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REVIEW PAPER

Cubosomes the pliant honeycombed nano-cargos in drug delivery applications

Lokesh Gupta [1, 2]*, Arun Mittal [2], Archita Katrolia [1], Abhay Raizaday [1], Lokesh Kaushik [2], Bishnu Kant Shukla [3]

[1]College of Pharmacy, JSS Academy of Techhnical Education, Noida, India 201301
[2]SRM Modinagar College of Pharmacy, SRM-IST, Delhi-NCR Campus, Ghaziabad, India 201204
[3]Department of Civil Engineering, JSS Academy of Technical Education, Noida , India 201301

ABSTRACT

Cubosomes are the aqueous dispersions of lipid-based liquid-crystalline bi-continuous phases, with the inner structure comprised of triply periodic, non-intersecting, curved, bilayers folded in cubic symmetry, and are organized to form two disjoined continuous water channels on an infinite periodic minimal surface. This review emphasized the peer findings about history and origin of cubosomes, including preparation, characterization and evaluation techniques, along with its promising features as a bio-therapeutics biodegradable cargo nano-material in descriptive manner. The structures of cubosomes, e.g. Q223, Q227, Q212, Q230 etc. are discussed here reflecting their versatile applicability. The automated cubosome preparation method in addition to the general preparation methods and assessment of cubosomes with the aid of both, ordinary visual characterization as well as sophisticated instruments like cryo-TEM, Cryo-FESEM, SAXS, LUMiFuge® have been described. Physical parameter's quantification approves the drug-carriers system fit in therapeutics, i.e stability analysis, permeation, entrapment efficiency (EE), loading capacity (LC), drug content of dispersions, *in-vitro* drug release studies, HPLC analysis, in-vivo studies, etc., which are framed here in detail. Cubosomes owes the versatility and desired characteristic of a nanoparticle for drug delivery and other biomedical applications. Therefore, we have described here the up to date wide area applications of cubosomes administered through various routes.

Keywords: *Biodegradable, Cubosome, Drug-delivery, Drug carriers , Nanoparticle*

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INTRODUCTION

Nanomedicine improves the available diagnostic and therapeutic tools, for encapsulation of drugs and/or imaging agents for its applications [1]. Though, nanoparticles have been designed with extensive efforts resulted in large number of "soft" and "hard" nano-carriers, like dendrimers, polymeric micellar nanoparticles, phytosomes, aquasomes, silica nanoparticles, iron oxide nanoparticles, liposomes, carbon nano-tubes, quantum dots, fullerenes, etc. [2–4]. Among several nanocarriers designed, cubosomes are the most recent incorporated nano-cargos [5–7].

Actually, since last few year cubosomes have emerged as gold standard as per as drug [8, 9] delivery is concerned [10–13]. Cubosomes are aqueous bi-continuous lipid-based liquid-crystalline dispersions, with the inner structure comprised of triply periodic, non-intersecting bilayers, and curved to form two disjoined continuous water channels folded on an infinite periodic minimal surface of cubic symmetry [14–16]. Their several properties make appeal as a promising vehicle for drug delivery [17–20] as evidenced by the studies conducted in the past few years [21–23]. The surfactant forms bilayers which are twisted as three dimensional, periodic, minimal surfaces forming structures which are tightly packed like "honeycomb" into bi-continuous domains of water and lipid [24–26]. Cubosomes can be prepared by mechanical fragmentation carried out in cubic lipid-water phase to three-phase region of a liposomal dispersion (unlike liposomes [27–30]. It could be differentiated from liposomes

* *Corresponding author: Email: lokesh_gupta88@yahoo.com; lokeshgupta@jssaten.ac.in*
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through their structures, which accommodates amphiphilic molecules, and lipid-soluble, water-soluble, simultaneously [31–34]. Larsson named these nanoparticles as 'cubosomes' to represent the cubic molecular crystallographic symmetry as well as demonstrated its similarity to liposomes [35–37]. Cubosomes are binary, highly twisted, viscous, isotropic, lipid bilayer systems with larger surface area (~400 m^2/g), and possess high adjuvant incorporating capacity, so that, these are also known as lyotropic liquid crystals [38, 39]. These bicontinuous cubic phases are optically isotropic, very viscous, and solid-like liquid crystals with cubic crystallographic symmetry, also termed as ''viscous isotropic phases'' [40, 41]. The cubic phase has received attentions of many researchers as a promising carrier for the delivery of low molecular weight drugs, proteins, peptides, amino acids, and nucleic acids [42, 43]. The present review has been prepared in the view of compilation of the studies reported in the area of cubosomes drug delivery. Cubic structure of cubosomes incorporates the unstable drug substances and protects them from physical, chemical or any other factor mediated degradation [44, 45]. For example, insulin was examined for agitation induced aggregation for 2 months at 37 °C and was found almost unaffected, while insulin in normal condition was aggregated and precipitated after 8 days only [46, 47]. Moreover, cefazolin and cefuroxime were also evaluated for stability profile in a GMO cubic phase gel and found the enhanced chemical stability of cefazolin and cefuroxime [47].

