Lipid Cubosome Nanoparticles for Drug Delivery

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Introduction to nanomedicine

The field of nanomedicine has been ever increasing over the past thirty or so years [1], with a major focus placed on the development of new materials for uses in disease diagnosis and treatment [2–4]. These materials can be formed by a process known as self-assembly, where introduction of a solvent (organic or aqueous) triggers the thermodynamic formation of structures in solution with sizes normally between 5 – 1000 nm. There has been intense development of these nanoparticles for a breadth of applications, expanding beyond just simple drug-carriers. We now have access to smart nanomaterials for stimuli-responsive drug-release, can introduce tailored groups for targeting specific cell types, and have begun investigating nanoparticle movements through the body and their interactions with cells [2]. Despite the broad range of materials available, liposomes emerge as the most common nanoparticles undergoing clinical trials [5]. Lipids are naturally occurring or synthetic small molecules that consist of a hydrophilic head group and a hydrophobic tail. An equation known as the packing parameter predicts the morphology obtained upon assembly and typical products are spherical micelles or vesicles. Liposomes are an attractive nanoparticle due to their biocompatibility and tunability. Their hydrophobic core is amenable to encapsulation of hydrophobic small molecules and these nanoparticles can withstand encapsulation and complexation of nucleic acids. In 2020, the ability of liposomes to deliver mRNA revolutionized COVID-19 vaccination efforts and transformed the field of vaccine development. However, there are challenges associated with the use of liposomes, notably their inherent instability and difficulties controlling their size and shape [6]. An alternative lipid-based nanoparticle becoming increasingly popular is the lipid cubosome, which consist of a crystallizable lipid, commonly monoolein, that forms an ordered cubic array upon introduction of water [7–10].

Advantages of cubosomes

The crystallization of the lipid creates a highly regular structure of intersecting water channels surrounded by a lipid bilayer, which confers greater stability upon dilution and temperature fluctuations, likely encountered as part of therapeutic handling and administration [11–13]. The final assembly can be one of many bicontinuous cubic phases, known as diamond (Q_{II}^{D}), primitive (Q_{II}^{P}), or gyroid (Q_{II}^{G}), with the majority reported as diamond or primitive [7]. The presence of water channels throughout the structure lends itself to the encapsulation of amphiphilic drugs. The cubic structure increases the overall surface area of the particle, relative to a sphere, to allow for attachment of a greater number of targeting groups and encapsulation of cargo. The surface charge of the particle can be modified through the incorporation of ionizable lipids during formation steps. Finally, the unique shapes accessible from self-assembly can influence the biodistribution properties within the body [14], allowing for potential access to locations that are not possible with simpler, spherical liposomes.

Effect of stabilizer

The stabilizing compound used during cubosome formation has a critical influence on the self-assembled properties of the resulting nanoparticles [15]. The stabilizer is essential for directing the assembly of the internal cubic structure and sterically potentiates aggregation between individual nanoparticles. The stabilizer has been demonstrated to play a key role in long-term colloidal stability and maintenance of structural integrity. Most commonly, the copolymer PEG_m-*b*-PPG_n-*b*-PEG_m (poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol)), known as poloxamer 407 or pluronic F127, is used owing to its ability to impart stealth properties to the cubosome due to the PEG units extending into solution. However, anti-PEG antibodies have been discovered and thus researchers are searching for promising alternatives. Furthermore, while PEG has advantageous biocompatibility properties, it also suffers from a lack of functionality, and thus the exploration of new stabilizer compounds has gained wide attention. Of note, other polymers including PNIPAM (poly(N-isopropylacrylamide)) [16], PCL (polycaprolactone) [15], and POx-based (poly(2-oxazoline) [17] structures have been investigated, along with lipids or surfactants [18], and inorganic compounds [19]. Importantly, each new stabilizer employed will impact the properties of the resulting cubosomes including the phase, size, polydispersity, surface charge, colloidal and temperature stability, and biological activity [15]. Results thus far have been promising, with new stabilizers identified that impart more desirable physicochemical properties onto the nanoparticles, such as biocompatibility, stability, cell targeting, and more favourable drug-release profiles.

Drug-carrying capacity and characterization

Cubosomes are thought to possess increased drug-carrying capacity due to the high volume-fraction of lipid material for integration with hydrophobic cargo [7]. Furthermore, the exposure of a high surface area of the lipid membrane to aqueous water channels enables the encapsulation of amphiphilic compounds. Several types of cargoes have been loaded into cubosomes, ranging from cancer therapeutics, antibiotics, and larger macromolecules, with encapsulation efficiencies reported as upwards of 60% in most cases [7,20–23]. Complexation of molecules onto the surface of the cubosome is also possible by having complementary charges between the cubosome and the therapeutic. Drug-loading characterization of cubosomes is commonly done by dynamic light-scattering (DLS), high-performance liquid chromatography (HPLC), and small-angle X-Ray scattering (SAXS). DLS is useful for providing information about the size of the nanoparticle upon integration of a cargo to ensure that the structural integrity is maintained. HPLC is powerful for obtaining quantitative data of drug-encapsulation if one can be confident that all the free drug has been removed. Finally, SAXS has been useful for determining whether the drug is encapsulated within the cubic phase or on the surface of the cubosome, as modifications to the cubosome-associated Bragg peaks can be observed. This method can also give information about the stability of the cubosomes upon integration of the drug and upon drug release. Additional techniques for cubosome characterization include small-angle neutron scattering, reflectometry, quartz-crystal microbalance with dissipation monitoring, and nuclear magnetic resonance spectroscopy [24]. Despite the plethora of approaches available to assist with drug loading characterization, ultimately cryo-EM is the only method that can concretely determine that the cubic structure persists before and after cargo encapsulation.

