Production and characterization of cationic cubosomes

Raphael Dias de Castro

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Production and characterization of cationic cubosomes

Raphael Dias de Castro

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*I dedicate this work to everyone who
supported me throughout the process.
I hope to inspire other people to search for knowledge...*

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*“We are not to tell nature what she’s gotta be...
She’s always got better imagination than we have.”
(Richard Feynman)*

ABSTRACT

CASTRO, R. D.. **Production and characterization of cationic cubosomes**. 2018. 88 f. Master dissertation (Master student em Biochemical and Pharmaceutical Technology) – School of Pharmaceutical Sciences (FCF-USP), São Paulo – SP.

Cubossomos não nanopartículas formas pela fase de auto-associação de um lipídio em solução aquosa, sob certas condições experimentais. Nanopartículas tem sido amplamente utilizadas como carreadores de fármacos hidrofóbicos, atuado como um método de entrega controlada, como potencial de resolver problemas relacionados ao fármaco, como baixa solubilidade e instabilidade. Esses benefícios estão especialmente relacionados com a estrutura interna cristalina dessas partículas, em particular, seu grande volume hidrofóbico. A produção de cubossomos baseia-se na auto-associação de lipídios em excesso de água, auxiliada pela presença de um polímero como estabilizador. As propriedades físico-químicas dessas partículas podem ser caracterizadas principalmente por técnicas de espalhamento, nomeadamente espalhamento de raios-X a baixos ângulos (SAXS), espalhamento de luz dinâmico (DLS), microscopia eletrônica de transmissão (TEM), criogênica ou por contrastação negativa, e potencial ζ (zeta). Este projeto tem como objetivo investigar a produção de cubossomos na ausência e presença de líquidos iônicos funcionalizados com cadeias alquílicas, objetivando a produção de nanopartículas catiônicas compatíveis com uso em terapia gênica, como no caso de transfecção de DNA. Cubossomos foram preparados com dois sistemas diferentes: monoleína e phytantriol estabilizados com Pluronic F127 em excesso de água ou tampão. O líquido iônico $[C_{14}mim][Cl]$ e o surfactante catiônico TTAB foram adicionados a essas formulações e foi verificado que as soluções finais eram compostas por cubossomos catiônicos. Partículas carregadas positivamente com tamanhos entre 300 nm e 350 nm foram obtidas e caracterizadas.

Key-words: Cubossomos, nanopartículas, entrega controlada, líquido iônico.

ABSTRACT

CASTRO, R. D.. **Production and characterization of cationic cubosomes.** 2018. 88 f. Master dissertation (Master student em Biochemical and Pharmaceutical Technology) – School of Pharmaceutical Sciences (FCF-USP), São Paulo – SP.

Cubosomes are nanostructures formed by the self-assembly of a lipid in an aqueous solution, under certain experimental circumstances. Nanoparticles have been widely used as carriers of hydrophobic drugs, acting as a drug delivery method, with the potential to solve problems related to the drug, such as low solubility and instability. These facts are specially related to the cubic crystalline structure of these particles, in particular their large usable hydrophobic volume. The production of cubosomes relies on the self-assembly of lipids in excess of water, aided by the presence of a polymer acting as an emulsifier. The physicochemical properties of such particles can be characterized mainly by scattering techniques, namely small angle x-ray scattering (SAXS), dynamic light scattering (DLS), conventional and cryogenic transmission electron microscopy (TEM) and ζ (zeta) potential. This project aims the investigation of cubosomes production in the absence and presence of ionic liquids functionalized with alkyl chains, in order to produce cationic nanoparticles suitable for gene therapy, as in the case of DNA transfection. Cubosomes were prepared with two different lipid/polymer/water systems: phytantriol or monoolein stabilized with Puronic F127 in excess of water or buffer. Ionic liquid $[C_{14}mim][Cl]$ and cationic surfactant TTAB was added to these formulations and it was verified that the final dispersions contained cationic cubosomes. Particles positively charged with sizes between 300 nm and 350 nm and cubic crystalline internal structure were obtained and characterized.

Key-words: Cubosomes, Nanoparticles, Drug delivery, Ionic Liquid.

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LIST OF ABBREVIATIONS AND ACRONYMS

β -XP The glycolipid 1-O-phytanyl- β -d-xyloside

C_{14} [$C_{14}mim$][Cl]

BU Bottom-up

DLS dynamic light scattering

DOPE dioleoylphosphatidylethanolamine

F127 Pluronic F127

GME 1-glycerol monooleyl ether

GMO glyceryl monooleate

IL Ionic liquids

IPMS *infinite periodic minimal surfaces*

LLC lyotropic liquid crystals

LNLS Laboratório Nacional de Luz Síncrotron

MLO Monolinolein

PDI polydispersity index

PEG(660)-MO PEGylated monoolein

PHY phytantriol

SAXS small angle x-ray scattering

SLN Solid lipid nanoparticles

TD top-down

TEM transmission electron microscopy

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INTRODUCTION

1.1 Motivation

Nanostructures have been used as delivery systems for carrying drugs in order to solve problems related to hydrophobicity, including low drug solubility, permeability, instability (like aggregation) and high toxicity (PACHIONI-VASCONCELOS *et al.*, 2016; SAHOO; PARVEEN; PANDA, 2007; AWASTHI *et al.*, 2016). In the last 30 years, drug-development researches concluded that transport is a key point for this field. Such fact justifies the high number of delivery systems that have been developed (DRUMMOND; FONG, 1999) in the last few decades.

