as TAG containing OA (OOO/POO, ECN 42) (Fig. 6C). A mean value of $26.74 \pm 0.27 \mu\text{g}/\text{plate}$ was measured for CO in control HeLa cells and similar values was detected in treated cells. The treatment with MO-based nanoparticles induced a significant increase in the level of TAG contain OA (Fig. 6D) that reached a value of $37.13 \pm 0.06 \mu\text{g}/\text{plate}$ at 4 h-incubation. HeLa cells incubated for 4 h with PF108 solution exhibited the same neutral lipid profile of control cells (data not shown).

3.6. Intracellular membranes and lipid droplets

Nile red (NR) is a fluorescent lipophilic dye characterized by a shift of emission from red to green according to the degree of hydrophobicity of lipids. NR fluoresces intensely in red in the presence of phospholipids constituting the membranes of the membrane-enclosed organelles (mitochondria, endoplasmic reticulum, Golgi Apparatus, endosomes and lysosomes), whereas neutral lipids, present in the LDs, are stained in green (Greenspan et al., 1985). Beside this property, quantitative fluorescent measurements were performed on whole cell to highlight variations of polar and neutral lipids after nanoparticle treatment in comparison with untreated control cells. Analysis of NR-loaded cells after cubosome treatment at 2 and 4 h incubation time (Fig. 7A) indicated a significant increase of polar and neutral lipids at both incubation times (Fig. 7B). Accordingly, the uptake of MO-based cubosomes gave rise to a significant increase of both cytoplasmic membranes and LDs. However, comparing the effects from two incubation times, whereas no variations in the amount of cytoplasmic membrane were detected, significant differences were found in the neutral lipids, indicating that the excess of lipids was rather stored into lipid droplets. Cell treated with PF108 solution showed the same level of cytoplasmic membranes and LDs as the control (data not shown). The ratio of red and green emission, previously used in the microscopy investigations to evaluate qualitative variations between polar and non-polar lipids (Diaz et al., 2008), was also applied in order to confirm the observed changes in the intracellular amount of polar and neutral lipids after cubosome treatment. Red/green ratio ranged from

5.6 for untreated cells to 3.6 and 2.5 for cubosome-treated cells at 2 and 4 h, respectively, indicating a progressive accumulation of hydrophobic lipids in the lipid droplets, so confirming the previously demonstrate increased of OA-containing TAG.

4. Discussion

NPs offer an extraordinary opportunity for application in pharmacology and medicine. However, NPs should no longer be viewed only as simple carriers for biomedical applications, since they can also play an active role in mediating biological effects (Kroll et al., 2009; Panariti et al., 2012; Przybytkowski et al., 2009). Actually, NPs interaction with living systems is currently originating growing interest under the perspective of improving drug delivery as well as implementing nanotechnologies in a safe way (Lesniak et al., 2013). Plainly, safe and effective utilization of such systems requires that they do not result in an adverse biological response. Lipid-based nanoparticles (LNPs) are well tolerated in living systems, since they are made from physiological compounds leading to the metabolic pathways (Severino et al., 2012), and among them MO-based cubosomes are innovative, promising drug delivery nanocarriers (Larsson, 1983; Larsson and Tiberg, 2005; Murgia et al., 2013; Caltagirone et al., 2014; Hinton et al., 2014). Particularly, MO, their main molecular constituent, is a well-

known lipid molecule commonly used as emulsifying agent and food additive. It is considered as a nontoxic, biodegradable, and biocompatible material classified as “generally recognized as safe” (GRAS) (Lim et al., 2014). However, the interaction of MO-based cubosomes with cell lipids is an essential focus in assessing and understanding toxicity and compatibility. Surprisingly, the literature on the impact of MO-based cubosomes on cell lipid profile is negligible (Falchi et al., 2015), despite the number of publications devoted to explore their potential applications for therapeutic and theranostic purposes (Caltagirone et al., 2014; Hinton et al., 2014; Mulet et al., 2013; Murgia et al., 2013, 2015).

Here, we investigated the changes in cellular lipid composition (total fatty acid profile, PL, FC, TAG, and CE) occurring in human carcinoma HeLa cells when exposed to short-term treatments (2 and 4 h) with MO/PF108 cubosomes. The treatments with a non-cytotoxic concentration of MO/PF108 cubosomes, corresponding to 83 $\mu\text{g/mL}$ of MO, significantly modulated HeLa cell lipid profile. After the addition to the culture media, MO/PF108 nanoparticles were immediately taken up by HeLa cells, inducing from 2 h-incubation a remarkable incorporation of OA in the phospholipid and triacylglycerol fractions, and accumulation of triacylglycerols in the LDs. The cellular uptake of MO/PF108 cubosomes was also confirmed by the significant increase in the level of OA observed from 2 h incubation in treated cells after lipid component