History

The history of cubosomes goes long back from 1962 when *Luzzati and Husson* recognized the cubic phase existence in lipid-water system through X-ray scattering measurement technique [18]. In 1965 *Lutton* found that the monoglycerides as polar lipids with poor water solubility exhibit hydrophilic behavior and reflects the similar structure as non-ionic surfactants [48]. Similarly, in 1968 Fontell et al. concluded the same in context to cubic phases into the ternary systems of amphiphiles, oils and water [49]. In 1979 Lindblom and co-workers reported the structure based lamellar bilayer cubic phase made up of monoglyceride–water systems [50]. Consecutively in 1980 Larsson et al., reported the possibility of cubic structures in biological systems along with the structural relationships between lamellar, cubic, and hexagonal phases in monoglyceride-water systems [28, 51]. In 1984 Hyde et al., reported the cubic structure consisting of a lipid bilayer forming an infinite periodic minimal surface of the gyroid type in the glycerol monooleate water system [10]. Parallelly in 1992 Landh and Larsson filed a patent entitled "method of preparing said particles and uses thereof" which they got in 1996 which consists the preparation of colloidal dispersions of non-lamellar lyotropic crystalline phases, termed as "cubosomes" [52, 53]. Gustafsson et al., 1996 investigated cubic lipid-water phase dispersed into submicron particles and in 1997 they have reported submicron particles of reversed lipid phases in water stabilized by a nonionic amphiphilic polymer [29, 30]. In 2001 Nakano et al., reported a new technique for the preparation of cubosomes based on dry film hydration which was made up of monoolein/ poloxamer along with an aqueous buffer [54, 55]. At the same time Spicer et al., developed some novel processes for producing cubic liquid crystalline nanoparticles with the concept of simple mixing of two water-like solutions using minimum energy and hydrotropic inclusion [34]. In 2002 Siekmann et al., investigated the the preparation of colloidal dispersions consisting of monoolein-rich monoglycerides with or without purified soya phospholipids, using monoglyceride/ phospholipid/ water cubic phase and Poloxamer 407 [56,57]. Later on, several researchers have put their remarkable effort to work on cubosomal drug delivery, which is further discussed in this review [58, 59].

Merits/Demerits

Cubosomes comprise three-dimensional mesoporous internal structure, which is unique invention in biological sciences and nanotechnology [60]. Cubosomal drug delivery possesses great potential in drug delivery, some of them are discussed here [61, 62]. Cubosomes have some exciting benefits such as thermodynamically stable cubic structure, easily bio-degradable, bio-compatible, and bio-adhesive.[63, 64] Ability to encapsulate all the three types of molecules viz. hydrophobic, hydrophilic and amphiphilic represents its evergreen application in delivery of all types of bio-active compounds [65, 66]. This nanocarrier is an excellent candidate for targeted as well as sustained/controlled-release delivery of drugs and other molecules having size ranges from 10 to 500 nm [67, 68]. The bi-continuous cubic

liquid crystalline phase of cubosomes possess excellent stability profile, and are excellent solubilizers with high drug loading capacity compared to other conventional lipid and non-lipid carriers [14, 15]. They provide excellent protection to the sensitive drugs and other molecules from any kind of degradations e.g., enzymatic or *in-vivo*. Cubosomal delivery increases bioavailability of drug molecules from twenty to hundred times [32]. Cubosomes preparation method utilizes minimal polymers and organic solvents which makes them non-toxic, non-allergic and non-irritating [69]. Glyceryl monooleate (GMO) is mainly used in formulations because of its rigid structure, stability and integrity at varied temperatures [70]. The preparation of cubosomes is much easy and could be conveniently used in topical and mucosal delivery of drug molecules [71].

Despite the aforementioned merits, cubosomes have some limitations as well such as drug leakage issue along with extremely high viscosity. These are responsible for the difficult parenteral delivery of cubosomes [21]. Another issue is the solubilization of drugs in amphiphilic monoglyceride's hydrated bilayer of cubosomes. Thus, phase transformations could occur for hydrophilic and lipophilic drugs, which might affect the release characteristics of the drugs as well as physical stability of the matrix. Irregular gelation and crystalline nature of the particles may further decrease shelf-life and desired therapeutic effect, respectively. The production of cubosomes is quite difficult because of their complex phase behavior, irregular gelation and viscous properties, and it also needs high pressure induced drug degradation of the particles in processing [72, 73]. Despite several limitations, researchers have explored drug delivery possibilities with cubosomes. However, there is scarcity of drug delivery studies with cubosomes.

Structure of cubosomes

Cubosomes are essentially complex three-dimensional lipid-based lyotropic liquid crystals. They can take the form of cubic phase liquid crystals or cubic phase mesostructured, which are three-dimensional or multiphase structures with a singular, curved bi-continuous lipid bilayer separating two congruent water channel networks. (Fig. 1) [21]. Cubosomes exist as self-assemble, discrete, sub-micron or nano-structured dispersed particles in the size range of 10-500 nm diameters,

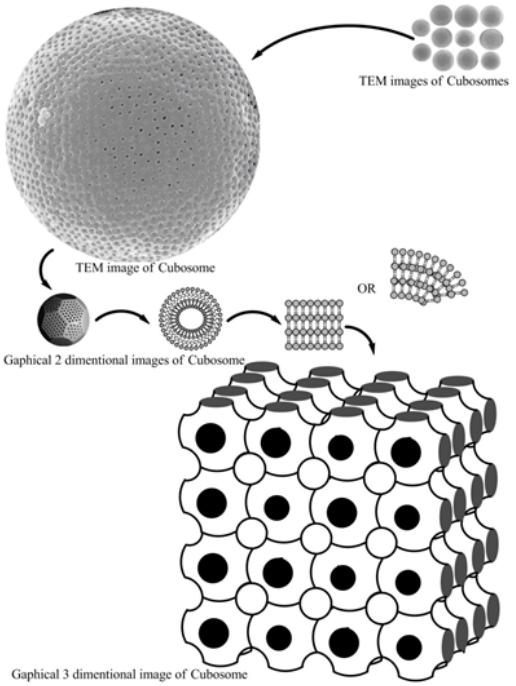

Fig. 1. Structure of Cubosomes

looks as dots, square shaped or slightly spherical shape [65, 66]. Cubosomes are found in the form of meso-structured cubic phase, may be observed in multiple phases which depends on its amphiphilic environmental condition. Typically, especially in nonionic surfactant systems, cubic phases are found in between lamellar and hexagonal liquid crystalline phases [74]. Cubosomes possess structural symmetric characterization in terms of degree of molecular orientation [75]. Based on X-ray crystallographic studies, there are three structures of cubosomes proposed by Luzzati et al., [18, 19] one is P*n*3*m* (D-surface/Diamond surface), second one is I*a*3*d* (G-surface/Gyroid surface), and third one is I*m*3*m* (P-surface/ Primitive surface), in terms of nodal surfaces (Fig. 2). When the bulk cubic phase is homogenized, a D-surface structure is created, which can then be converted into a P-surface by the addition of polymers [57, 76, 77]. 'Schwarz' discovered three types of surfaces in cubic phases based on the concept of curvatures, differential geometry and periodic minimal surfaces by soap film analogy [73, 78]. In monoolein-water system he observed that D-surface was formed at high water level, while G-surface was formed at lower levels and last one i.e. P-surface was formed after addition of caseins or amphiphilic block copolymer [79]. Based on