The ideal concentration of a drug at its target is a key feature to be achieved in the development of a drug delivery system. If the drug concentration is above the optimum value, it may cause intoxication, whereas when below, the drug might be inefficient (CAI *et al.*, 2017). Considering *in vivo* situations, the actual concentration can vary abruptly to extremes. In addition to that, aggregation caused by poor solubility can also be related to limited efficiency of most drugs (SAHOO; MISRA; PARVEEN, 2017). Biological barriers are responsible for premature drug liberation or degradation, limiting its action on target cells or tissues. Oral, parenteral and intravenous administration offer several obstacles for the drug to achieve its target (JURJ *et al.*, 2017, 2017). In this scenario, the use of nanocarriers with the ability to diffuse through biological

barriers, allowing the drug to reach its target tissue, presents itself as a suitable solution to the points aforementioned (MENG *et al.*, 2018; AWASTHI *et al.*, 2016). The diffusion behavior of such particles is influenced by several factors such as drug solubility and molecular geometry (AKHTER *et al.*, 2018). Combining these previous facts with the interaction between the carrier and the drug — namely the particle porosity, related to the drug release — a scenario of active investigation is laid out. All these aspects make nanocarriers a promising technology to improve therapeutic efficiency of drugs (RADAIC *et al.*, 2016).

Nanocarriers include a vast range of systems such as lipidic complexes (like liposomes, solid lipid nanoparticles, cubosomes, among others), polymeric micelles, polymersomes, dendrimers and so on (PACHIONI-VASCONCELOS *et al.*, 2016). The choice of a particular nanocarrier system must consider the drug physicochemical characteristics. In the case of a protein drug, for instance, parameters such as pH and thermal stability must be taken into account. On top of that, each system chosen to delivery some specific drug has its advantages and disadvantages (WAKASKAR, 2018; BLANCO; SHEN; FERRARI, 2015). A few examples might be considered. Solid lipid nanoparticles (SLN), for instance, are biocompatible and biodegradable, having high melting point, remaining in solid phase in temperatures close to 37 °C. However, their production often involve the use of organic solvents or high pressure homogenization, and their loading efficiency is limited (LUO *et al.*, 2015). Liposomes are spherical structures that could form a single or multiple stacked bilayers (also known as Multilamellar Vesicles, or MLVs), allowing these particles to carry hydrophobic or hydrophilic drugs. On the other hand, during the production process, liposomes can become unstable, which could cause premature release or aggregation.

A different kind of nanoparticle rises as a solution to some of these issues. Cubosomes are lipidic nanoparticles with cubic inner crystalline structure formed by the self-assembly of an amphiphilic molecule in excess of water and in the presence of a stabilizer (like a non-ionic polymer) (KARAMI; HAMIDI, 2016). Noteworthy, it is quite often the need to use an external mechanical energy to create the dispersion (PAN *et al.*, 2013). Applications for these nanoparticles have been intensely studied in the last few years in particular their ability to

incorporate hydrophobic and hydrophilic drugs, consequently providing a reasonable method for controlled drug delivery (ANSELMO; MITRAGOTRI, 2017). In particular, cubosomes offer large hydrophobic volume, due to its peculiar cubic structure (DRUMMOND; FONG, 1999; SPICER *et al.*, 2001). These particles are also highly thermodynamically stable and have well established production protocols (GARG; SARAF; SARAF, 2007).

Having all the attributes aforementioned, cubosomes are presented as a remarkable drug delivery system that could be used for specific applications, such as a DNA carrier intended for gene therapy (CORTESI *et al.*, 2014). However, since DNA is an anionic molecule, the interaction between cubosomes interfaces and DNA could be slightly improved by some modification on the particles like the addition of a cationic molecule. Such structural change on the nanoparticle surface could improve the binding of DNA into the nanoparticle. In that context, cationic surfactants may provide a reasonable solution to that specific application, tailoring cubosomes surface charge to load DNA strands also allowing other hydrophobic drugs intended to cancer therapy, to be loaded in the inner large hydrophobic area of such particles.

1.2 The State of the Art

1.2.1 Nanotechnology and nanomedicine

Nanotechnology can be defined as the science and engineering involved in the development, synthesis, characterization and application of materials and devices which smallest functional parts are in the range of hundreds of 1×10^{-9} m (SAHOO; PARVEEN; PANDA, 2007). Such systems configure a multidisciplinary field of investigation gathering engineering, physical, chemical and biological techniques in the same system. Richard Feynman, in his lecture “*There’s plenty of room in the bottom: An Invitation to Enter a New Field of Physics*”, presented in the annual meeting of the American Physical Society, in Dec. of 1959 predicted the possibility of manufacturing a material down to molecular or atomic levels, imagining the entire Encyclopedia Britannica written in a pin’s head, foreseeing the rising ability of examining and controlling matter in nanometric scale (SAHOO; PARVEEN; PANDA, 2007). It is then clear that nanotechnology provides the means for the application of several new materials, namely in

the development of drugs, water decontamination, exchange of information and communication and the production of lighter and durable materials (CHENG *et al.*, 2016).

Among the possibilities named before, nanomedicine has gained great attention in recent years, with the search for more efficient tools in diagnosis, prevention and treatment of several diseases (AWASTHI *et al.*, 2016). This rise occurred specially in the last two decades following the improvement in the production of materials in nanometric scale. The number of studies published in the field of nanomedicine have abruptly raised since the 1990's, as long as the investment in nanoscaled drugs production, which is predicted to surpass the US\$ 100 billion in the next few years (PACHIONI-VASCONCELOS *et al.*, 2016).

Since the 1970's (AWASTHI *et al.*, 2016), nanoparticles have been used successfully as drug delivery systems, with the delivery of vaccines as a pioneering application. Since then, several other applications have been developed with the use of nanoparticles in medicine, not only as nanocarriers, extending to cancer therapy, bioanalytical diagnose, imaging techniques and tissue engineering (RIZVI; SALEH, 2018; MUDSHINGE *et al.*, 2011).