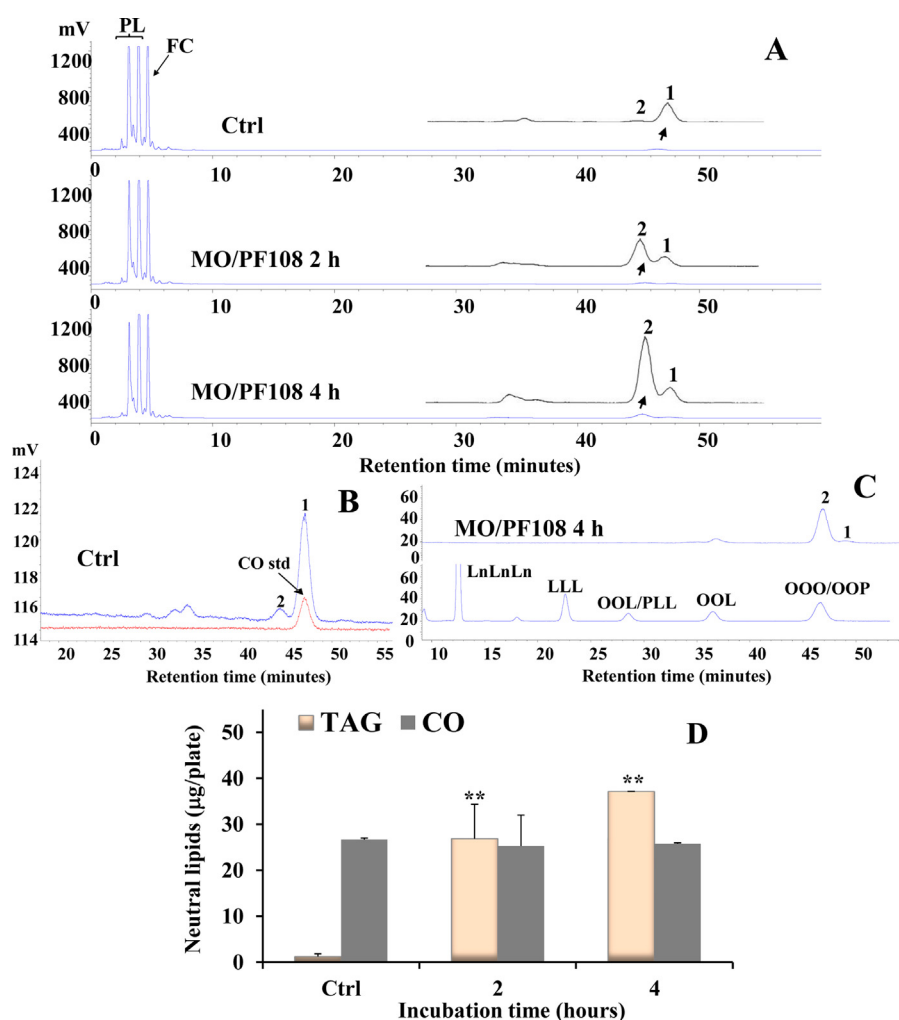


Fig. 6. Chromatographic profile of lipid classes (phospholipids, PL; free cholesterol, FC) measured in control HeLa cells and cells treated for 2 h and 4 h with MO/PF108 cubosomes obtained by HPLC-ELSD analysis at 2 mL/min and details of the main peaks 1 and 2 detected at 45 and 43 min (A). Identification of peak 1 as cholesteryl oleate (CO) (B) and peak 2 as triacylglycerols containing OA (C). Values of TAG and CO measured in control and treated cells (D); results were expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving duplicate analyses for each sample. ** $P < 0.01$ versus Ctrl.

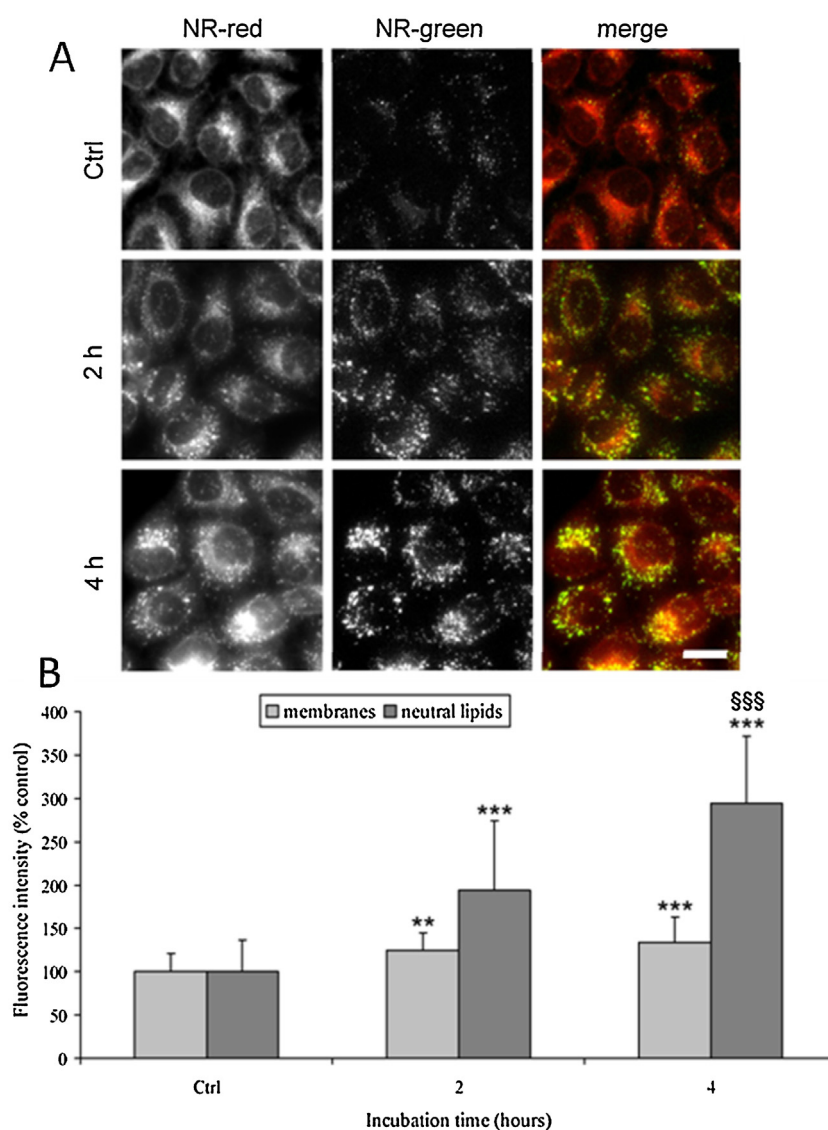


Fig. 7. The panel shows representative images of red (cytoplasmic membranes), green (lipid droplets, LDs) and merged emissions of untreated-control and cubosome-treated HeLa cells loaded with Nile red (A). NR-fluorescent quantification of cytoplasmic membranes and lipid droplets of cells exposed to cubosome treatment for 2 and 4 h (B). The percent of treated cells was normalized to the control cells. Results were expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving duplicate analyses for each sample. *** $P < 0.001$; ** $P < 0.01$ versus Ctrl. \$\$\$ $P < 0.001$ versus 2 h incubation time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

