Recent advances of non-lamellar lyotropic liquid crystalline nanoparticles in nanomedicine

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Abstract

Non-lamellar lyotropic liquid crystalline nanocarriers such as hexosomes and cubosomes are relatively unexplored lipid-based nanoparticles in nanomedicine that can be specifically formulated to match specific medical applications, thus exploiting the majority of the possible routes of administrations. A growing number of papers are evidencing intriguing features that make them good candidates as nanocarriers for therapeutically active molecules or imaging probes. Yet, important aspects, including pharmacokinetics, hemocompatibility, toxicity, and delivery properties are not completely clarified, so that their full potential as nanomedicines remains to be assessed.

This article reviews recent advances in the field and suggests possible new routes forward.

Keywords: Cubosomes, Hexosomes, imaging guided drug delivery

1. Introduction

Phospholipids and monoglycerides possibly represent the most prominent examples of amphiphilic lipids, a class of molecules with a natural propensity to self-assemble in water originating various kinds of aggregates, spanning from micelles to lyotropic liquid crystalline phases (LLC), and showing either normal or reverse curvature of the interface.[1–3] The self-assembly process that leads to the formation of such variety of nanostructures is promoted by the hydrophobic effect that acts to reduce or, at best, totally avoid the unfavorable interactions among the hydrophobic tails of the amphiphiles and water. The supramolecular architecture of the aggregates is mainly dictated by the local constraint imposed by the shape of the amphiphile, as can be rationalized in terms of a geometrical parameter known as the critical packing parameter $P = v/a_0 l_c$, where v represents the volume of the hydrophobic chain, a_0 is the headgroup area, and l_c is the critical hydrocarbon chain length, approximately equal to the fully extended chain length.[4,5] Since P relates the geometry of the amphiphile with the preferred curvature of the interface, it can be used to predict which phase will be preferentially formed. For example, self-assembling of amphiphilic lipids originates planar interfaces (lamellar structures) for P values equal to 1, while reverse aggregates form for P values larger than 1. It should be observed that alterations of the original nanostructure can be provoked by variations in ionic strength or temperature or by addition of components that may modify the amphiphile headgroup area or the hydrophobic chain splay, in turn causing changing of the effective P value.[6,7]

Among the nanostructures originated by amphiphilic lipids in water, the Lyotropic Liquid Crystals (LLC) phases deserve a special mention. These are peculiar nanostructures that exhibit the typical long-range order of solids still maintaining a certain fluidity characteristic of liquids. The most emblematic LLC phases are the lamellar, the hexagonal, and the bicontinuous cubic. The former is constituted by mono-dimensional stacked bilayers, while in hexagonal phases water cylinders surrounded by a lipid monolayer are organized in a two-dimensional hexagonal array (see Figure 1). Finally, in bicontinuous cubic phases two continuous but not interconnected water channels are formed by a three-dimensional and non-intersecting bilayer that extends in space superimposed over

an Infinite Periodic Minimal Surface (IPMS), being the primitive (body-centered lattice, Im3m), the gyroid (body-centered lattice, Ia3d), and the double diamond (primitive lattice, Pn3m) the most important IPMSs in lipid-based systems (see Figure 1).[8] Far from being exclusively engineered by researchers and confined within labs, these exotic nanostructures have been widely found in nature[3] in cell membranes under physiological/pathological (infection, tumours) states, or as a result of induced stress (starvation, drug treatments),[9] as well as in wing scales or cuticle of green butterflies and beetles.[10]