1.2.2 Cationic nanoparticles and gene therapy

Gene therapy research has experienced a noticeable growth since early 2000's (GINN *et al.*, 2013) and has shown itself as an efficient method for treatment of serious diseases, such as cancer (IBRAHEEM; ELAISSARI; FESSI, 2014), by replacing an unhealthy for a healthy gene, therefore restoring normal cellular functions. When compared to other treatments, gene therapy can be potentially safer for its reduced toxicity and fewer side effects (PATIL; RHODES; BURGESS, 2005). However, it is important that this gene reaches the intracellular environment in order to be pharmaceutically functional. In that context, delivery systems for gene therapy have been investigated over the last years as carriers that will protect the gene from degradation, allowing passage through the cellular membrane targeting the nucleus.

Various carriers intended for gene delivery, also known as vectors, have been investigated, namely viral and nonviral (LI *et al.*, 2015). Somia and Verma (2000) proposed that the properties

of the ideal gene therapy vector, stating that they must be: of easy and sustained production, immunologically inert, selective to target specific cells, of ideal size, appropriate to promote site-specific integration and capable of transducing non-dividing cells. Viral vectors have been first used successfully for gene therapy. However, this method brings at least two practical drawbacks: too difficult and expensive to be produced and limited load capacity (IBRAHEEM; ELAISSARI; FESSI, 2014).

Nanoparticles comprehend the nonviral class of vectors used for gene delivery. Since DNA is anionic, these nanoparticles must be positively charged in order to interact with DNA, since this interaction is mainly electrostatic. In that way, DNA strands can bind to the surface of these charged particles (LONEZ; VANDENBRANDEN; RUYSSCHAERT, 2008; CORTESI *et al.*, 2014). Nanoparticles produced from cationic lipids have been reported as efficient DNA carriers (LI *et al.*, 2015; CORTESI *et al.*, 2014; LONEZ; VANDENBRANDEN; RUYSSCHAERT, 2008; IBRAHEEM; ELAISSARI; FESSI, 2014), specially liposomes. However, liposomes also have limited loading capacity considering both hydrophobic and hydrophilic portions of such particles.

1.2.3 Self-assembly and cubic crystalline phase

The self-assembly behavior of amphiphilic molecules, such as surfactants and lipids, has been investigated since the second half of the 20th century, in both theoretical and experimental fields (LUZZATI *et al.*, 1968; SCRIVEN, 1976; ISRAELACHVILI; MITCHELL; NINHAM, 1976; LARSSON, 1989; NAGARAJAN; RUCKENSTEIN, 1991; HYDE *et al.*, 1996; GARG; SARAF; SARAF, 2007; MARIANI; LUZZATI; DELACROIX, 1988). Amphiphiles are molecules that possess both hydrophobic and hydrophilic regions in the same molecule. In general, these systems have a so-called polar head group (hydrophilic) and an apolar (hydrophobic) region, generally formed by a hydrocarbon chain (NETO; SALINAS, 2005). The self-assembled phases of these molecules in aqueous environment is highly diverse, depending on the water content and temperature (LARSSON, 1989). These crystalline structures have been mathematically modeled (SCRIVEN, 1976; HYDE *et al.*, 1996; ANDERSSON *et al.*, 1999; LARSSON, 2000; GÓŹDŹ, 2015) in order to understand the disposition of hydrophobic and

hydrophilic regions inside the lyotropic phases.

1.2.3.1 A brief overview on the physics of self-assembled phases

The theory of self-assembly of amphiphilic molecules, relies on (ISRAELACHVILI; MITCHELL; NINHAM, 1977): (i) the interaction free energy of the systems' components, (ii) the geometry of the amphiphiles and (iii) a thermodynamic analysis of the system.

This phenomenon is closely related to the hydrophobic and hydrophilic effects that forces the system to self-assemble in the presence of water. In such case, it can be explained in terms of the entropy of the water and free energy of the molecules (NETO; SALINAS, 2005; ISRAELACHVILI; MITCHELL; NINHAM, 1976; ISRAELACHVILI; MITCHELL; NINHAM, 1977). Israelachvili, Mitchell and Ninham (1977) explain that, in thermodynamic equilibrium, the chemical potential of all molecules in aggregated systems will be the same, given by:

$$\mu_N^0 + \frac{k_B T}{N} \ln \left(\frac{X_N}{N} \right) = \mu_M^0 + \frac{k_B T}{M} \ln \left(\frac{X_M}{M} \right) = \text{const.} \quad N, M = 1, 2, 3, \dots \quad \text{and} \quad N \neq M \quad (1.1)$$

From Equation (1.1), it follows that:

$$X_N = N \left(\frac{X_M}{M} \right)^{\frac{N}{M}} \exp \left\{ \frac{N}{k_B T} (\mu_M^0 - \mu_N^0) \right\} \xrightarrow{M=1} X_N = N X_1^N \exp \left\{ \frac{N}{k_B T} (\mu_1^0 - \mu_N^0) \right\} \quad (1.2)$$

where X_N is the molar fraction of the molecules participating in the aggregate, N is the aggregation number of each aggregate ($N = 1$ corresponds to isolated molecules), k_B is Boltzmann's constant and T is temperature. Assuming that the dominant interactions between lipids are related to the hydrocarbon-water interface and polar heads region, contributions to the free energy per molecule μ_N^0 will be: (i) attractive — hydrophobic or from interfacial tension forces, the later represented by an interfacial free energy per unit area γ — and (ii) repulsive — mainly related to polar head electrostatic of steric repulsion and hydrocarbon side-chain repulsion. The contribution from

the hydrophobic interaction to the free energy μ_N^0 is γa , where a is the molecular area measured at the hydrocarbon-water interface, and the repulsive contribution for zwitterionic or ionic head-groups is proportional to a^{-1} . Thus, μ_N^0 can be expressed in terms of these contributions and a_0 , which is the optimal surface area per molecule related to the minimum of free energy per molecule (ISRAELACHVILI; MITCHELL; NINHAM, 1977):

$$\mu_N^0 = \gamma a + \frac{\gamma a_0^2}{a} = 2a_0\gamma + \frac{\gamma}{a}(a - a_0)^2 \quad (1.3)$$