saponification. The intracellular uptake of LNPs involves, in general, a mechanism of endocytosis, by which localised regions of the plasma membrane wrap around the material to be internalized, fold in and detach to form endocytotic vesicles (Oh and Park, 2014; Panariti et al., 2012). After incorporation in the endolysosomal compartments, LNPs are susceptible to rapid enzymatic hydrolysis mediated by lysosomal lipases (Maier et al., 2013). Presumably, after the addition to the culture media, MO/PF108 nanoparticles were immediately taken up by HeLa cells by endocytosis, and OA, maybe derived from the lipolysis of MO in the endolysosomal compartments, was utilized for the synthesis of phospholipids and triacylglycerols incorporating OA. At short term-incubation, the OA increase in treated cells was mainly derived from an intracellular hydrolysis process of MO more than from the MO hydrolysis in the external medium. In fact, the extent of hydrolysis phenomena in MO/water based systems resulted negligible at short incubation times (Murgia et al., 2002). The OA increase measured in MO/PF108 treated HeLa cells at 2 and 4 h of incubation corresponded approximately to the 10% and 12% of the

OA amount in MO/PF108 cubosomes, respectively. The cellular internalization of MO/PF108 cubosomes caused a modification of the HeLa cell PL profile, inducing a significant synthesis of the PL fraction incorporating OA (PL containing 18:1–18:1 and 18:1–16:0). A significant increase of polar lipids was also observed in treated cells by NR measurements (red emission) at both incubation times, indicating that the uptake of MO-based cubosomes induced a significant increase of cytoplasmic membranes as a consequence of the endocytotic process. A good negative correlation (correlation coefficient $r = -0.9239$, with $r = \text{Cov}(X,Y)/\sigma_X \cdot \sigma_Y$) was found between TC/PL ratio values and values of NR-fluorescent quantification of cytoplasmic membranes. The PL fatty acid modification observed is physicochemically relevant. Plasma membrane and organelle membranes consist of proteins embedded in a fluid PL bilayer. PL, together with membrane proteins and cholesterol, are involved in many cell functions such as in the control of cell shape, compartmentalization, storage of compounds, ion transport, metabolism, cell signalling processes and cell fusion processes (Monteiro et al.,

2014). The lipid composition of membranes is not homogenous throughout the cell; each organelle has been found to have a unique lipid composition allowing optimal functionality (Hagen et al., 2010; Vance, 2014). As a matter of fact, alterations in cell membrane lipid composition, like changes in the ratio between saturated and monounsaturated fatty acid chains of the PL, can directly influence the biophysical properties of biological membranes (cytoplasmic and organelles' membranes), deeply altering fluidity, the lipid raft thermodynamic properties and microdomain organization (Hagen et al., 2010; Ariyama et al., 2010). Therefore, changes in lipid composition influence many cellular functions such as signal transduction, cell adhesion, lipid trafficking, ion channel function, receptor mobility/interaction, and the biological response to the drugs (Hagen et al., 2010).

In response to MO/PF108 nanoparticle treatments, our study also evidenced a significant increase of neutral lipids stored into the lipid droplets observed in the cytoplasm of the cells. In turn, as indicated by the good correlation coefficient found between TAG/neutral lipid values ($r=0.9668$), the consequent accumulation of the TAG fraction incorporating OA (OOO/OOP) was ascertained. Here, it deserves noticing that intracellular LDs basically accumulate when cells are cultured in the presence of high concentrations of various exogenous lipids (Hsieh et al., 2012). In many cell cultures of non-adipocytes, only a variable number of LDs exist under normal conditions, but the addition of unsaturated fatty acids such as OA to the medium induces abundant TG-rich LDs, whereas the treatment with cholesterol induces increased CE-rich LDs (Hsieh et al., 2012; Suzuki et al., 2012). It is worth to note that no significant changes in the cellular levels of FC and CO were observed in cells treated with MO/PF108 nanoparticles, indicating the activation of specific lipid biosynthetic pathways in HeLa cells.

5. Conclusions

This study showed that exposure of HeLa cells to MO-based cubosomes stabilized by Pluronic F108 (MO/PF108) can affect the intracellular metabolism of lipids. Results evidenced that MO/PF108 cubosome formulation was promptly taken up by the HeLa cells and influenced the cellular lipid profile. Particularly, a significant synthesis of PL and TG rich in OA was observed along with a marked increase in the synthesis of intracellular membranes and accumulation of LDs. Given the importance of lipid composition in membrane fluidity, cell signaling modulation and protein function, the data of the present study furnish evidence of the impact of lipid-based cubosomes, at a low non-cytotoxic concentration, on the biochemistry and physiology of cancer HeLa cells. It should be largely taken into account when evaluating the in vitro and in vivo biological response to encapsulated drugs. Moreover, we highlight the potential role of lipid-based nanoparticles not only as simple carriers for in vivo drug delivery but also as an in vivo platform to modulate the lipid biosynthetic pathways of specific cell target. Further studies will be needed to establish also the impact of lipid-based cubosomes on the lipid profile of normal cell.

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Conflict of interest

The authors declare that they have no conflict of interest.

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VIII

Near infra-red fluorescent cubic liquid crystalline nanoparticles for optical imaging applications *in vivo*.