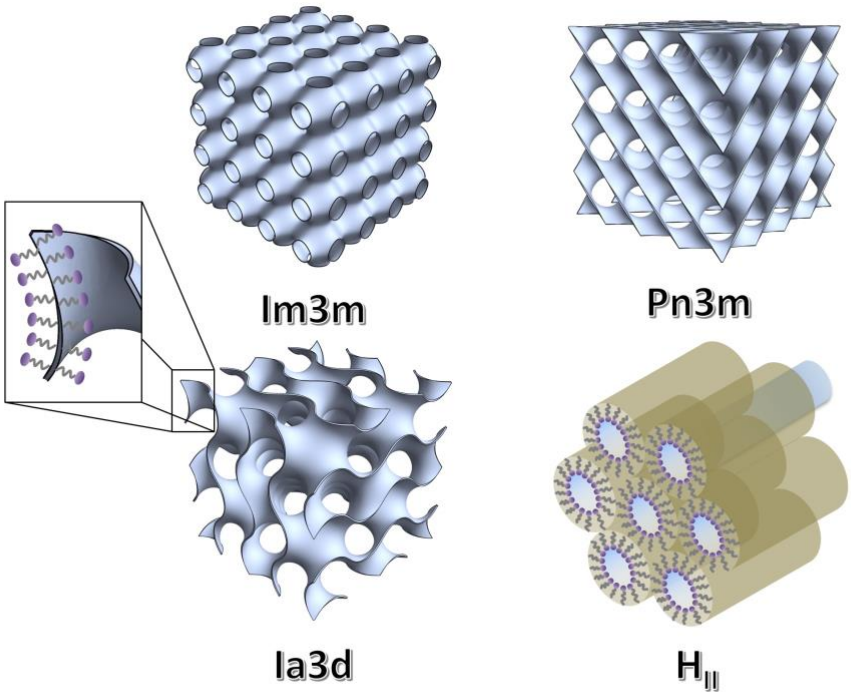

Figure 1. *Schematic illustration of the Infinite Periodic Minimal Surfaces most representative for lipids self-assembly in water (primitive Im3m, double gyroid Ia3d, and double diamond Pn3m) and of the reverse hexagonal arrangement (H_{II}).*

Since lamellar, hexagonal, and bicontinuous LLC phases can be formulated as colloidal dispersions, they are extremely appealing for the pharmaceutical industry.[11–13] Indeed, nanoparticles (NPs)

characterized by LLC core possess both hydrophobic and hydrophilic compartments where molecules of therapeutic/diagnostic interest can be hosted, while their surface can be easily decorated with targeting agents to properly address the NPs toward the pathological tissue.[14,15] Therefore, they are often engineered for drug delivery or diagnostic purposes, sometimes combining these two features to obtain theranostic tools. The colloidal dispersions of lamellar phases are well represented by the ubiquitous liposomes, while their much less investigated hexagonal or bicontinuous cubic homologues are respectively known as hexosomes and cubosomes, and often referred to as the non-lamellar LLC NPs.

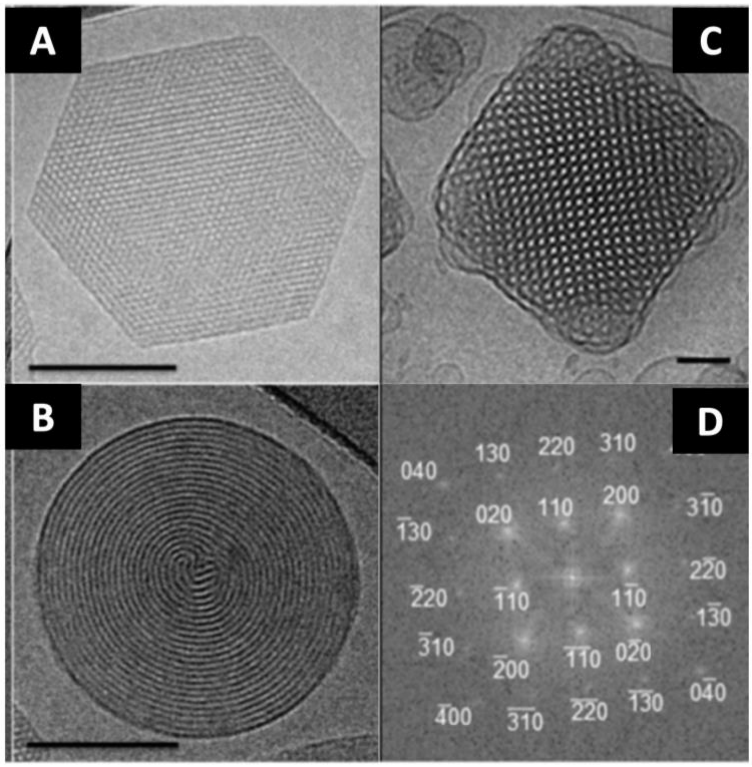

Figure 2. *Representative images of hexosomes (A, B)*[16] *and a cubosome (C) along with its Fourier transform with the corresponding indexation of the reflections (D). Figures C and D are reproduced with permission from ref.* [17] *(further permissions related to the material excerpted should be directed to the ACS).*

In Figure 2 are reported the cryo-TEM images of a single hexosome and a single cubosome NP showing the classical hexagonal and cubic morphology and the honeycomb structure of their interior. Remarkably, as shown in Figure 2B, hexosomes may have rounded shaped architecture. In addition, this NP is characterized by an inner structure characterized by curved striations that represent deformed water cylinders. Such configuration should be hypothetically adopted to allow a homogeneous hydrophobic surface of the NP that can be better stabilized by a more favorable adsorption of the dispersant used to stabilize the formulation (see below).[18] It is also worth mentioning, that cubosomes may be present with spherical or quasi-spherical morphology, though their inner structure is always characterized by the alternating bright spots (the water channels) and the dark matrix (the lipid bilayer) as seen in Figure 2C.