Equation (1.3) implies that there is a variation of the free energy about its minimum, resembling an elastic potential. This mathematical fact also implies that lipids will aggregate in structures with surface areas about a_0 , leading entropy to favour the structure with the smallest aggregation number — Equation (1.2).

1.2.3.2 The cubic phase

Figure 1 – Phase diagrams of (a) monoolein-water and (b) phytantriol-water binary systems. Under 37 °C and for over hydrated systems, both lipids self-assemble into cubic crystalline phases, with $Pn3m$ symmetry. L_2 and L_α stand for inverse lamellar and direct lamellar phases respectively, and H_{II} represents the hexagonal phase. Adapted from Greaves *et al.* (2007).

Several lipids have amphiphilic properties and self-assemble into different phases in the presence of water. These systems have the property of associate in an organized structure possessing long range order and high degree of disorder at atomic scale (LARSSON, 1989).

A lyotropic liquid crystalline phase is formed depending on the weight ratio between the lipid and water, and also on the temperature. Detailed binary phase diagrams of the lipids glyceryl monooleate (GMO), also named monoolein, and phytantriol (PHY), also named *3,7,11,15-tetramethyl-1,2,3-hexadecanetriol*, are displayed in Fig. 1.

Figure 2 – Mathematical surfaces representing self-assembly cubic crystalline structures: (a) *Ia3d*, (b) *Pn3m* and (c) *Im3m*. Surfaces plotted with *Mathematica 10.3* using equations from Andersson *et al.* (1999) and Gózdź (2015)

The phase diagrams (Figure 1) of such molecules have several aggregation phases. However, in particular, two lyotropic phases cover the greater part of these diagrams: hexagonal (H_{II} at temperatures higher than 80 and 40°C, for GMO and PHY, respectively) and cubic (*Pn3m* and *Ia3d*). These phases are closely related to the molecule's geometry (NETO; SALINAS, 2005) — the wedge shape of the molecules and the way they pack together determine the structure (LARSSON, 1989).

In the late 1960's, Luzzati *et al.* (1968), among others (MARIANI; LUZZATI; DELACROIX, 1988; LUZZATI *et al.*, 1993; LUZZATI *et al.*, 1997), proposed that the cubic structure for organic lipidic systems is highly dependent on the amount of water. Later, Scriven (1976) has proposed geometric models to these systems. Figure 2 shows mathematical representations of *infinite periodic minimal surfaces* (IPMS) related to each cubic phase found in several lipids' phase diagrams. In the case of monoolein and phytantriol, for instance, the *Im3m* phase exists only in the presence of at least a third component in the system, such as a protein (MAZZONI *et al.*, 2016) or a polymer (LARSSON, 1989). From the experimental point of view, these systems have the same optical properties as solid crystals, for they also have Bragg diffraction peaks under

small X-ray scattering (NETO; SALINAS, 2005).

Since the beginning of the last decade, several applications have been proposed for the cubic phase of lipids (KARAMI; HAMIDI, 2016; SHAH; SADHALE; CHILUKURI, 2001; LARSSON, 1999). In particular, the cubic phase can be used to produce nanoporous materials, since its properties resemble biological membranes (NETO; SALINAS, 2005). The cubic phase of lipids like monoolein (Fig. 3a) and phytantriol (Fig. 3b) and water systems are of special interest due to their highly internal surface area (SPICER *et al.*, 2001) and broad range of the cubic crystalline phase in the phase diagram, as shown in Figure 1. Since the groundbreaking works of Luzzati and colleagues, several studies investigated these systems and their applications. Larsson (1989) was the first to use the word “cubosomes” as a reference to the particles in an aqueous dispersion of the cubic crystalline phase. A recent publication by Barriga, Holme and Stevens (2018) brings an extensive review on these particles, their production and properties, and also several of the most recent applications. These systems, which are the main focus of this project, will be detailed in the next sections.

1.2.4 Lipids and polymer of choice

Cubosomes have been investigated since the late 1990's (GUSTAFSSON *et al.*, 1996; LARSSON, 1999; NETO *et al.*, 1999) and early 2000's (SPICER *et al.*, 2001; BARAUSKAS *et al.*, 2005). Early works focused in protocols using monoolein as the base lipid and Pluronic F127 (F127) (also named Poloxamer 407) as steric stabilizer. The monoolein-water phase behavior was one of the most studied systems (QIU; CAFFREY, 2000).

The choice of lipids and polymers to be used on sample preparation followed the indication of several works that some combination of lipids and polymers should assemble into the desired cubic structures, varying the protocol for sample preparation and therefore some physicochemical aspects of the resultant particles. Some reviews have extensively reported the most used combinations, but in Table 1, adapted from Yaghmur and Glatter (2009), it is possible to verify that monoolein have been extensively used to form cubosomes, most of times with F127 as stabilizer.

Table 1 – Types of nanostructured dispersions formed by amphiphilic lipids or polymers - adapted from Yaghmur and Glatter (2009).