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Near infra-red fluorescent cubic liquid crystalline nanoparticles for optical imaging applications *in vivo*

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Abstract

Herein we provided the first proof-of-principle for *in vivo* fluorescence optical imaging application using monoolein-based cubosomes in a healthy mouse animal model. This formulation, administered at a non cytotoxic concentration, was capable of providing both exogenous contrast for NIR fluorescence imaging with very high efficiency and chemospecific information upon lifetime analysis. Time-resolved measurements of fluorescence after the intravenous injection of cubosomes revealed that the dye rapidly accumulates mainly in the liver, while lifetimes profiles obtained *in vivo* allowed discriminating between the dye free or embedded within the cubosome nanostructure after injection. Plasmatic elimination of the dye-loaded cubosomes occurs following a pseudo first-order kinetic.

Introduction

Spontaneous self-assembly of lipids in water allows formulation of liquid crystalline nanoparticles of the inverted type, showing inner nanostructure characterized by lipid bilayers wrapped on triply periodic minimal surfaces of cubic symmetry.¹⁻⁴ Discovered in the eighties, such nanoparticles are universally known as cubosomes.^{5,6} They are considered an emerging platform for a wide range of nanomedicine applications by virtue of unique features.⁷ These include a high hydrophobic volume (higher than liposomes),⁸ the possibility of simultaneously host both diagnostic agents and therapeutics (cubosomes are also proposed as theranostic tools),⁸⁻¹⁰ storage stability, and a viscosity of the formulation close to water, a fundamental characteristic for bolus delivery.¹¹ Recently, their superior efficacy in the siRNA delivery (cuboplexes) with respect to traditional lipoplexes was also discussed.¹² The necessary colloidal stability of cubosome formulations is typically achieved by using polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide triblock copolymers (Pluronic) as stabilizing agents, although other kinds of stabilizers have been proposed.¹¹⁻¹⁶ As a consequence, cubosomes are surrounded by a PEO corona that, similarly to stealth liposomes, should retard the clearance from the bloodstream of these nanoparticles via the mononuclear phagocytic system by inhibiting opsonization.^{17,18} Furthermore, their surface can be decorated with targeting residues to properly address cubosomes towards specific tissues.^{19,20} Despite their remarkable potential as nanocarriers, to the best of our knowledge, so far no papers have appeared describing the successful exploitation of cubosomes for *in vivo* imaging. Differently, several authors demonstrated that the hexagonal counterpart of cubosomes (namely, hexosomes) can be effectively labeled with radiotracers (such as ⁹⁹Tc)^{21,22} or MRI contrast agents (paramagnetic nitroxide lipids),^{11,23} providing valuable imaging tools for *in vivo* diagnostics. Compared with traditional structural and functional imaging modalities, fluorescence optical imaging (FOI) offers several advantages, requiring a relatively simple instrumental setup, and showing high signal-to-noise ratios *in vivo*, owing to

the development of novel synthetic fluorescent probes with high brightness and photostability in the near infra-red spectral region (NIR, 650-900 nm).²⁴⁻²⁷ Therefore, innovative FOI methods hold promise as radiation-free, portable, and potentially cost-effective screening technology extremely useful in imaging studies *in vivo*.²⁸ In particular, Time-Domain optical imaging (TD-OI) technology allows for NIR fluorescence lifetime analysis, which is based both on the specificity of fluorescence probes and the sensitivity of their emission lifetime, related to environmental characteristics.²⁹⁻³⁴ In this context, here we provide the first proof-of-principle for FOI application using monoolein (MO)-based cubosomes *in vivo* in a healthy mouse animal model.

Results and Discussion

To favor its encapsulation within the MO bilayer, the fluorescent probe used, a Cyanine 5.5, was modified by adding a long hydrocarbon chain (Cy5.5-C₁₄, see Supplementary Information for details). The fluorescent cubosome formulation was easily prepared by dispersing MO and Cy5.5-C₁₄ in a Pluronic F108 (PF108) aqueous solution. The physicochemical characterization of the formulation was performed by dynamic light scattering (DLS), electron transmission microscopy at cryogenic temperature (cryo-TEM), and small-angle x-ray scattering (SAXS). According to DLS results, the formulation was a homogenous dispersion (polydispersity index of 0.12) of nanoparticles characterized by a mean diameter of 144 ± 1 nm and a ζ -potential of -21 mV. The nanoparticles morphology, as seen by cryo-TEM, revealed the co-existence of cubic shaped and quasi-spherical objects both displaying an inner structure made of a dark matrix and alternate bright spots (Supplementary Fig. S1a), as classically observed in liquid crystalline nanoparticles. It deserves noticing the complete absence of the vesicular material that is commonly found in this kind of formulations.³⁵ The nature of the liquid crystalline phase was assessed by SAXS. The diffraction pattern was characterized by at least five Bragg peaks with relative positions $\sqrt{2}:\sqrt{3}:2:\sqrt{6}:\sqrt{8}$ (Supplementary Fig. S1b), confirming that the inner