The vast majority of hexosomes and cubosomes described in literature were prepared using monoolein (2,3-dihydroxypropyl (*Z*)-octadec-9-enoate) or phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) as molecular building blocks, although other lipids can be used to formulate non-lamellar LLC NPs (some recent examples can be found in refs. [19,20]). Sometimes, the lipid constituents of the NPs are at the same time pharmaceutically active ingredients, as in the case of the omega-3 polyunsaturated fatty acids monoglycerides eicosapentanoic acid, docosapentanoic acis, and docosahexanoic acid monoglycerides that were used to formulate hexosome dispersions.[21,22]

Hexosomes and cubosomes preparation implies either the fragmentation in water of a previously prepared bulk LLC phase (top-down approach) in the presence of an emulsifier or the dissolution and mixing of the lipids and the additives, also with the aid of an hydrotrope,[23] to obtain an aqueous solution to which another water solution containing a dispersant is added (bottom-up approach). As a general rule, top-down methods provide formulations characterized by smaller NPs and lower polydispersity index with respect to bottom-up methods, but the heat produced during the fragmentation process may damage temperature-sensitive cargos.[12,24] As an alternative, a recently

introduced bottom-up method make use of a microfluidic device to prepare colloidally stable cubosomes endowed of small size of the NPs and narrow size distribution.[25,26]

Differently from liposomes, hexosomes and cubosomes formulations requires a stabilizing agent to avoid collapse of the colloidal dispersion. Stabilization in water of non-lamellar LLC NPs is typically achieved by steric repulsion using tri-block copolymers (poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide)) known as Pluronics, being the F127 and the F108 the most used. The hydrophobic block anchors in the lipid bilayer, while the hydrophilic arms surround the NP originating a polyethylene oxide corona that protect the formulation against flocculation and subsequent phase separation. However, other polymers were also proposed for stabilization of hexosomes and cubosomes, including amphiphilic brush copolymers[27] or Tween 80,[28] and sporadic examples of non-lamellar LLC NPs stabilized in water without the use of polymers can be found in the literature.[29–31]

Apart from the evident morphological and topological differences and the requisite of a stabilizing agent for their formulation, non-lamellar LLC NPs differentiate from liposomes also for another aspect that may be critical in view of their use as nanomedicines: because of the highly convoluted volumes of the lipid chains in hexagonal or bicontinuous cubic morphologies with respect to liposomes shells, the cubosomes and hexosomes possess a larger hydrophobic volume then their lamellar counterpart. For example, under the constraints imposed by the use of an identical molecular building block (specifically, monoolein) and NP volume, it was calculated that the hydrophobic portion of cubosomes characterized by the *Im3m* symmetry of the bicontinuous nanostructure and a lattice parameter of 130 Å is more than three times larger than that of liposomes of the unilamellar kind, and that the surface exposed to water by cubosomes is much smaller (about 60 %) compared to that of liposomes.[32] Interestingly, such peculiarity of LLC NPs can be exploited for transfection of functional biomacromolecules. For example, hexosomes were used for controlled sustained release of plasmid DNA,[33] whereas siRNA was encapsulated in both hexosomes[34] and bicontinuous cubic NPs, [26], in the latter case originating gene silencing cubosomes called cuboplexes. As an

answer to the challenge of siRNA delivery to target cells, cubosomes functionalized with zinc coordinated lipids were also prepared.[35]

A remarkable aspect of NPs constituted by self-assembled lipids is that their internal structure may be finely tuned in a desired way by introducing molecules that properly alter the critical packing parameter. An example of pharmaceutical interest is given by the local anesthetic drug bupivacaine, responsible of the lamellar to non-lamellar transition of NPs prepared using a mixture of citrem (citric acid esters of monoglycerides and diglycerides made from sunflower oil) and soy phosphatidylcholine.[36] Increasing of the tunability of bicontinuous bulk lipidic cubic phases and their NP dispersions was the subject of a recently published article. Using high-throughput protocols, in that paper authors investigated the phase behavior of the quaternary system monoolein/cholesterol/phospholipid/water providing a library of physiologically relevant bicontinuous cubic materials also in the form of colloidal dispersions.[37] Another important recent achievement is the formulation of highly swollen cubosomes, a result that assumes particular relevance taking into account the growing need for large molecules (e.g. proteins or peptides) loading within nanocarriers for *in vivo* delivery. Barriga and co-workers engineered lipid-based bicontinuous cubic membranes of Im3m symmetry characterized by lattice parameters up to 480 Å.[38] Successively, Zabara *et al.* succeeded in swelling also bicontinuous cubic phases of Ia3d and Pn3m symmetries to levels at which the inner water channels reached five times the diameter found in conventional lipidic mesophases, and used the ensued ultra-swollen mesophases to crystallize membranes proteins with large extra-cellular domains, otherwise inaccessible to conventional in-meso crystallization.[39] More recently, Barriga and co-workers were successful in manufacturing monoolein-based cubosomes characterized by lattice parameters of about 340 Å and water channel diameters exceeding 170 Å (over 4 times larger than classical cubosome structures).[40]