Type of nanostructured dispersion	Space group of the inner structure	Stabilizer	Lipid system	Reference
Cubosomes	Pn3m (Q^{224}) or Im3m (Q^{229})	F127	GMO/water	(LARSSON, 1989; GUSTAFSSON <i>et al.</i> , 1996; GUSTAFSSON <i>et al.</i> , 1997; LARSSON, 1999; LARSSON, 2000; TERASAKI, 2004; LARSSON, 2009; NETO <i>et al.</i> , 1999)
Cubosomes	Not reported	F127	GMO/ethanol/water	(SPICER <i>et al.</i> , 2001)
Cubosomes (coexisting with L_3 phase)	Not reported	PEG-based copolymers bearing lipid-mimetic anchors	GMO/glycerol/water	(RANGELOV; ALMGREN, 2005; ALMGREN; RANGELOV, 2006)
Cubosomes (coexisting with L_3 phase)	Not reported		GMO/sodium cholate/water	(GUSTAFSSON <i>et al.</i> , 1999)
Cubosomes	Im3m (Q^{229})	F127	GMO/oil/water	(NAKANO <i>et al.</i> , 2002)
Cubosomes	Pn3m (Q^{224}), Im3m (Q^{229})	F127	GMO/GME/water	(POPESCU <i>et al.</i> , 2007)
Cubosomes	Im3m (Q^{229})	D-alpha-tocopheryl PEO 1000 succinate (vitamin E TPGS)	PHY/water	(BARAUSKAS; JOHNSON; TIBERG, 2005)
Cubosomes	Pn3m (Q^{224})	F127	PHY/water and	(RIZWAN <i>et al.</i> , 2007; DONG <i>et al.</i> , 2006; BOYD <i>et al.</i> , 2007)

Several protocols have been used for GMO cubosomes preparation and most of them include the use of a non-ionic tri-block co-polymer, namely F127, and whose molecular structure is shown in Figure 3c. Dong *et al.* (2012) have investigated the influence of F127 in the structure of PHY and GMO cubosomes, stating that in the case of PHY cubosomes, F127 molecules were absorbed to the surface of the nanoparticles. However, in the case of GMO cubosomes, F127 molecules were integrated to cubic phase (DONG *et al.*, 2012). The molecular structures of GMO, PHY and F127 are shown in Figure 3.

GMO cubosomes have been extensively investigated regarding their applications. Karami and Hamidi (2016) and Esposito *et al.* (2016) have published two extensive and recent reviews on the therapeutic applications of these particles. These particles are in an advanced research stage, with several studies published containing results of *in vivo* and *in vitro* tests. Naming a few: Biffi *et al.* (2017) have shown that GMO cubosomes can be used for fluorescence optical imaging; Nasr, Ghorab and Abdelazem (2015) have verified that GMO cubosomes loaded with anticancer drug 5-fluorouracil are efficient in the treatment of liver cancer; Luo *et al.* (2015)

Figure 3 – Chemical structure for (a) GMO, (b) PHY and (c) F127: $a = 100$, $b = 65$.

showed that the cubic liquid crystalline nanoparticles could be a potentially nanocarrier in the delivery of gambogic acid for cancer therapy; and Ali *et al.* (2017) have shown that GMO cubosomes are efficient as spironolactone or nifedipine — model drugs used as treatment for hypertension — carriers. From the toxicity point of view, Falchi *et al.* (2015) have recently investigated the effects of the interaction of GMO cubosomes with HeLa cells, providing more information on cell behavior alterations, such as modifications on the cell lipid profile and lipid droplet accumulation, and thus concluding that GMO cubosomes stabilized Pluronic F127 or F108 have low toxicity against HeLa cells (FALCHI *et al.*, 2015).

PHY, when compared to GMO, has a very similar phase behavior as shown on Figure 1b. This lipid has been commonly used in cosmetics industry and can be considered as an alternative to GMO (RIZWAN *et al.*, 2007). Since the publication of the review by Yaghmur and Glatter (2009), several works have been published with results from the preparation and application of PHY cubosomes. Recently, Akbar *et al.* (2017) published an extensive review on the phase behavior of PHY in water and some therapeutic applications. Akhlaghi *et al.* (2016) have also investigated the influence of the preparation method in PHY cubosomes internal structure. In an alternative to F127, Azhari *et al.* (2016) have shown that the non-ionic surfactant Tween-80 (Polysorbate 80) can be used as stabilizer in the production of cubosomes, possibly enhancing the capability of these particles to get through the blood-brain-barrier.

1.2.4.1 An alternative to cationic lipids: ionic liquids

Ionic liquids (IL) are amphiphiles formed by an “ionic bond” between charged (and generally organic) molecules that have special chemical characteristics, namely the stability on liquid phase up to temperatures close to 100 °C (HE; ALEXANDRIDIS, 2015). Other features of IL have attracted the attention of recent researches, such as low vapor pressure, non-combustibility, high ionic conductivity and dissolution of several substances (SUSUMU *et al.*, 2006). Another important feature of IL is the possibility to self-assembly in several different structures (GREAVES *et al.*, 2007; ŁUCZAK *et al.*, 2008), resembling the behavior of surfactants, as reported by Mulet *et al.* (2010) where systems containing GMO and IL self-assembled into cubic structures under specific circumstances. Considering this fact, the study of the interaction between nanoparticles and ionic liquids opens the possibility of modifying structural and physicochemical properties of these particles, aiming to specific applications. Hayes, Warr and Atkin (2015) have published an extensive review on IL properties and the formation of nanostructures, even presenting some applications on nanomedicine stating that, although IL were once generally considered toxic, there are several medical applications under active investigation. For instance, Abbaszadegan *et al.* (2015) have shown that silver nanoparticles protected with imidazolium-based ionic liquid were efficient as antibacterial. In another work, Banerjee *et al.* (2018) presented an oral insuline formulation using an IL as delivery vehicle. Recently, Shamshina *et al.* (2018) have written a review on the role of IL in the pharmaceutical industry, pointing out the use of IL as solvents in the drug synthesis and as a component of drug delivery systems.