nanoparticle was made of a bicontinuous cubic double diamond phase (space group $Pn3m$), having a lattice parameter of $89.1 \pm 0.4 \text{ \AA}$. Such a value is about ten percent smaller than that recorded in pristine cubosomes (not containing the fluorescent probe) obtained using the same components.¹⁹ This fact implicitly indicates that, although the nanoparticle is retained, accommodation of Cy5.5-C₁₄ within the lipid bilayer alters the MO effective packing parameter,³⁶ inducing a slightly higher curvature at the MO/water interface, and a consequent decrease in the lattice parameter. The photophysical characterization of the dye and the cubosome formulation was performed by UV-Vis and fluorescence spectroscopy. As shown in Supplementary Fig. S2 and reported in Table S1, the free dye Cy5.5-C₁₄ showed, in EtOH, an absorption band centered at 685 nm ($\epsilon = 140000 \text{ M}^{-1} \text{ cm}^{-1}$) with a shoulder at 650 nm ($\epsilon = 14000 \text{ M}^{-1} \text{ cm}^{-1}$). A strong emission at 709 nm was observed ($\Phi = 0.27$). Once encapsulated inside the cubosomes (Cy5.5-C₁₄@cubosomes, aqueous dispersion), both the absorption ($\lambda_{\text{abs}} = 696 \text{ nm}$ and 665 nm) and the emission bands ($\lambda_{\text{emis}} = 719 \text{ nm}$) of the dye were slightly red-shifted (Supplementary Fig. S3), probably due to the more apolar environment experienced by the fluorophore inside the nanoparticles with respect to EtOH. The differences in fluorescence anisotropy values ($r = 0.008$ and $r = 0.100$ for Cy5.5-C₁₄ and Cy5.5-C₁₄@cubosomes, respectively) clearly confirmed the internalization of the fluorophore inside the nanoparticles. Before the evaluation *in vivo* as diagnostic tool, the formulation was tested *in vitro* for its toxicity. Indeed, several recent *in vitro* investigations drew attention to the potential cytotoxicity of cubosomes and, particularly, to their hemolytic properties.^{37,38} Accordingly, the proposed formulation was investigated for its cytotoxicity against 3T3 cells and erythrocytes. Although the cytotoxicity of cubosomes was found to be strongly dependent on the cellular line investigated, the lower concentration threshold was around $20 \text{ }\mu\text{g/mL}$ of MO.^{9,39-41} The formulation here tested was found to induce no cytotoxicity in 3T3 cells up to $55 \text{ }\mu\text{g/mL}$ of MO (Fig. 1a). The incubation of Cy5.5-C₁₄@cubosomes with human red blood cells evidenced the capacity to

induce direct lysis of erythrocytes in a dose-dependent manner modifying a standard hemolytic assay⁴² and using 10^9 erythrocytes (Fig. 1b).^{43,44} These results are in line with experiments previously reported,^{41,45} and evidenced that the cubosomes formulation here proposed did not show lysis of erythrocytes below 62 $\mu\text{g/mL}$ of monoolein.

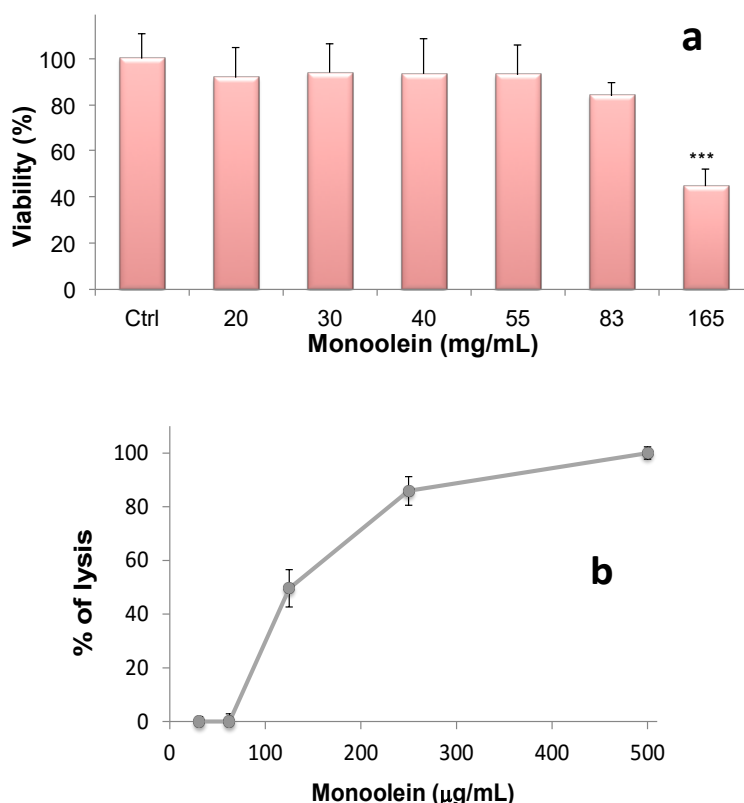


Figure 1. Evaluation of the formulation cytotoxicity. (a) Viability, expressed as % of the control, induced by incubation for 4 h with different concentrations of cubosomes (corresponding to 20-165 $\mu\text{g/mL}$ of MO) in 3T3 fibroblasts by MTT assay. Data are expressed as a mean $\pm$ standard deviation (SD) of three independent experiments involving quadruplicate analyses for each sample; *** = $p < 0.001$ versus Ctrl. (b) Hemolysis test of human red blood cells incubated with different amounts of cubosomes in a standard hemolytic assay using 10% erythrocytes. Data were expressed as % of lysis $\pm$ SD.

Consequently, *in vivo* experiments were performed using a MO concentration of about 50 μg per mL of plasma. After the *in vivo* injection of Cy5.5- C_{14} @cubosomes into the tail vein (i.v.) of a healthy mouse, the distribution of the fluorophore was monitored using TD-OI. This technology helped understand the lifetime distribution, and provided a convenient route of analyzing the biodistribution and plasmatic

elimination of a fluorescent cubosomes formulation after intravenous administration in mice. Optical imaging was performed two times during the first day, then every 24 hours for the next two days: the results showed that, following injection, Cy5.5-C₁₄@cubosomes were rapidly taken up by the liver (Fig. 2a). To further investigate the extent of Cy5.5-C₁₄@cubosomes and/or free dye accumulation in the organs and tissues, mice were sacrificed at 48 hours after injection of the cubosomes for *ex vivo* TD-OI analysis of explanted organs (i.e., kidneys, thymus, heart, spleen, brain, lungs, stomach, intestine, and liver). This approach excludes any potential influence due to autofluorescence and scattering within the body and fur, thereby increasing both specificity and sensitivity of the probe detection. *Ex vivo* evaluation of organs at 48 hours post-injection showed that the highest fluorescence intensity was localized in the liver and stomach, emphasizing that Cy5.5-C₁₄ is excreted through these organs (Fig. 2b). Cy5.5-C₁₄@cubosome plasmatic elimination after single i.v. administration was investigated by analyzing the fluorescence signal of plasma samples at different times post-injection, which could be interpreted using a pseudo first-order kinetic (Supplementary Fig. S4).