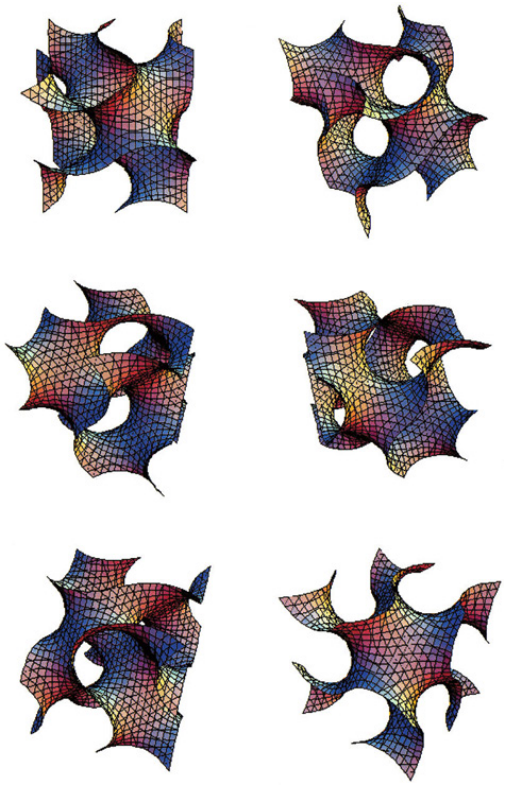

Fig. 2. Representation of perspectives of the bicontinuous cubic liquid crystalline unit cell [16]

the schoen gyroid (G) minimum surface, the Q230 cubic phase makes up the Ia3d crystallographic space group symmetry; Based on the Schwartz diamond (D) minimum surface, Q224 cubic phase makes up the Pn3m crystallographic space group symmetry; Based on the Schwartz primitive (P) minimum surface, Q229 cubic phase makes up the Im3m crystallographic space group symmetry [80, 81]. In addition, four more cubic phases as well as crystallographic space group symmetries have been reported. With two exceptions (Q223 and Q227), which were discrete or discontinuous micellar cubic phases, these include Pm3n (Q223), P4332 (Q212), Fd3m (Q227), and Fm3m (Q225), which may all be classified as bi-continuous cubic phases [20, 82–84]. Based on its three-dimensional hexagonal close-packed arrangement of inverse micelle's space group symmetry, one new lyotropic liquid crystalline phase, P63Immc, has recently been reported [85, 86]. Currently, the most often used liquid crystal forms in drug delivery are unsaturated monoglycerides or phytantriol (PT), which make up cubic mesophases [87]. The bulk phase typically consists of a transparent, viscous, semi-solid gel with properties comparable to cross-linked polymer hydrogels [16], whose high viscosity makes handling it challenging and restricts its use. Also, it can cause the irritation reaction to biological epithelia [88]. All the aforesaid properties of cubosomes makes them favorable in drug delivery applications, so, in this review the applications of cubosomes have been discussed.

Methods of preparation

Cubosomes have been prepared through various techniques by different researchers which are more or less similar to the nanoparticle preparation. Cubosomes possesses one special form i.e., precursor form which will be discussed in the following paragraphs along with the methods of preparation, characterization and evaluation of cubosomes which will give the concise information about this novel nanocarrier (Fig. 3).