The IL *1-tetradecyl-3-methylimidazolium chloride*, also known as $[C_{14}mim][Cl]$ (C_{14}), is an imidazolium-based IL with 14 carbon atoms in its carbonic chain (Fig. 4) with properties similar to surfactants, being capable of aggregate into lyotropic liquid crystals (LLC) (ZHAO; GAO; ZHENG, 2010). Moreover, as reported in a review published by He and Alexandridis (2015), this class of IL can be used to functionalize the surface of nanoparticles.

Thus, in the present study we are evaluating the effect of a cationic IL, (*1-tetradecyl-3-methylimidazolium chloride*, also known as $[C_{14}mim][Cl]$ (C_{14})) on the inner structure and

Figure 4 – [C₁₄mim][Cl] chemical structure.

physicochemical stability of cubosomes composed by GMO or PHY at different molar ratios. To do so, we explored some of the most used biophysical tools to access the IL-Cubosomes interaction, like, Small angle X-ray Scattering, Dynamic Light Scattering, Cryogenic (and Conventional) Transmission electron Microscopy and ζ -potential. As it will be shown latter in the text, [C₁₄mim][Cl] (C₁₄) was able to distabilize the cubosomes at concentrations larger than 5% w/w.

1.3 Goals and objectives

1.3.1 General Goal

To study the structural influence of cubosomes in the absence and presence of an ionic liquid and produce cationic nanoparticles with internal cubic crystalline structure.

1.3.2 Specific Goals

A set of goals and objectives have been established in the original project:

- (i) Obtain a stable aqueous dispersion of cubic crystalline nanoparticles;
- (ii) Characterization of physical and chemical parameters of such particles in solution;
- (iii) Investigate the effect of the addition of ionic liquids to these structures.

MATERIALS AND METHODS

2.1 Materials

Materials used in this project are presented in Table 2. Ultra pure Milli-Q water was used in the preparation of all samples. Samples were prepared at the Biosystems Laboratory located at Institute of Physics (University of São Paulo - USP), using the equipments bellow:

- Precision Balance – BEL Engineering model M124A
- Ultrasonic bath Emasonic E30H - Elma Schmidbauer GmbH - Potency 240 W
- Heat Bath – Novatécnica Equipamentos para Laboratório – NL1225
- Digital Termo-Hygrometer model 7666-02-0-00 - Incoterm
- Rotary evaporator – Fisatom model 804 70-130 W 60 Hz
- Magnetic Stirrer – LGI Scientific – 0-2500 rpm 500 W
- Pipette series – LabmatePro – HTL Lab Solutions
- Solution stirrer AP56 – Phoenix Luferco – 3800 rpm

Table 2 – Materials used in sample preparations.

Product	Supplier	Purity	Density (g cm ⁻³)	Molecular weight (g mol ⁻¹)
Monoolein (1-Oleoyl- <i>rac</i> -glycerol - M7765)	Sigma-Aldrich®	≥ 99%	0.969	356.6
Pluronic® F127 (P2443)	Sigma-Aldrich®	—	1.095 at 25 °C	102.1
TTAB (Myristyltrimethylammonium bromide - T4762)	Sigma-Aldrich®	≥ 99%	1.133	336.4
[C ₁₄ mim][Cl]	Io-Li-Tec®	> 98%	—	314.9
Phytantriol	Avanti® Polar Lipids Inc.	> 98%	0.932 at 25 °C	330.5
Myverol 18-99K®	Kerry® Bio-Science Inc.	—	—	356.3

2.2 Sample preparation

Samples were prepared following two different methods, as described in the literature (SPICER *et al.*, 2001; AKHLAGHI *et al.*, 2016). Both methods are essentially based in the dispersion of lipid in excess of water, in the presence of F127 as stabilizer. However, they differ in the way that the mixture is prepared. The diagram of Figure 5 shows the steps for each method. The top-down (TD) method consists in the use of external mechanical energy to break the bulk crystalline phase in a high water content solution (LANDH; LARSSON, 1996). Bottom-up (BU) method relies on the use of a hydrotrope molecule, like ethanol (LYNCH; SPICER, 2004), to solubilize monoolein, avoiding the use of high external mechanical energy in order to break the bulk into a dispersion. The protocols are described below.

At the end of the process, all samples da similar visual aspect — milky-like and opaque, besides GMO(BU) samples, which had visible (naked eye) aggregates as compared to GMO(TD) samples.

2.2.1 Top-down (TD) method

The top-down method was used for the preparation of samples following slightly modified procedures described by Spicer *et al.* (2001). Briefly, 55 mg of a GMO/F127 mixture, in a mass ratio of 92% GMO to 8% F127 w/w, were melted at 60 °C until a clear solution was formed. Water was added up to a final volume of 5 ml, reaching the following ratios: 98% water, 1.8% GMO and 0.2% F127 (w/w). The final solution was homogenized on a ultrasonic temperature controlled bath at 60 °C for 60 minutes. This sample was labeled as GMO(TD) and submitted to further analysis.

Figure 5 – (a) Top-down protocol. (b) Bottom-up protocol. Adapted from Karami and Hamidi (2016).