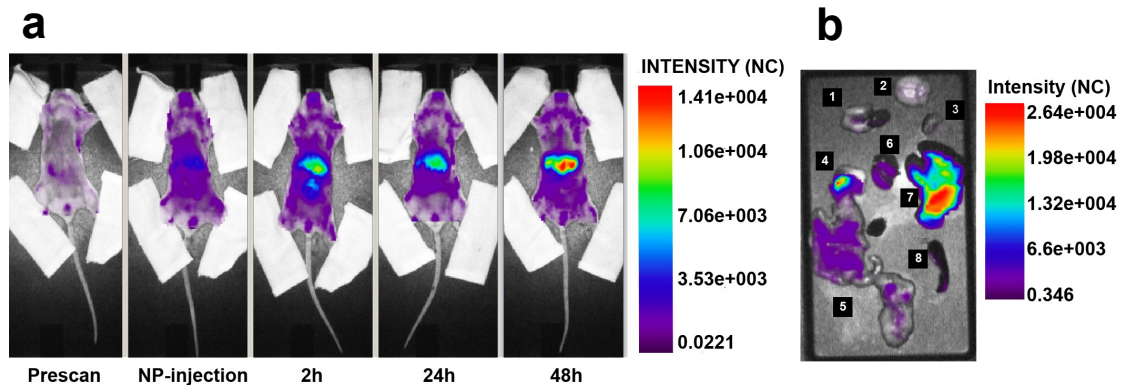


Figure 2. *In vivo* and *ex vivo* Time-Domain optical imaging experiments. (a) Whole body fluorescence intensity distribution of a representative healthy mouse that received an i.v. injection of cubosomes (1.5 µL stock solution + 148.5 µL PBS, corresponding to ~0.4 nmol Cy5.5-C₁₄ and 50 µg MO), displayed in normalized counts (NC). Following injection, cubosomes were rapidly taken up by the liver. (b) In accordance to the *in vivo* results, *ex vivo* analysis clearly showed that the highest signal of fluorescence emission was associated with liver. A strong intensity specific signal was also detected in the stomach. 1. Lungs and heart; 2. Brain; 3. Thymus; 4. Stomach; 5. Intestine; 6. Kidney; 7. Liver; 8. Spleen.

Fluorescence lifetime information is complementary to intensity measurement and can be used to improve signal-to-background contrast and provide environment-sensing capability. Some biological events not available from fluorescent intensity images can be monitored directly from the fluorescence lifetime map. The fluorescence decay of Cy5.5-C₁₄@cubosomes and Cy5.5-C₁₄ were measured *in vitro* and single-exponential models were appropriately fitted. Cy5.5-C₁₄ clearly changed its fluorescence lifetime properties once loaded in the cubosomes formulation (Fig. 3a).

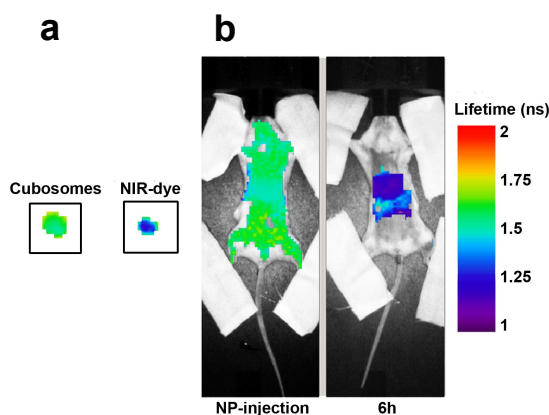


Figure 3. *In vitro* and *in vivo* fluorescence lifetime experiments. (a) *In vitro* fluorescence lifetime analysis with equimolar aliquots of the cubosomes preparative and the control NIR-dye. The samples were placed on a paper substrate before analysis of the lifetime. Fluorescence lifetime maps were created from temporal point-spread function (TPSF) data acquired using the time-domain diffuse optical imaging system. (b) *In vivo* fluorescence lifetime maps of whole body mice injected with cubosomes. The images were taken a few minutes and 6 hours after the injection.

This provided the possibility to discriminate *in vivo* between the Cy5.5-C₁₄@cubosomes and the free dye based on distinct lifetime profiles. Whole body imaging analysis at a few minutes after i.v. injection revealed a diffuse distribution of cubosomes throughout the body of the mouse and, after the first 6 hours, a strong signal associated with the free dye was clearly detectable mainly in the liver and intestine, decreasing over time (Fig. 3b).

To cross-validate the results obtained *in vivo* and *ex vivo*, the localization of Cy5.5-C₁₄ within the liver was confirmed by fluorescence microscopic examination of

tissue cryo-sections obtained from the explanted organ at 48 hours after Cy5.5-C₁₄@cubosomes injection (Fig. 4). The close examination of the liver cryo-sections revealed the presence of several spherical fluorescent aggregates (indicated by the arrows) with size in the micrometer range, which could reasonably be identified as the lipid droplets (cytoplasmic organelles involved in neutral lipid storage) since the highly hydrophobic Cy5.5-C₁₄ dye should preferentially stain structures of this kind in the cells. It deserves here noticing that MO-based nanoparticles (cubosomes, or vesicles) are readily internalized after exposure to cells, causing growth in size and number of the lipid droplets.^{19,39,46}