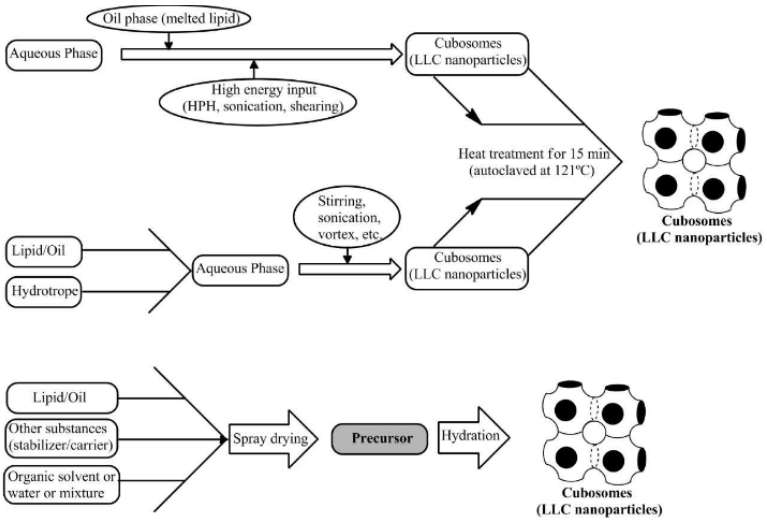

Fig. 3. Manufacturing techniques of cubosomes

Table 1. Drug delivery applications of Cubosomes

S. No.	Drug	Composition/Constituent	Use	Route
1.	Water monoolein- alcohol mixture	Ethanol	Vehicle for biologically active substances	-
2.	Triclosan	Glyceryl monooleate	Skin	*In vitro*
3.	Baicalin/KiOM-C	Glyceryl monooleate	Anti-viral, Anti-inflammatory	*In vitro* (small intestine adsorption)
4.	Tacrolimus	Glyceryl monooleate	Eczema (atopic dermatitis)	Intradermal
5.	Odorranalectin/streptavidin	Glyceryl monooleate	Pretargeted Immunotherapy	Intranasal
6.	Bromocriptine	Glyceryl monooleate	Anti- Parkinson	Intraperitoneal
7.	Earthworm fibrinolytic enzyme (protein)	Glyceryl monooleate/propylene glycol	Thrombosis, Fibrinolytic, Anti-Ischemia	Intra-tympanic
8.	Propofol	Soy phosphatidylcholine/glycerol dioleate	Sedative	Intravenous
9.	Somatostatin	Soy phosphatidylcholine/glycerol dioleate	Anti-cancer, Haemorrhage	Intravenous
10.	Clotrimazole	Glyceryl monooleate	Anti-fungal	Mucosal
11.	Cyclosporine A	GMO/F127/water	Immuno-suppressants, Anti-inflammatory	Ocular
12.	Dexamethasone	Glyceryl monooleate	Anti-inflammatory vitiligo	Ocular
13.	Flurbiprofen (FB)	-	Anti-inflammatory	Ophthalmic
14.	fluorometholone	glycerol monooleate	anti-inflammatory agent	Ophthalmic
15.	Brimonidine tartrate	glyceryl monooleate	Glaucoma	Ocular
16.	Cinnarizine	GMO/F127/water and PT/F127/water	Vertigo	Oral
17.	Amphotericin B	PT/F127/water	Anti-fungal	Oral
18.	Curcumin with piperine	Phytantriol	-	Oral
19.	Ibuprofen	Phytantriol	Anti-inflammatory	Oral
20.	Insulin	Glyceryl monooleate	Anti-diabetic	Oral
21.	Simvastatin	Glyceryl monooleate	Anti-hyper lipidimic	Oral
22.	Omapatrilat	Glyceryl monooleate		Oral
23.	Coenzyme Q10	Glyceryl monooleate	Antioxidant	Oral
24.	Paclitaxel	Soy phosphatidylcholine/glycerol dioleate	Anti-cancer	Oral, Intravenous
25.	Indomethacin	Glyceryl monooleate	Anti-inflammatory	Percutaneous
26.	5- fluorouracil	Pluronic F127 MO	Liver targeted drug delivery	Subcutaneous
27.	Polysaccharide	phytantriol	Immunization	Subcutaneous
28.	Silver Sulfadiazine, Monoolein	Glyceryl monooleate	Second degree Burn	Topical
29.	Herbal extracts (Poria cocos, Thuja orientalis, Espinosilla, Lycium chinense Mill, Coix lacryma-jobi, and Polygonum multiflorum Thunberg)	Glyceryl monooleate	Hair re-growth	Topical
30.	Diclofenac sodium	-	Anti-inflammatory	Transdermal
31.	capsaicin	Phytantriol- and glycerol monooleate	skin-targeted	Transdermal

Precursor forms

Cubic phase can be found in three different macroscopic forms: precursor, bulk gel, and particle dispersion. The precursor form is a solid/ liquid substance that transforms into a cubic phase in response to stimuli, such as contact with a liquid

[24, 25]. The cubosomal precursor's development results through production of *in-situ* cubosomes that avoids expensive and difficult high energy procedure and protects the thermo-sensitive molecules such as proteins [69]. There are mainly two types of cubosomal precursor forms: the first is liquid precursor and the other is powdered precursor [89].

The liquid phase precursor produces cubosomes after applying a strong driving force and could produce more stable cubosomes of desired size, as well. Cubosomal particles could be produced from hydrotropic dilution method via nucleation and growth mechanism, which is quite similar as crystallization and precipitation technique [90]. Mostly, liquid phase precursors are utilized in the preparation of mouth washes, hand washes, etc., in which cubosomes are prepared during rinsing, washing process respectively. This method does not require high shear, and it minimizes the degradation of active moiety in cubic crystals. Therefore, these precursors provide easy scale-up techniques for the cubosome preparation and also makes easy the handling of bulk solids as well as avoids the drug degradation which generally occurs through high energy process techniques [77, 91].

The powdered cubosomal precursors consists of some dehydrated surfactants along with coating polymer, prepared by spray drying technique. In this process, the liquid droplets in emulsion and dispersed solid particles in concentrated aqueous polymer solution, forms the encapsulated particles, followed by the spray drying technique, especially in case of foods, detergents and similar consumer goods. This process assists in preloading of the potent drug in cubosomes before drying, which provides process and performance-based benefits and liquid phase cubosomal precursors. When the powdered precursors are reconstituted using water, average particle size (600 nm) cubosomes are formed, which could be conformed through characterization techniques like cryo-transmission electron microscopy (cryo-TEM), light scattering technique etc. [92].

Preparation technique

The complex process of cubosomes preparation has been attempted by several groups in different ways. The usual methods includes the use of heat or sonication or homogenization techniques to develop cubosomes. The use of homogenization techniques is observed to be most convenient. The advantages and disadvantages of mostly followed techniques are mentioned in Table 2.

Top-down and Bottom-up technique

The top down and bottom up method-based preparation needs three major constitutes viz. aqueous phase (water or buffer), lipid phase and steric stabilizer [93–95]. Top-down technique needs viscous lipid or bulk cubic phase dispersed into an aqueous phase by sonication, which creates high energy, high-pressure homogenization and shearing [51]. The bottom-up approach involves dilution of a single-phase solution into a two-phase regime of cubosomes co-existing with additional water phase, which need less energy input due to the presence of hydrotrope like chloroform, ethanol, etc. which prevents lyotropic liquid crystals formation [61, 96].

High-pressure Homogenization

High-pressure homogenization is the most suitable technique for the preparation of cubosomes which provides low polydispersity index, high stability and long shelf-life. This technique consists of three step process viz. gel preparation, shearing and homogenization [73, 97]. Gel preparation includes dissolution of lipids and surfactants in suitable organic solvents followed by mixing and evaporation. Shearing involves formation of micro dispersion using gel and aqueous phase. Homogenization includes dispersion using high pressure homogenizer at

Table 2. Promises and Challenges in Cubosome's formulation techniques

S. No.	Name of Technique	Promises	Challenges
1.	Top-down approach	Less Aggregation, longer stability up to one year	Needs High-energy inputs for dispersion of the aggregates into Cubosomal nanoparticles
2.	Bottom-up approach	Needs less energy inputs, safer with temperature-sensitive substances	Suitable for thermo-sensitive substances only, less stabile formulations
3.	Solvent evaporation method	Smaller sized Cubosomes and higher physical stability	High polydispersity index due to large-scale mixing of ethanol and water
4.	Spray-drying method	Highly versatile, economic, and scalable technique, also suitable for drying labile products, viz. proteins, vaccines and other biological products	Difficulty in immediate spray-drying of cubic phase (hydration of monoolein)

optimal thermal conditions [54, 56].