2.2.2 Bottom-up (BU) method

Samples with the GMO-F127 system were prepared with the bottom-up method, using an adapted procedure from Spicer *et al.* (2001). Sample GMO(BU) preparation was performed with following these steps: 100 mg of a GMO and F127 mixture (both powder), in a ratio of 92% GMO to 8% F127 w/w, were put in a glass vial where ethanol and water were added (59 μ l and 17 μ l respectively) to form a lyotropic solution with cubic crystalline structure. Water was added to complete a 0.5 ml volume and sample was manually agitated, forming a cubic nanoparticles dispersion.

The PHY-F127 system was also used to prepare cubosomes with the BU protocol, following the procedures described by Akhlaghi *et al.* (2016). Briefly, 10 ml of ethanol were added to 100 mg of PHY to produce a clear translucent solution. Separately, as aqueous solution of F127 was produced by adding 25 mg of polymer to 22.5 ml of water. Both solutions were kept at 40 °C and were mixed at the same temperature. The final solution was magnetically stirred at 40 °C for 5 minutes and then the excess of ethanol and water was evaporated in a rotary evaporator (20 to 40 rpm) at 45 °C until reach a final volume of approximately 2.5 ml. Water was added to complete a final sample volume of 5 ml.

2.3 Characterization

2.3.1 Small angle X-ray scattering

Small angle X-ray scattering is a powerful technique to identify the crystallographic structure of the lyotropic liquid crystalline systems. If the system has any kind of periodic structure of any given direction, this technique is capable of generating diffraction patterns that can be used to determine the space group and lattice parameter of the liquid crystal. This feature is accomplished by measuring fluctuations in electron density in a material over the size range of 1 to 100 nm (LYNCH; SPICER, 2004). Another important feature is the ability of performing measurements of systems in solution, simulating the natural environmental conditions in which the liquid crystal dispersions would be used (RADAIC *et al.*, 2016)

Variations of important parameters such as temperature, pH and ionic strength might add new information about the system behavior. A typical setup for a SAXS experiment is shown in Fig. 6. The experimental principle of SAXS is based on the scattering of a monochromatic X-ray beam generated by a source (conventional or synchrotronic radiation) (BARBOSA *et al.*, 2013). The beam ($\vec{k}_i$) hits the sample and is scattered ($\vec{k}_o$) reaching the detector, while it also passes through the sample ($\vec{k}_i'$) going directly to the beam stopper, also known as direct beam.

Figure 6 – Typical SAXS setup. In general, conventional or synchrotron sources are followed by very similar systems that resemble this setup (BARBOSA *et al.*, 2013).

The scattering vector ($\vec{q}$) is thus defined as the difference between $\vec{k}_o$ and $\vec{k}_i'$ (Figure 7). SAXS output data will then relate X-ray scattering intensity with values of q , determined mathematically by simple geometric deduction (Eq. [2.1]) given that $k = 2\pi/\lambda$ and considering that the initial wavelength of the X-ray radiation is not altered after scattering ($\lambda_o = \lambda_i' = \lambda_i$ — see Fig. 7).

Figure 7 – Scattering vector geometry.

$$\vec{q} = \vec{k}_o - \vec{k}'_i \implies q = \frac{4\pi}{\lambda} \sin \theta \quad (2.1)$$

The main underlying concept of SAXS technique is the scattering of X-rays by electrons of the atoms in a crystal lattice. These electrons absorb the X-ray photons energy ($E = h\nu$; being h the Planck's constant and ν the radiation frequency) and new photons are emitted with such an intensity that is determined by the existence of constructive or destructive interference. This phenomenon is explained by the law of diffraction deduced by W. H. Bragg and W. L. Bragg in the beginning of the 20th century. Fig. 8a shows the interaction between X-ray radiation and a 2D crystal lattice and the Bragg “reflection” phenomenon, governed by Equations (2.2). Constructive interference will occur in the detector if both beams are in phase. This condition is satisfied when δ is equal to an integer multiple n of the radiation wavelength λ . Therefore, δ is the path difference between the two X-ray photons reflected by successive planes distant a length d (Fig. 8b). The distance between the planes is related to the lattice parameter a (the distance between two consecutive lattice points - Fig. 8b) by the expression of d in Eq. (2.2), where h , k and l are the Miller indices that specify the lattice plane where the scattering occurred.

$$2d \sin \theta = n\lambda \quad d = \frac{a}{\sqrt{h^2 + k^2 + l^2}} \quad (2.2)$$

In a crystal lattice, the SAXS scattering vector $\vec{q}$ matches with the reciprocal vector of the lattice. If constructive interference occurs, the lattice parameter a can be determined. The interaction between the X-ray beam and the crystallographic planes of the tri-dimensional structure, determined by intensity peaks in SAXS data, provides a relation between values of the scattering vector and the respective Miller indices of each allowed reflection. Therefore, the

Figure 8 – Diffraction theory: (a) Geometry of Bragg's diffraction. The path difference is given by $\delta = d \sin \theta$. (a) Simple cubic lattice unit cell. The colored area corresponds to one of the Bragg's planes.

lattice parameter can be determined by Eq. (2.3).

$$q = \frac{2\pi}{a} \sqrt{h^2 + k^2 + l^2} \quad (2.3)$$

SAXS data for crystalline structures typically present peaks that can be related to Bragg reflection planes. Figure 9 presents two examples of typical SAXS plots for a lyotropic cubic phase portraying various peaks, each one with Miller indices (Fig. 9a) or irrational numbers (Fig. 9b) assigned. These irrational numbers, calculated with $\sqrt{h^2 + k^2 + l^2}$, represent a constant proportional to the magnitude of the reciprocal vector of lattice — in this case, and in general, the first reflection does not have a discernible intensity peak. The interpretation of these numbers can lead to the identification of the space group related to the crystalline structure of the sample. In the case of cubosomes produced from a GMO-poloxamer system, the space groups that are likely to be found are $Pn3m$ or $Im3m$ (see Fig. 2b and 2c). Table 3 presents each space group with its respective allowed reflection planes Miller indices.