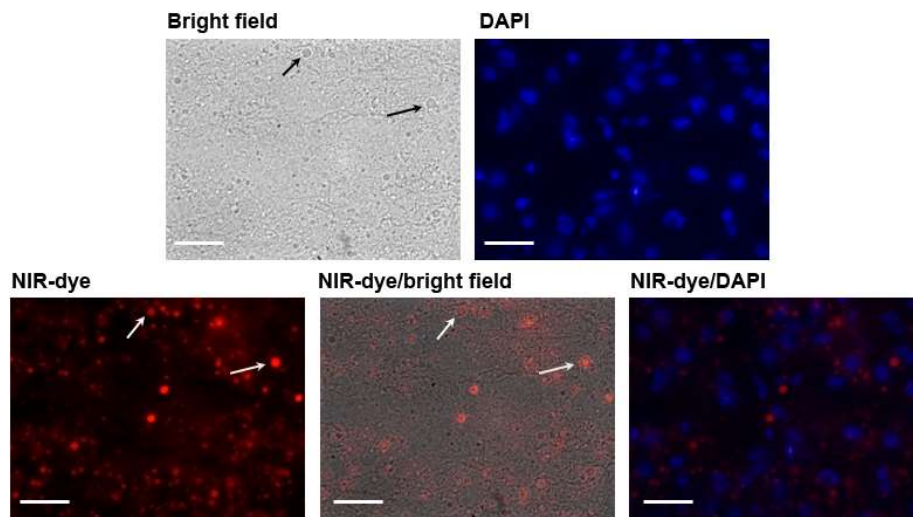


Figure 4. Microscopic examination of liver cryo-sections. Representative microscopy images of cryo-sections obtained from liver explanted from the cubosomes injected mice. Sections are imaged under bright field and fluorescence mode under x63. DAPI staining was carried out to visualize cell nuclei, which appear in blue, while Cy5.5-C₁₄ appears in red. The arrows indicate droplet structures in the hepatic parenchyma (bar = 15 μ m).

Because of its high sensitivity, FOI has been increasingly applied to assess tissue pathology, such as in image-guided surgery, and for sentinel lymph node fluorescence mapping. In this respect, several intraoperative NIR fluorescence systems are now available for pre-clinical and clinical studies and some of these systems have been approved by the FDA for use in humans.⁴⁷ From a molecular imaging perspective, it was here noted that cubosomes have great applicative potential in medicine as fluorescence and lifetime probes. For this purpose, more

progresses of fluorescent cubosomes towards *in vivo* applications will depend on the introduction of targeting properties that will increase the specificity of the cubosomes to be used for both imaging and therapeutic applications.

Methods

Cubosomes preparation and characterization. Cubosomes were prepared by dispersing melted monoolein (MO, 1-monooleoylglycerol, 98.1 wt%, kindly provided by Danisco A/S, DK-7200, Grinsted, Denmark) in an aqueous solution of Pluronic F108 (PF108, PEO₁₃₂-PPO₅₀-PEO₁₃₂, Sigma-Aldrich) using an ultrasonic processor UP100H by Dr. Hielscher, cycle 0.9, amplitude 90%, for 10 min. To obtain fluorescent cubosomes, the fluorophore Cy5.5-C₁₄ was dispersed in the melted MO with the help of an ultrasonic bath before dispersion in Pluronic F108. The typical sample volume was 4 mL with 96.39 wt % of water, 3.3 wt % of MO, and 0.31 wt % of Pluronic F108. The molar concentration of Cy5.5-C₁₄ was 2.7×10^{-4} . Dynamic light scattering (DLS) measurements for the determination of the nanoparticles size and ζ -potential were performed at 25 °C using a ZetaSizer Nano ZSP (Malvern Instruments Ltd., Worcestershire, U.K.). DLS measurements were also performed after the cubosome formulation was passed through a 30G needle (the same used for cubosome i.v. administration for *in vivo* tests), to confirm that injection did not alter the physicochemical characteristics of the formulation. The characterization of the nanoparticles internal structure at 25 °C was carried out by small angle x-ray scattering (SAXS) using a Ganesha 300XL (SAXSLAB ApS, Skovlunde, Denmark). The morphology of the nanoparticles was observed at 120 kV acceleration voltage in an FEI Tecnai T12 G² transmission electron microscope at about -175 °C in the low-dose imaging mode to minimize electron-beam radiation-damage. Images were digitally recorded with a Gatan US1000 high-resolution CCD camera.

Photophysical measurements. Cubosomes solutions were diluted with Milli-Q[®] water. UV-vis absorption spectra were recorded at 25 °C by means of a Perkin-Elmer Lambda 45 spectrophotometer. Quartz cuvettes with optical path length of 1

cm were used. Corrected fluorescence emission and excitation spectra (450 W Xe lamp) were obtained with a modular UV-vis-NIR spectrofluorimeter Edinburgh Instruments FLS920 equipped with both a Hamamatsu R928P photomultiplier tube (for the 500-850 nm spectral range) and an Edinburgh Instruments Ge detector (for the 800-1600 nm spectral range). Luminescence quantum yields (uncertainty $\pm 15\%$) were recorded on air-equilibrated solutions and cyanine IR-125 (from Acros Organics) in Ethanol ($\Phi = 0.132$).⁴⁸ The same instrument connected to a PCS900 PC card was used for the TCSPC (Time-Correlated Single Photon Counting) experiments (excitation laser $\lambda = 640$ nm). Corrections for instrumental response, inner filter effects and phototube sensitivity were performed.⁴⁹ Fluorescence anisotropy measurements were performed on an Edinburgh FLS920 equipped with Glan-Thompson polarizers. Anisotropy measurements were collected using an L-format configuration, and data were corrected for polarization bias using the G-factor. Four different spectra were acquired for each sample combining different orientation of the excitation and emission polarizers: I_{VV} , I_{VH} , I_{HH} , I_{HV} (where V stands for vertical and H for horizontal; the first subscript refers to the excitation polarizer and the second subscript refers to the emission one). The spectra were used to calculate the G-factor and the anisotropy r : $G = I_{HV}/I_{HH}$, $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$.