Probe ultra-sonication

Probe Ultra-sonication is utilized in case of small volume samples, even though 600 µl sample can be used with quick results. It involves preparation of gel with stabilizer, cubic phase generation with solvent equilibrium and finally ultra-sonication [97]. The variables involved in this process are amplitude and frequency, which have to be maintained to avoid overheating and phase transition problems [69].

Automated cubosome preparation

This process involves production of large amount of cubosomes using fully automated robotic systems with probe sonicator and 96 well plate with a capacity of 600 µl of solvent. The cubic phases partitioning could carry lipophilic, hydrophilic or amphiphilic molecules [98, 99].

Heat treatment

Using homogenization and heat treatment, non-cubic vesicles are converted into well-ordered cubic particles in the heat treatment technique, a disused cubosome production method. It creates some cubic phases with a narrow particle distribution and improved colloidal stability, as well as small particle size fractions that correlate to vesicles [76].

Spray drying

Development of dry powder precursor is needed for cubosome preparation to overcome less flexibility of liquid precursors. So that spray drying technique is utilized to prepare starch/ dextran encapsulated monoolein precursors. It has have reported that the payload/encapsulation was decreased due to high proportion of polymers such as 75% starch or 60% w/w dextran, so that its utilization is restricted for potent medicament, vitamins, flavors etc. [100, 101].

Miscellaneous methods

Some important methods for preparation of cubosomes have been discussed above. Apart from that some researchers tried beyond them. Emulsification technique have been tried in the preparation of cubosomes. It involves dilution of omapatrilat monoolein-ethanol mixture aqueous solution of using poloxamer-407 [102,103]. Solvent dilution technique has been also utilized because it needs less energy in submicron particles production [90, 104, 105].

Characterization

Visual evaluation, cross-polarized light microscopy, scanning electron microscopy, cryo-field emission scanning electron microscopy (Cryo-FESEM), cryo-transmission electron microscopy (cryo-TEM), dynamic light scattering, small angle X-ray scattering (SAXS), stability analyzer LUMiFuge®, differential scanning calorimetric analysis, etc. are all methods for characterizing cubosomes [106].

The morphology of colloidal particles including cubosomes are used to study through TEM and SEM, here we have mentioned TEM images of colloidal cubosome nano-particles in Fig. 4 [107]. Polarizing microscopy depends on optical birefringence phenomena of cubosome. Polarizing images of the hexagonal and layered liquid crystalline's anisotropic molecular arrangement showed fan-shaped/cone-shaped mosaic and striated/cross pattern shapes, respectively. The non-birefringence molecular arrangement of cubic liquid crystalline shows dark area in polarizing images, which indicates the liquid crystalline phase [108]. Gustafson and co-workers utilized Cryo-TEM technique, which provided the solution to several hurdles of ordinary electron microscopy in terms of three-dimensional structure of cubosomes and in detection of phase transition mechanism as well [36, 98, 99]. It is based on freeze and fracture of the sample, forwarded by molding of the fracture surface on a platinum-carbon grid and

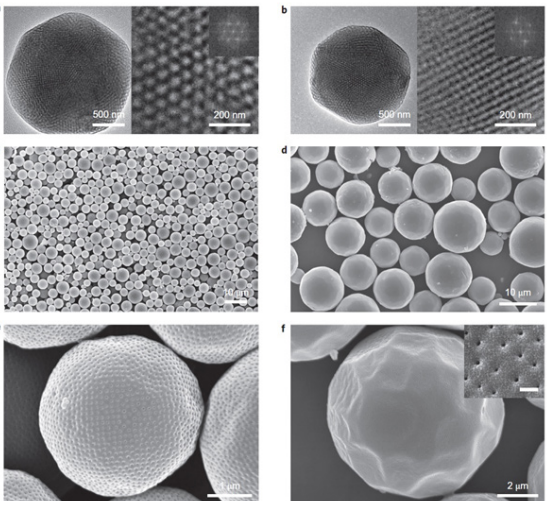

Fig. 4. Representative SEM and TEM images of cubosomes. a and b TEM, c-f SEM [107]

then illustration of inner structure of cubosomes via detection of the carbon film through electron microscopy [109–111], also used with combination of SAXs [112–114]. Some advanced and modified microscopic tools have been also used for characterization of cubosomes [115, 116] including confocal laser scanning fluorescence microscopy (CLSFM), freeze-fracture electron microscopy (FF-EM), two-photon microscopy, confocal microscopy, etc. [117, 118]

For the purpose of measuring the phase-transition temperature of a binary liquid crystalline system as well as looking into its stability, differential scanning colorimetry (DSC) determines the phase transition through thermal effects, typically accompanied by endothermic or exothermic energy changes [119–121].Whereas, The 1~10° small angle X-ray scattering is used due to low long-range order of liquid crystals, which is based on the Bragg's formula [122, 123].

$$S=\frac{2\sin\theta}{\lambda}=\frac{q}{2\pi}$$

Here *S* is scattering vector, θ is the scattering angle, λ is the wavelength of X-ray, and *q* is the scattering factors technique helps in determination of crystallographic structure using scattering vector ratio of each corresponding peak [61,96]. Moreover, some of the researchers have also used wide-angle X-ray scattering (WAXS) technique for the description of particle morphology and internal structure [124, 125]. X-ray diffraction measurements (XRD) technique records the diffraction patterns using several detectors and data can be collected at room temperature [126, 127]. The X-ray diffraction patterns derives structural information after identification of lipid phase's symmetry and calculates the unit cell dimension using Bragg's law.

$$n\lambda = 2d\sin\theta$$

Where λ is wavelength of the rays, θ is angle between incident rays and surface of the crystal and *d* is spacing between layers of atoms [128, 129]. Similarly powder X-ray diffraction (PXRD) can scan the images slowly and optimizes the formulation based on variables, process parameters, drug-free control, etc. The rotating crystal method along with monochromatic X-ray beam situated on surface of imaginary cones is used in which the sample is placed under vacuum, then after diffraction patterns are recorded via detector and crystallographic database is calculated through software (Jade 8.0, Materials Data Inc., USA) [125, 130, 131].