Drug release and phase-transitions

Due to the high volume-fraction of the hydrophobic lipid environment, drug-release from cubosomes normally occurs through a burst mechanism [25]. It has been reported that approximately half of all the drug can be released in the first 1-6 hrs of analysis [7]. This is not ideal for *in vivo* applications as it is likely that the majority of cargo will be released prior to the cubosomes reaching their cellular targets. To overcome this, several groups have investigated the introduction of stimuli-responsive lipids into the cubosome formulation to temper early release [26]. This can be triggered in response to changes in pH, reactive oxygen species, and enzymes which may be present in the physiological environment, and has shown to be able to prevent burst release in a few cases [23,26–28]. The specific cubic mesophase can

influence drug loading and release rates. It is also known that microenvironmental changes can cause the particle to undergo a phase transition that can trigger premature cargo release. This emphasises the need for thorough characterization of the cubic phase under biologically mimicking conditions to determine how the phase will change under such circumstances. This will be essential for understanding the circulation and drug-delivery capabilities of the nanoparticle *in vivo*.

Cell uptake

An important consideration for nanoparticle drug-delivery is the uptake mechanism into cells as this will dictate subcellular cargo trafficking. It has been recently reported that cubosomes undergo micropinocytosis in the majority, as determined by inhibiting components of the endocytotic pathway [29]. This mode of entry is advantageous for drug-delivery because it is believed to bypass lysosomal fusion, a harsh acidic environment that can cause cargo degradation. Cubosomes also undergo a fusogenic mechanism of uptake, where they can directly fuse with and migrate through the cell membrane [29,30]. This again avoids unwanted interactions such as lysosomal degradation and may provide a direct route of cargo release within the cell interior for increased therapeutic efficacy. The uptake into cells will be dependent on cell type, the chemical composition of the nanoparticle, and the phase of the cubic structure.

Success *in vitro* and *in vivo*

Various levels of success have been reported for cubosome-mediated drug delivery *in vitro*, with few reports of demonstrable activity *in vivo*. Drug delivery and cytotoxicity will depend on cell type, inherent drug toxicity and activity, experimental conditions (*in vitro* and *in vivo*), and sample quality (dispersity, presence of contaminants). Several reviews exist covering the full extent of cubosome-mediated therapeutic-delivery [7,31,32], and thus this perspective will instead briefly focus on general trends observed upon nanoparticle drug-delivery. Several chemotherapeutics have been delivered to cells using cubosomes. In particular, paclitaxel, doxorubicin, and cis-platin have been highly investigated, but others have been loaded, characterized and evaluated for activity [31]. Commonly, drug-loaded cubosomes will exhibit a lower IC_{50} than the free drug. It has been reported that in 2D cell culture the free drug can outperform the nanoparticle-drug combination, but that in 3D models or *in vivo* studies the loaded cubosomes produce higher killing efficacy than the free drug. This is likely due to favourable properties of nanoparticle circulation, reducing non-specific distribution that occurs for the free drug to improve accumulation in the tumor. Delivery of antibacterial agents has also been investigated [33,34].

Interestingly, cubsomes were found to be beneficial for targeting of intracellular infections caused by mycobacterium tuberculosis [35]. This again may be due to the fusogenic mechanism of cell uptake by cubosomes, allowing them to encounter intracellular bacteria without needing to break free from an endo-lysosome. These unique properties position cubosomes in an interesting space where their unique mechanism of uptake may unlock high activity for distinct applications.

Challenges and next steps

Lipid cubosomes clearly exhibit favourable properties for drug-delivery but are yet to undergo the scrutiny of clinical trials. Further investigations are needed to determine how these particles behave *in vivo.* Information is needed about how the inevitable phase transitions that occur as the nanoparticles circulate throughout the body impact their physiological properties. Significant changes in morphology, dispersity, or integrity may contribute to erroneous and off-target effects that limit their clinical success. Furthermore, these transitions will contribute to changes in drug distribution and delivery, especially as the hierarchy of complexity increases as experiments move from *in vitro*, to mice and eventually humans. While liposomes pave the way for clinical success and, importantly, scale-up of nanoparticles, cubosomes need to meet similar requirements. The assembly pathway to drug-loaded cubosomes is relatively straightforward and can occur at reasonably high concentrations especially relative to more nuanced nanoparticle formulations. However, difficulties in accurate and largescale characterization can occur due to the necessity of using high-intensity and low throughput methods of analysis like cryo-EM and SAXS. A robust and straightforward method of generating low dispersity and morphologically pure cubosomes is required and may be possible through microfluidic methods [29]. Commonly, publications report the dispersity of cubosomes to be upwards of 0.2 (measured by DLS), with liposomes clearly observed as contaminants within the cryo-EM micrographs. This hinders our ability to determine the true capabilities of cubosomes as drug-delivery vehicles. Overall, cubosomes offer a versatile platform to generate smart nanoparticles that will find applications as therapeutics, but to date have not overcome common challenges experienced by the entire field of nanomedicine.

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