It deserves noticing that lamellar forming lipids (with critical packing parameter close to 1) such as cholesterol and several phospholipids, may play a fundamental role in the preparation of swollen

cubosomes since they cause an increase of the curvature of the monoolein bilayer, and consequently favor bicontinuous cubic nanostructures having larger lattice parameters.[41]

Cubosomes and hexosomes were discovered in the early 80s but, for many years, they were basically investigated for their physicochemical peculiar characteristics although, similarly to the LLC bulk phases to which they belong, their application in the pharmaceutical field were immediately predicted. Therefore, only after a rather long period of latency, in recent years, we are witnessing a huge growth of publications dedicated to the study of these NPs under a pharmaceutical perspective, particularly as an alternative to liposomes. Definitely, as will be discussed in the following, these NPs possess numerous appealing features that can be exploited for their use as nanocarriers for *in vivo* delivery of drugs or imaging agents. The present article attempts to review recently published papers on hexosomes and cubosomes emphasizing on their possible pharmaceutical use.

2. Hexosomes and cubosomes as nanomedicines

2.1 Drug delivery applications

Given their peculiar characteristics, hexosomes and cubosomes are considered as an emerging platform for the *in vivo* delivery of molecules of pharmaceutical interest and imaging probes. Numerous examples of application of non-lamellar LLC NPs as nanomedicines along with important related features are reported in ref. [15]. They are formulated as stable colloidal dispersions of NPs with sizes in the nanometric range, therefore suitable for intravenous administration, although topical and oral administrations are also suggested. Since their hydrodynamic diameter is typically in between 100 nm and 400 nm, these non-lamellar LLC NPs are large enough not to pass kidneys filtering units thus eluding fast bloodstream clearance, but at the same time they are small enough to be useful in oncology by exploiting the so-called Enhanced Permeation Retention (EPR) effect, the most important passive mechanism for the accumulation of NPs in tumor tissues. In addition, when prepared using Pluronics as stabilizing agents, hexosomes and cubosomes possess some stealth properties, being enveloped within a hydrophilic polyethylene oxide corona that, at least in principle,

should hinder opsonization and, consequently, retard their clearance from the systemic circulation. Moreover, the surface of these NPs can be decorated with targeting agents such as folate residues or epidermal growth factor receptor (EGFR) antibody fragment, to confer them active targeting properties toward cancer cells,[42,43] or with dyes to impart imaging abilities. To this aim, cubosomes functionalized with an azide group were recently prepared to participate in copper-free click chemistry, paving the way for drug delivery and imaging applications via metabolic labelling with cubosomes. [44] The addressing properties of folate-conjugated cubosomes loaded with doxorubicin were revisited investigating their internalization into cancer cells expressing different levels of folate receptor (FR) protein.[45] This study evidenced that drug accumulation straightforwardly depends by FR protein levels exhibited by the cells. The encapsulation of doxorubicin into LLC phases duly modified to switch symmetry at different pH, further enable these systems desirable triggered release features[46] in response of the different pH environment at which cancer cells proliferate.

Cytotoxicity features and interactions with biological components of self-assembled lipids into LLC NPs also in relation to their internal structure were extensively reviewed recently.[47] It was highlighted that interactions with biological fluids may alter the lattice dimension, sometimes provoking phase transitions. Since there is a straightforward connection between drug delivery performances and nanostructure, from this analysis emerges the urgency for the investigation of truly representative phase behaviors in biological environments. Furthermore, several investigations demonstrated that cubosomes stimulate the activity of the immune system with marked differences in the immunogenicity depending on the lipid used, being monoolein-based cubosomes less immunogenic than phytantryol-based cubosomes. A comparison among hexosomes and liposomes containing the same immunopotentiator (monomycoloyl glycerol-1) was performed by Hubert *et al.*. Their results demonstrated that, with respect to liposomes, hexosomes induces stronger MOMP (Chlamydia trachomatis major outer membrane protein) specific response but, on the other hand, elicited lower levels of effector T cells. Therefore, this study showed that liposomes and hexosomes

induce different immune response and demonstrated, at the same time, the potential of these NPs in engineering of customized vaccine formulations.[48] A recent publication reported about the biocompatibility of monoolein-based cubosomes with respect to Organ of Corti derived cell line (OC-k3), opening the way for the use of these NPs for the treatment of sensorineural hearing loss.[49]