The particle size could be determined using dynamic light scattering (DLS) technique and the mean hydrodynamic diameter could be computed using the cumulant approach, in which polydispersion index (PDI) indicates the width of hydrodynamic diameter of distribution [55, 127] Particle size distributions of cubosomes could also be determined through DLS using the zeta potential or particle size analyzer instruments. Data could be calculated using average volume-weight-size and polydispersity index in triplicate mode usually [132, 133]. Quasi-elastic light scattering (QELS) is employed to measure the particle size of cubosomes using a Nanosizer apparatus (e.g. Nano-ZS90, MALVERN Zetasizer software) fitted with a helium-neon laser unit. The analysis is carried out manually for the measurement of parameters like temperature, scattering angle, refracting index, environment medium viscosity, hydrodynamic diameter, translational diffusion coefficient, etc. [115, 116].

The rheological parameters like shearing measurements, viscosity can be done using rheometer (TA instruments) with cone-plate geometry and Peltier plate (temperature control unit). The sol-gel phase transition temperature is measured at different variables to analyze the overall gelling profile [134]. Whereas, Langmuir-Blodgett Trough is used to investigate interfacial behavior (surface pressure) of adjuvants and lipid monolayers at ambient temperature [117, 118]. For measuring the resonance frequency of quartz crystals, the Quartz Crystal Microbalance (QCM-D) technique is used, which provides information about the mass of the adsorbed substance in a time-resolved way. Main acoustic constraints associated with it are resonant frequency and dissipation factor, through which we can estimate thickness and viscoelasticity of the adsorbed layer, permitting imminent stability and surface coverage of cubosomes [109, 135–137]. Neutron Reflectivity (NR) technique is utilized to measure the specular reflection of neutrons at an interface, which can give structure and composition of adsorbed layers in cubosomes and distinguish the allocation of polymers-lipids inside the adsorbed layer of cubic nanoparticles [138–142].

Beside these techniques, 13C NMR Spectroscopy is utilized to evaluate the accessibility of ions inside aqueous layers of the cubic nanoparticles through the values of chemical shift and ion accessibility

experiments [54]. For moisture determination combination of Karl-Fischer titration and thermal gravimetric analysis is employed for characterization of dry powder precursors of cubosomes [34].

The entrapment or encapsulation efficiency (EE %) of cubosomes could be determined by several techniques such as ultrafiltration [143], column separation [144], centrifugation [145], size exclusion chromatography, gel chromatography [146,147], membrane dialysis method [148,149], etc. The aforesaid steps are followed by analytical methods such as HPLC, UV-Visible Spectrophotometry, etc. [150]. The encapsulation/ entrapment efficiency (EE) could be calculated by the following formulas [132, 133, 143].

$$EE = \frac{\text{Total Drug} - \text{Free Drug}}{\text{Total Drug}} \times 100$$

$$EE = \frac{\text{Encapsulated/Entrapped Drug}}{\text{Total Drug}} \times 100$$

Drug loading capacity (DLC)is determined by dialysis technique using dialysis tubing cellulose membrane ultrafiltration and dialysis [95,124,126], followed by UV−Visible spectroscopy. The drug loading capacity (%DLC) could be calculated by the following formulas [132, 133].

$$DLC = \frac{\text{Amount of drug in cubosomes}}{\text{Cubosomes weight}} \times 100$$

For calculation of drug release profile, various mathematical equations have been followed by researchers such as Higuchi-Connor's, Hixson–Crowell, Fick diffusion, first-order, Weibull, zero-order equations, etc. [148]. Drug release profile of cubosomes is evaluated by dialysis method using a dialysis tubing cellulose membrane and then HPLC method) or UV−Visible spectroscopy2or equilibrium dialysis method (MWCO membrane) [151, 152] in optimum conditions. Due to the hydrophobic drug's affinity for the cubosomal hydrophobic domain, the release of hydrophobic drugs from cubosomes is found to be rather challenging, making the evaluation of cubosomes' *in vitro* drug release a laborious process [153, 154]. The factors which might affect drug release profile of cubosomes are solubility and partition/ diffusion coefficient of drug; geometry, pore size and interface curvature of cubosomes; conditions of release media like temperature, pH and ionic strength [155]. The *in-vitro* permeation/ penetration studies of cubosomes is carried out by some researchers using dorsal hairless skins of mice (SKH type) or stillborn piglet skin in a diffusion cell assembly under PBS at body temperature. The test samples are evaluated by fluorescence spectroscopy, confocal scanning laser microscopy (CSLM) or WSE using HPLC [156, 157].

The stability of cubosomes could be evaluated in terms of stress testing of physical stability as mentioned in Chinese Pharmacopoeia 2010 (part 2, Appendix XIX C), in which cubosomal samples are placed in drug stability testing chamber under high temperature (60 °C) and strong light (4,500±500 lx) and morphology are checked in several durations [148, 158]. The stability of cubosomes in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) based media is evaluated using HPLC method [159,160]. Another researcher put their efforts and evaluated the stability after placing the sample in tightly closed umber colored container at refrigerated temperature (3-4 °C) for 3 months and then carried out mean particle size analysis and entrapment efficiency (%) measurements in triplicate mode [161]. Elnaggar et al., 2015 reported the short-term stability testing of cubosomal dispersions at room temperature, dark conditions and 3 months, subjected for PS, PDI, ZP, and %EE in triplicates [143]. Chong et al., 2014 reported the stability evaluation of colloidal systems (cubosomes and hexosomes) using Pluronic F127 followed by stability assay with 384-well plates and multimode microplate reader [61,162]. Serum Stability tests were also used to evaluate cubosome stability in biological media, because their diffusion in serum may lead to protein adsorption and alter the structure or disintegration. The parameters to be examined are size of RF and RFPεL using DLS method or nanoparticle tracking analysis (NTA) [163, 164]. Cytotoxicity Assays estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Cell viability tests), so that in case of cubosomes this method is mostly employed. The protocol for MTT assay is followed and statistical analysis is applied to evaluate relevant toxicity of cubosomes [145, 165].