Cytotoxicity experiments. Mouse 3T3 fibroblasts (ATCC collection) were grown at 37 °C in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL⁻¹), and streptomycin (100 µg mL⁻¹) (Invitrogen) in a 5% CO₂ incubator at 37 °C. The cytotoxic effect of the nanoparticle formulation was evaluated in 3T3 fibroblasts by the MTT assay.⁵⁰ 3T3 cells were seeded in 96-well plates at a density of 2×10^3 cells/well in 100 µL of serum-containing media. Experiments were carried out two days after seeding when cells had reached 70% confluence. Cubosomes were added to the cells at different concentrations (corresponding to 20, 30, 40, 55, 83, and 165 µg/mL of MO) in 100 µL of serum-free medium, and incubated at 37 °C for 4 h. The cell culture medium was then

removed from each well of the 96-well plates; an 8 μ L portion of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL in H₂O) was added to cells in fresh medium and left for 2 h at 37 °C. The medium was aspirated, 100 μ L of DMSO was added to the wells, and color development was measured at 570 nm with an Infinite 200 auto microplate reader (Infinite 200, Tecan, Austria). The absorbance is proportional to the number of viable cells. All measurements were performed in quadruplicate and repeated at least three times. Results are shown as percent of cell viability in comparison with non-treated control cells. Evaluation of statistical significance of differences was performed using Graph Pad INSTAT software (GraphPad software, San Diego, CA, USA). Comparison between groups was assessed by one-way analysis of variance (One-way ANOVA) followed by the Bonferroni Multiple Comparisons Test.

Hemolytic assays. These assays were done as previously described.⁵¹ Briefly, hemolytic activity was performed by mixing different amount cubosomes in Veronal buffer with 50 μ L of 10% human erythrocytes (EA) suspended in a final volume of 250 μ L. After incubation at 37 °C for 30 min, red cell lysis was calculated by measuring the OD415. Hemolytic activity was expressed as a percentage of lysis $\pm$ SD.

Animal model and treatment. Pathogen-free female BALB/c mice (4- to 6-weeks old) were purchased from Harlan Laboratories (San Pietro al Natisone, Italy). Mice were housed in a controlled environment and maintained with *ad libitum* food and water. Mice were treated intravenously (i.v.) with 150 μ L of cubosomes solution, corresponding to $\sim$ 0.4 nmol Cy5.5-C₁₄ and 50 μ g MO. Mice were followed by optical imaging images acquisition over time to evaluate the probe biodistribution and accumulation in specific organs, and the animals were also subjected to blood samples for the plasma collection.

In vivo and ex vivo time domain optical imaging analysis. Experimental animals were anesthetized with isoflurane at 1.8 - 2.0 volume %, shaved in the abdomen to

avoid laser scattering caused by fur, placed in the small animal time-domain Optix MX2 preclinical NIRF-imager (Advanced Research Technologies, Montreal, CA) and maintained under vaporized isoflurane for the entire imaging session. Two-dimensional regions of interest were selected and laser power, integration time and scan step size were optimized according to the emitted signal. A blank image was acquired before i.v. administration of cubosomes to record background signal intensity and after i.v. treatment the animals were monitored at multiple time points. In all imaging experiments, a 670 nm pulsed laser diode with a repetition frequency of 80 MHz and a time resolution of 12 ps light pulse was used for excitation. Fluorescence emission was collected at 700 nm and optical imaging results were analysed by reporting fluorescence intensity values in normalized counts (NC) representing the photon count for unit excitation laser power and unit exposure time, allowing comparison among different images. The fluorescence lifetime results were obtained by fitting every fluorescence decay curve corresponding to each pixel measured by Optix using the Levenberg Marquet least squares method.⁵² For *ex vivo* imaging, the last *in vivo* whole body imaging session was followed by euthanasia of animals and the explanted organs were collected, washed in PBS and analysed with the same Optix system. Following, explanted organs were snap-frozen in isopentane and dry ice, and cooled at -80 °C for tissue analysis.

Ex vivo tissue analyses. For fluorescence microscopy, sections of 7 and 12 µm thickness were cut from frozen organs with a cryostat at -20 °C. Glass slides were kept at 4 °C at dark and Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA), diluted 1:1 with PBS, was used to stain cell nuclei. Fluorescent images were acquired by using an inverted microscope with a CCD camera (DVC-1412AM monochrome digital camera QE > 62% at 550 nm) and the objectives lens 10X, 40X and 63X (NA 1.63) immersion oil.

Ethical statement. All animal procedures were used to minimize animal pain and suffering. All the experimental procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of the Laboratory Animals

of the National Institutes of Health and in compliance with the European (86/609/EEC) and Italian (D.L.116/92) laws. The protocols for mice experimentation were approved by the Institutional Animal Care and Use Committee of the Cluster in Biomedicine (CBM) of the Area Science Park of Trieste and by the Italian Ministry of Health (DM 17/2001-A dd. 02/02/2011).

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Author Contributions

S. M. and S. B. conceived and designed the study. S. B, L. A. and C. G. performed the optical imaging experiments. A. M. F. and A. R. carried out the cytotoxicity tests. C. C. and V. L. synthesized the fluorophore. V. M. and M. M. prepared the cubosomes formulation. L. Prodi and L. Petrizza performed the photophysical characterization of the fluorophore/formulation. P. M. conducted the hemolysis tests. A. L. was in charge for the animal care. Y. T. and J. S. performed the

electron microscopy experiments. M. O.-R. performed the small angle x-ray experiments. S. M. and S. B. mainly wrote the manuscript but, accordingly to their specialties, all the authors contributed in writing the paper.

Additional Information

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