More obscure appears the role of the nanostructure with respect to cellular toxicity since interpretation of data is complicated by the type of NPs constituents. For example, it was reported that pristine monoolein-based cubosomes stabilized by Pluronic F127 enters HeLa cells by an energy-independent cholesterol-dependent mechanism, while poly-*e*-lysine coated cubosomes enter the same cells by energy-dependent endocytosis.[50] Furthermore, it was found that hexosomes prepared using phytantriol and mannide monooleate are taken up by HeLa cells via a mechanism dependent on the cellular membrane tension (while liposomes preferentially enter HeLa cells by endocytosis) that implies bilayer destabilization.[51] Remarkably, the distinct route through which cubosomes enter the cells was successfully used to increase the bovine serum albumin uptake both *in vitro* in renal tubular cells and *in vivo* in renal experiments in *Clcn5* knockout mice characterized by defective receptor-mediate endocytosis. These experiment proved cubosomes as efficient nanocarriers to transport pharmaceutical cargos passing cells membranes also in case of genetically malfunctioning endocytic receptors.[52] Two publications were devoted to shed some light on the interaction between phytantriol-[53] or monoolein-based[54] cubosomes with model cell membranes. Mainly, these studies respectively evidenced the change in bilayers lipid organization due to cubosomes incorporation,[53] and that interaction of cubosomes with supported bilayers occurs via membrane fusion events similar to those observed with small intestine and STO fibroblast cell lines.[54]

To better understand *in vivo* interactions between cubosomes and cells an important step was recently taken realizing an environment mimicking the human vascular systems, i.e. a capillary glass device coated with collagen-extracellular matrix supporting HUVECs.[55] This device, coupled with a peristaltic pump, was used to study the interaction of cubosomes with endothelial cells under flow in a three-dimensional setting. Because of different Brownian motion and laminar flow, authors mainly

evidenced clear differences among cubosomes-cells interactions under static, and venous or arterial flow.

Although it was demonstrated that the high interfacial area of cubosomes causes burst release of both hydrophobic[56] and hydrophilic[57] drugs, as mentioned before, the most popular application envisaged for non-lamellar LLC NPs is their use as carriers for drug delivery. To this aim, numerous papers have recently appeared especially focusing on encapsulation of anticancer drugs, including 5-fluoruracil,[58] doxorubicin[45,59] also co-encapsulated with *Brucea javanica* oil,[60] docetaxel,[42] 2-hydroxyoleic acid,[61] lumefantrine,[62] methotrexate,[63] and paclitaxel.[43] As a general result, researchers observed an encouraging increase of toxicity of the drug towards cancer cell lines *in vitro* when administered encapsulated within the NPs. In at least one example, the anticancer drug was simultaneously loaded within hexosomes whose surface was decorated with imaging (rhodamine) and a targeting (folate) agents thus providing a theranostic nanomedicine.[64] Cubosomes loaded with paclitaxel were injected intraperitoneally in ovarian cancer xenograft mice. This study evidenced that the cubosome formulation was significantly more cytotoxic than the free drug, but, quite surprisingly, bioconjugating the NPs with EGFR antibody fragments did not offer additional advantages in terms of tumor mass reduction.[43] As briefly mentioned above, LLC can be engineered by doping the main lipid with amphiphilic acids[65] or bases [46] in order to provide a pH-responsive switch of symmetry associated with a change in release rate of encapsulated drugs. Similarly, non-lamellar LLC NPs can be designed in the same way to provide pH-responsive phase behavior,[59–62] a characteristic of relevance when engineering nanomedicines for oncology use, since extracellular pH of tumors may be more acidic (up to 5.8) than extracellular fluids in normal tissues.[46,66] Bilewicz *et al.* doped cubosomes with magnetic (hydrophilic mixed ferrite and hydrophobic magnetite) NPs realizing in this way hybrid NPs, called magnetocubosomes, that can be addressed to the target site by a magnetic field and be useful for magnetic hyperthermia.[67] The same group loaded magnetocubosomes with methotrexate and demonstrated this drug is released in a controlled manner applying an alternate magnetic field.[63]

As may be expected, hexosomes and cubosomes were also formulated to increase the bioavailability of numerous water-insoluble compounds useful in medical applications different from cancer treatments. For example, Angelova *et al.* explored the neuroprotective potential of curcumin, fish oil, and catalase loaded monoolein-based cubosomes showing their good performances in attenuating ROS accumulation in differentiated human neuroblastoma SH-SY5Y cells.[68,69] Hexosomes containing undecylenic acid (an antifungal agent) were successfully used to greatly reduce the activity of *Candida albicans* at a non-toxic concentration for human A594 cells,[70] while fluorescent squaramides (a class of molecules formally derived by squaric acid) were incapsulated in cubosomes for possible *in vivo* cell imaging.[71]