Biomedical applications of cubosomes

Cubosomes are attractive for drug delivery due to their ideal characteristics such as nano-scale pore size, solubilizing efficiency, biodegradability, bio-adhesiveness, penetrability, bio-compatibility, etc. This nanocarrier have been proved as a potential carrier in drug delivery system for ocular,

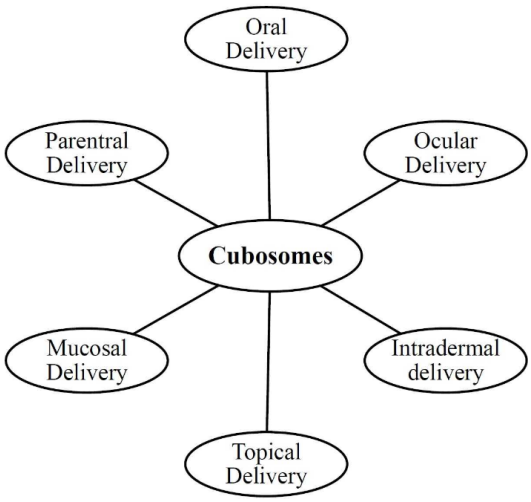

Fig. 5. Versatile applications of cubosomes as evidenced through different administration routes

dermal, intradermal, mucosal, intranasal, oral, percutaneous, intraperitoneal, intratympanic, intravenous routes of administration, etc. because they have high tolerance against environmental stresses and provides controlled release of drugs (Fig. 5) and also reported with cell membranes and blood components [166].

Cubosomes in oral drug delivery

Cubosomes possess promising advantages in oral drug delivery for drugs/compounds having poor solubility/absorption because of their self-emulsifying liquid crystalline nanoparticulate technology as well as sustained release of drug molecules [167, 168]. Yang et al., 2012 prepared cubosomes of Amphotericin-B (AmB) using the SolEmuls technology and enhanced the oral bioavailability (approx. 285%) of AmB via successful encapsulation into cubosomes [159]. Similarly, Yang et al., 2011 encapsulated AmB in reproducible cubosome based oral formulation using phytantriol (PYT), which is an alcohol (polyol) widely used in cosmetics and personal care products [132]. Tu et al., 2014 encapsulated curcumin and piperine into the cubosome to improve their oral bioavailability and tissue distribution significantly [144]. Boyd et al., 2007 reported the improved oral bioavailability of cinnarizine, a poor aqueous-soluble drug, entrapped in various types of liquid crystalline cubic phases [112]. Chung et al. 2002 prepared glyceryl mono-oleate (GMO) based cubosomes of insulin for its hypoglycemic effect through oral administration [169]. Similarly, Simvastatin was encapsulated in GMO-based cubosomes for oral administration and found the relative bioavailability of 241% with sustained release effect over 12 hrs. [154]. Cubosomes were also reported as sustained/ controlled released carrier for rifampicin. Alternatively, cubosomes could encapsulate large proteins for local activity in GIT with controlled release and targeting functionalities as well. 5-fluorouracil (5-FU) loaded cubosomes have been significantly utilized for liver targeting in treatment of liver cancer [161].

Cubosomes in mucosal drug delivery

Promising features like explicit morphology, bio-adhesiveness, particle size, and bio-compatibility with tissues and cells propound the cubic nanoparticles as a noble candidate for mucosal drug delivery [170] like ophthalmic [171], buccal, vaginal and nasal mucosa. Propantheline bromide and oxybutynin hydrochloride were delivered vaginally through the GMO-based gel with sustained release behavior throughout an 18-hr *in vitro* period [153]. Han et al., 2010 prepared ophthalmic delivery of cubosome mediated Flurbiprofen to reduce ocular irritancy and improve bioavailability significantly [136]. Gan et al., 2010 reported cubosomal dexamethasone ocular treatment with enhanced apparent permeability and bioavailability [172]. Wu et al., 2011 prepared small odorranalectin-bearing cubosomes, administered via nasal to brain route of S14G-HN for the treatment of Alzheimer's disease with enhanced therapeutic effects [133].

Cubosomes in topical applications

Cubosomes have shown excellent potential as an appropriate candidate for topical delivery of drugs and other macromolecules due to their properties like sustained release characteristics, bio-adhesiveness, solubility, protection ability from physical/enzymatic degradation, nontoxic nature, permeation enhancer, etc. Bender et al. 2008 reported the incorporation of sulphorhodamine-B in GMO-based and PT-based cubic mesophases for applications on human skin *in vitro* and have found better penetration and skin absorption. GMO–water was used for topical applications of d-aminolevulinic acid and showed fast penetration as compared to basic ointment [173, 174]. Cys-A was incorporated into GMO-based cubosome significantly for its transdermal delivery [175, 176]. Carbomer-Indomethacin were incorporated into cubosomes and applied topically in UVB-

induced erythema [177]. Helledi and Schubert reported 6-fold enhanced penetration of acyclovir via cubosomes as compared to the marketed product [178]. Morsi et al., 2013 prepared silver sulfadiazine loaded cubosome dispersions for the treatment of deep second degree burn and compared it with the marketed product (Dermazin), which resulted in an excellent healing preparation with least side effects and better patient compliance [179]. Cubosomes have been reported for topical delivery of small molecules like fluconazole, cyclovir, paeonol, δ-aminolevulinic acid, sulphorhodamine B, calcein, diclofenac salts, etc. [180–182], acetazolamide-loaded cubosomes occular delivery for the treatment of glaucoma [183], brimonidine tartrate, fluoromethane loaded cubosomes to improve ocular bioavailability for the treatment of glaucoma [184, 185].