Bacterial resistance to antibiotics is becoming a major challenge for global health, and antimicrobial peptides (AMPs) are considered a valid alternative for the treatment of bacterial infections. However, they may undergo proteolytic degradation and sometimes display low water solubility, therefore application in clinic of bare AMPs are limited. Among others, to carry, protect, and deliver AMPs non-lamellar LLC NPs have been suggested. The post-loading onto the surface of cubosomes of three different AMPs, namely AP114, DPK-060, and LL-37, and their antimicrobial effect were tested.[72] Particularly, this study showed that association with cubosomes effectively protected LL-37 against enzymatic attack, although reduced its spectrum in bacterial killing. Differently, the antibacterial effect was preserved or promoted for AP114 and DPK-060, respectively. LL-37 was also encapsulated in non-toxic stabilizer-free cubosomes, a formulation that, additionally, favors cellular proliferation and can therefore boost wound healing.[73] Interestingly, from both these works it appears that coupling of LL-37 with cubosomes reduces its activity only against Gram-negative bacteria strains. Investigation of the mechanism of interaction of cubosomes carrying LL-37 with *Escherichia coli* highlighted that the NPs adsorbed and distorted the bacterial membrane causing the bacteria death.[74] In another work it was shown that oleic acid self-assembles in the presence of LL-37 forming a variety of pH and composition dependent nanostructures that encompass normal emulsions, micellar (*Fd3m*) cubosomes, hexosomes, and vesicles.[75] Similarly, incorporation of the

AMPs gramicidin A', melittin, and alamethicin and the structural alteration they provoked in cubosomes formulated using different monoacylglycerols were described earlier.[76] To make non-lamellar LLC NPs formulations containing AMPs useful for routes of administration different from parenteral, AP114 was added to a freeze-dried powder that upon hydration formed hexosomes. Among the various lyo-protectant tested trehalose was found the best choice, allowing the best performances of the AMP in terms of antibacterial activity.[77]

Oral and topical (including ocular) administration of non-lamellar LLC NPs present several favorable qualities, such as enhanced bioavailability, ease of use, and compliance of the patients that encourage their practice. However, only few papers describing the application of hexosomes and cubosomes for these kinds of use have appeared in the last three years. Specifically, cubosomes loaded with oridonin, a diterpenoid with promising anticancer activity extracted from *Rabdosia rubescens*, were administered orally to rats and investigated from the pharmacokinetic point of view. Results of this study proved that the formulation allowed a sustained release of the drug increasing its adsorption *in vivo*.[78] Spray dried cubosomes containing ovalbumin (as model antigen) and Quil-A (as adjuvant) were loaded within polymeric microcontainers coated with a pH-sensitive lid (Eudragit® S100). Authors reported that the microcontainers effectively protect cubosomes against degradation by the intestinal fluids, though they are released as hexosomes because of interaction of cubosomes with components of the lid. However, *in vivo* experiments showed this formulation did not elicit an immune response after oral administration in mice.[79] Cubosomes loaded with the model drug Cefpoxodime proxetil (a bitter antibiotic) were successfully proposed as taste-masking formulation for pediatric oral drug delivery.[80]

The *stratum corneum* is the outermost layer of the skin and acts as a front line of body defenses against environmental injuries. To accomplish this task corneocytes arrange in a characteristic “brick and mortar” configuration avoiding entry of exogenous materials. However, lipids may fluidize the *stratum corneum* facilitating the passage of drugs through the skin. Various lipid-based nanoparticles have been demonstrated useful for topical administration of drugs, and non-lamellar LLC NPs are

among them. Polymer-free cubosomes loaded with two different photosensitizing dyes were developed for the photodynamic therapy of human skin malignant melanoma. Bioimaging investigations proved the strong cytotoxicity on MeWo and Me45 cells exerted by the formulation encapsulating Chlorin e6 after photoirradiation.[81] The skin irritation and bacterial (*Staphylococcus aureus*) killing properties in *ex vivo* pig skin wound infection model of two different cubosomes formulation pre- or post-loaded with the AMP LL-37 were also studied showing that the formulations are not irritant to the skin, and evidencing the higher antibacterial LL-37 activity of pre-loaded cubosomes. [82] With the aim of targeting apoptotic cells in damaged retinal tissues, phytantriol- or monoolein-based cubosomes containing phosphatidylserine (PS) and annexin (ANX) were formulated. Since PS is exposed on cells surface during apoptosis,[83] and ANX has a great affinity with PS in the presence of Ca^{2+},[84] this protein was added to impart cubosomes targeting properties to apoptotic cells. Testing the phytantriol-based cubosomes on a rat model, this work demonstrated they specifically targeted damaged rather than healthy ocular tissues *in vivo*, showing their potential as drug delivery system for eye diseases.[85] Cubosomes encapsulating timolol maleate, a drug used in the therapy for glaucoma, were proposed also for the treatment of increased intraocular pressure. *Ex vivo* and *in vivo* experiments proved that the cubosome formulation is not cytotoxic and reduced the intraocular pressure in rabbits better than commercial timol maleate eye drops.[86]

2.2 Image-guided drug delivery

The progressive breakthroughs in nanomedicine have supported the development of multifunctional and multimodal nanostructured probes that can simultaneously provide imaging contrast, contain more than one modality-specific contrast agent, target specific cell populations, and carry a wide range of therapeutics.[87] Image-guided drug delivery refers to the combination of drug targeting and imaging.[88] Each of the imaging modalities has its advantages and limitations in sensitivity, resolution, and penetration depth. Combining multiple imaging modalities in a single nanoparticle

system can overcome limitations of the individual techniques while maximizing its advantages. Imaging can be used for several different purposes, in particular for monitoring biodistribution and target site accumulation of drugs and drug delivery systems, off-target localization, drug release, and drug efficacy.[88] Besides, imaging techniques can be used to trigger and quantify drug release from stimuli-responsive nanocarrier materials, such as temperature-sensitive liposomes, and ultrasound-responsive microbubbles.[89]

Furthermore, image-guidance is well suited to study drug delivery across biological barriers as mucosal, endothelial, blood-brain, cellular membrane, and numerous other barriers and to design strategies that aim to improve this process.[90] For instance, at the preclinical stage, the assessment of intratumoral barriers combined with a quantitative analysis of the intratumoral distribution of nanomaterials would be instrumental in defining more effective strategies to improve their clinical translation.[91] Finally, the combination of drug targeting and imaging could allow profiling patients that are likely to benefit from the nanoparticle-based drug delivery, thereby paving the way for personalized nanomedicine.[88]

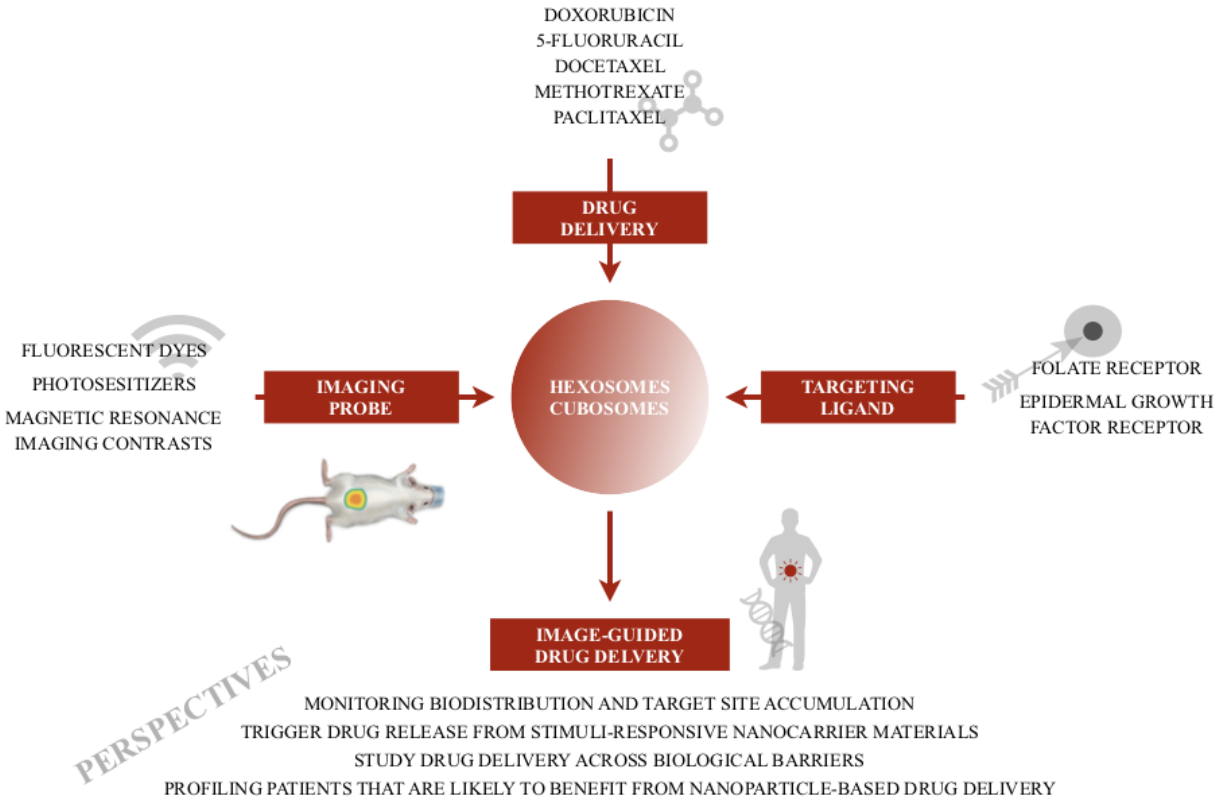

Figure 3. *Cubosomes and hexosomes as versatile platforms for image-guided drug delivery. Schematic representation of the most representative functional components investigated so far. The perspectives of their application are summarized.*