Cubosomes in injectable/parenteral drug delivery

Cubosome is an attractive carrier for drug delivery, particularly because of its unique behavior and it has been found suitable for parenteral route as well. Cervin et al. reported the somatostatin loaded cubosomes, which were injected through intravenous route in rats and the outcome was found 6-fold significant [142]. Cubosomes also increases payloads of peptides, proteins and other small molecules, which make it as an ideal carrier for parenteral route [24, 186]. Apart from these, several reports show the hemolytic nature of monoglycerides and GMO. Therefore, parenteral administration of cubosomes got restricted [77].

Miscellaneous biomedical applications of cubosomes

Beyond the aforesaid applications of cubosomes, there are several reports which exhibit some more applications but with scarcity of significances (Table 1). Cubosomes are used in personal care and consumer products, i.e., L'Oréal utilizes the cubosomes as o/w emulsion stabilizer and pollutant absorbent in some cosmetic products [33, 88, 187]. Alpha lipoic acid (ALA) have been used to treat photo-damaged skin through poloxamer (P407) gel based cubosomal nanoparticulate system, which results in improved skin color and texture with no adverse effects like irritation, peeling etc. [188]. Periodontal drug delivery is another interesting application of cubic phase gel for delivery of antibiotics injected into the periodontal pocket [189]. Cubosomes of GMO/phytantriol were prepared using vitamin-E for hydrophilic compounds like Allura Red and FITC-dextran were found with increased therapeutic effect [42]. Cubosomes with fluorescein probe and quercetin have been significantly exploited for anti-cancer therapy [190]. Moreover Gaetano et al., 2011 investigated cubosome mediated delivery of ruthenium-based molecule (DOPURu) for antineoplastic action [163]. Rizwan et al. reported the cubosomes as a potential and effective sustained release delivery carrier of vaccine (model antigen Ova and peptide/protein-based) using imiquimod and monophosphoryl lipid [114]. Recently, cubosomes have been used for siRNA/gene delivery in the form of "PEGylated

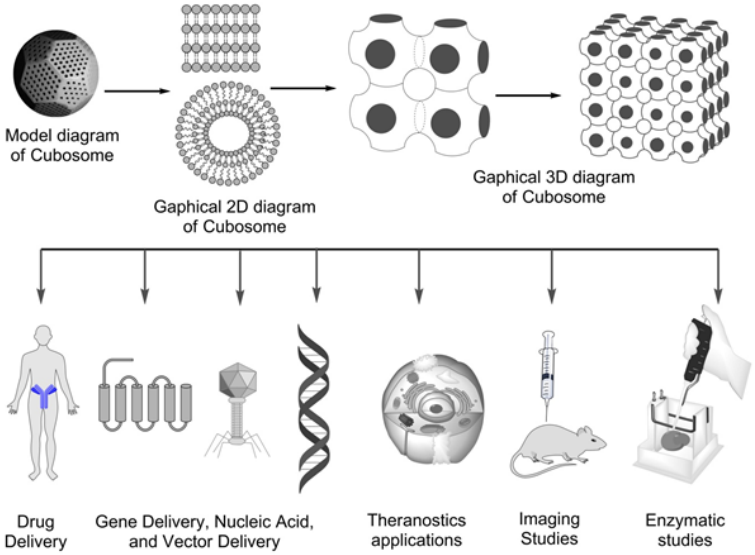

Fig. 6. Graphical Abstract

cuboplexe", which were sterically stabilized lipid-based nanoparticles and found superior than the traditional lipoplex systems, [191–193]. Metallo-cubosomes for RNA therapeutic delivery [194], hybrid cubosomes have been reported for the NIR-Induced photodynamic treatment of tumor lesions [131], enhance the ability of immunostimulants as promising adjuvant for vaccines [147], theranostic combinatorial approach for high efficacious and targeted delivery in cancer cells [195].

CONCLUSION AND FUTURE PROSPECTS

The review is mainly focused on the properties of cubosomes for the drug delivery applications till now including preparation, characterization and applications. Its bicontinuous lipid-based liquid-crystalline nature with three-dimensional mesoporous internal structure along with promising advantages like thermodynamic stability, bio-degradability, bio-compatibility, bio-adhesiveness, have opened new avenues for encapsulation and targeting of drugs to the sites like brain in sustained and controlled release mode. Another additional property which proves its immense potential is because of the nanoscopic size making it an equivalent carrier to other nanoplatforms. The preparation of cubosomes involves several methods such as top-down and bottom-up technique, High-Pressure Homogenization, Probe Ultra-sonication, Automated Cubosome Preparation, Heat treatment approach, Spray drying approach, etc. but top-down and bottom-up technique are mostly employed among them. The characterization and evaluation of cubosomes involves microscopy, dynamic light scattering, SAXS, stability, DSC analysis, entrapment efficiency, loading capacity, *in-vitro* drug release studies, etc. Cubosomes have been utilized as delivery carrier for various drugs, proteins, immunogenic substances, blood components, cosmetics, etc. through various routes such as ocular, dermal, intradermal, mucosal, intranasal, oral, percutaneous, intraperitoneal, intratympanic, intravenous routes of administration, etc. The cubosomes are relatively new in the area of nanotechnology and it have wide scope of research in drug delivery and formulations with industrial and commercial approach. The future prospects might attract and excite the researchers of both academic and industrial sectors for further investigations with its clinical applications and commercialization. In the commercial sphere, we might see increased exploration of cubosomes for delivering a variety of drugs, from traditional pharmaceuticals to biologics. They show potential in cosmetic and food industries as well. Imagine personalized skincare products tailored to your skin's specific needs or enhanced bioavailability of nutrients in functional foods. So, in both clinical studies and commercial applications, the future appears bright, with innovation and novel solutions on the horizon. Cubosome is an interesting candidate for researchers of interdisciplinary research in engineering, biology, chemistry, material science, medicine, cosmetics and consumer products, and it is crucial to provide a consistent understanding of these fascinating nanoparticles.

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CONFLICTS OF INTEREST

Authors have no conflict of interest to declare.

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