As newly emerging nanostructured probes for drug delivery, a range of fluorescent cubosomes suitable for image-guided drug delivery has been designed and tested on cell-based models. Using three-dimensional (3D) confocal microscopy and edge-to-edge compartment analysis, Furia *et al.* showed that cubosome nanoparticle systems labeled with the fluorophore DiD can function as intracellular carriers of the anticancer drug elesclomol.[92] Flow cytometry was used for the quantification of cubosomes cellular uptake. Moreover, label-free time-lapse multiphoton fluorescence lifetime imaging microscopy (MP-FLIM) enabled to measure the fluorescence of endogenous NADH and NADPH cofactors, thus monitoring pharmacodynamic changes in response to drug therapy. Another study has reported the successful application of two new fluorescent squaramide-based receptors (L1 and L2) for cellular imaging, showing their internalization inside the cells upon encapsulation inside the cubosome nanoparticles.[71] The free compounds exhibit different behavior concerning delivery across the cell membrane. L1 was able to pass through the cell membranes of living tumoral (Caco-2) and non-tumoral (293T) human cell lines, while L2 was localized on the cell membranes but did not enter the tested cells.[71] These results suggest a potential *in vivo* application for this kind of nanostructured imaging probes, and authors are currently working on the development of more hydrophobic fluorescent squaramides to improve the loading efficiency inside cubosomes. In another study, Chlorine 6 (Ce6) or meso-Tetraphenylporphine-Mn(III) (TPP-Mn) chloride photosensitizing dyes were encapsulated in cubosomes for the photodynamic therapy (PDT) of human skin melanoma cells.[81] FluoView FV1000 confocal laser scanning microscopy was used to image intracellular internalization, localization, and distribution of the photosensitizer

delivery vehicle. Results showed that Ce6 and TPP-Mn photosensitizers hold promises for effective bioimaging and maximum tumor damage via photoactivation.

To the best of our knowledge, so far, only very few studies have been reported in which cubosome formulations have been labeled with imaging probes, and in which their biodistribution and accumulation to the targeted site is imaged and quantified *in vivo.* Tran *et al.* described the first application of near-infrared fluorescent (NIRF) imaging and gadolinium lipid-based magnetic resonance (MR) imaging modalities combined in cubosomes and hexosome.[93] High throughput screening of a compound library identified two novel cubosome and hexosome systems with optimized MRI sensitivity and improved *in vitro* toxicity. When the authors investigated the ability of these formulations to provide both MRI and fluorescence contrast in a murine model, they showed it was feasible to study the nanoparticle biodistribution using fluorescence imaging, and enhanced contrast MR images of the liver and the spleen were obtained upon either cubosomes or hexosome administration. Biffi *et al.* provided the first proof of principle for *in vivo* time-domain fluorescence optical imaging application using monoolein-based Cyanine5.5-labeled cubosomes in a healthy mouse animal model. Cyanine5.5 was modified by adding a long hydrocarbon chain, to favor its encapsulation within the monoolein bilayer. Fluorescence imaging after the intravenous injection of cubosomes showed that the dye rapidly accumulated mainly in the liver, while chemospecific information upon lifetime analysis allowed for discriminating between free dye or dye embedded within the cubosome nanostructure after injection.[94]

3. Conclusions and outlook

Hexosomes and cubosomes are sometime indicated as the non-lamellar counterparts of liposomes. Indeed, similarly to the latter, they have been shown to properly encapsulate and transport, also simultaneously, different kinds of drugs and imaging probes and, due to their favorable physicochemical properties, promise to be a new, effective bioactive platform. However, with respect

to liposomes, they are much less studied. As a consequence, although investigation on the pharmaceutical applications of non-lamellar LLC NPs are growing exponentially, materials collected in this review highlighted that much work remains to be done to completely understand fundamental aspects concerning drug delivery properties, nanoparticles-biological tissues interactions, and *in vivo* biodistribution. Particularly, such delay in hexosomes and cubosomes pre-clinical development reflects in the lack of global marketed products. While several liposomes formulations are on the market or in clinical trials (Doxil® and Lipo-Dox® are few emblematic examples),[95] pharmaceutical regulatory approval of non-lamellar LLC NPs is yet to come. In the effort to efficiently generate bioactive hexosomes and cubosomes and translate them into clinical practice, the development of multifunctional nanoplatforms has been gaining considerable interest as a promising approach to integrate drug delivery and imaging.

Conflict of interest statement

Authors declare no conflict of interest

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Graphical Abstract