Table 3 – Miller indices correspondent to each cubic space group. Adapted from (GARTI, 2012).

$(h^2 + k^2 + l^2)^{1/2} =$	1	$\sqrt{2}$	$\sqrt{3}$	$\sqrt{4}$	$\sqrt{6}$	$\sqrt{7}$	$\sqrt{8}$	$\sqrt{9}$
Pn3m (<i>hkl</i>)	—	110	111	200	211	—	220	221
Im3m (<i>hkl</i>)	—	110	—	200	211	—	220	—
Ia3d (<i>hkl</i>)	—	—	—	—	211	—	220	—

Figure 9 – Typical SAXS charts with identified peaks. **(a)** Each peak is associated to Miller indices (hkl) related to reflection planes which **(b)** can be associated with a constant.

Given that each detected diffraction peak is related to a value of the scattering vector — each peak named as q_1, q_2, q_3 and so on — it is possible to calculate the ratios between the i^{th} peak (q_i) and q_1 one as follows, using Equation (2.3):

$$\left. \begin{aligned} q_i &= \frac{2\pi}{a} \sqrt{h_i^2 + k_i^2 + l_i^2} \\ q_1 &= \frac{2\pi}{a} \sqrt{h_1^2 + k_1^2 + l_1^2} \end{aligned} \right\} \Rightarrow \frac{q_i}{q_1} = \sqrt{\frac{h_i^2 + k_i^2 + l_i^2}{h_1^2 + k_1^2 + l_1^2}} \quad (2.4)$$

Now, with Equation (2.4), one can calculate the “spaces” between peaks. These spaces are therefore related to the ratios between the allowed Bragg reflections for each spacegroup, thus defining a method for the crystal lattice identification. In the case of $Im\bar{3}m$ and $Pn\bar{3}m$, the first two ratios are (see Table 3):

$$\begin{aligned} Im\bar{3}m &\rightarrow \frac{q_2}{q_1} = \sqrt{2} & \frac{q_3}{q_1} = \sqrt{3} \\ Pn\bar{3}m &\rightarrow \frac{q_2}{q_1} = \sqrt{\frac{3}{2}} & \frac{q_3}{q_1} = \sqrt{2} \end{aligned}$$

2.3.1.1 SAXS of GMO/F127 samples

SAXS experiments were performed at line SAXS1 of Laboratório Nacional de Luz Síncrotron (LNLS) at Campinas - SP. Beam wavelength of the synchrotron source was reported

to be 1.544 Å and the sample-detector distance was set to 908 mm. The acquisition system was calibrated with a silver behenate sample, using its diffraction pattern for calibration. Data acquisition time for each sample was set to 100 s. Data for GMO/F127 (BU and TD) had good overall quality.

2.3.1.2 SAXS of PHY/F127 samples

Samples prepared with PHY and F127 were submitted to SAXS analysis at the Crystallography Laboratory at the University of São Paulo in Nanostar equipment which has a conventional x-ray source with wavelength reported at 1.54 Å. Acquisition was programmed to be realized in 3 runs of 1 hour for each sample. The best data collection for each sample was chosen for analysis. The conventional x-ray source impacts directly the intensity of scattering data, when compared to a synchrotron source. However, data quality and its analysis have not been impacted.

2.3.1.3 SAXS analysis with computational tools

Indexing each peak as q_1 , q_2 and q_3 respectively — from the lowest to the highest value of q for the detected peaks — it was possible to use computational tools to analyze these peaks positions in SAXS data and their related parameters. For that purpose, a Python program (Fig. 10) was developed by the author to automatically calculate the lattice parameter for each sample. This program uses internal Python libraries such as `numpy` and `scipy`, and `fit2d`, a program developed by Dr. Andy Hammersley at European Synchrotron Radiation Facility (ESRF), to integrate synchrotron raw data.

It is important to mention that the calculations performed to obtain the results of lattice parameter depend on parameters related to a Gaussian adjust to each diffraction peak. Therefore, these results vary in function of these parameters.

2.3.2 Transmission electron microscopy

Complete characterization of amphiphile self-assembly structures cannot be achieved only through “indirect” techniques, such as light scattering (dynamic or static) or X-ray scat-

Figure 10 – Graphical interface of the program developed to analyze SAXS data.

tering (namely SAXS - Section 2.3.1). These indirect techniques can provide inner structural information by relaying the data analysis in previously determined models, but fail to provide detailed information about aggregate size and polydispersity, and existence of different structures in the same sample.

In order to achieve a full analysis of the self-assembly structure, direct imaging techniques can be associated with scattering experiments. Light microscopy could be used as a first attempt to perform direct observation of the sample, but most surfactant or lipid self-assembly structures are in the dimension range of a few nanometers, which makes transmission electron microscopy a more suitable technique (DANINO; BERNHEIM-GROSWASSER; TALMON, 2001), due to electrons wave properties. Conventional TEM is an important technique to verify the existence of particles in the dispersion and, at the same time, estimate their size and shape.

Direct observation of the internal morphology of self-assembly systems in aqueous solution, such as cubosomes, using a vacuum chambered TEM system implies that the sample must retain its inner structure stability during preparation and measurement. This feature is difficult to be achieved since samples must be dried, resulting in the complete removal of water and deformation or entire destruction of the structure (CUI *et al.*, 2007). In order to perform observations with a sample dispersed in an aqueous solution, an alternative method of fixation must be used. In that way, cryogenic fixation is best suited to dispersions in aqueous solutions.

This is performed by plunging the liquid dispersion into a suitable cryogen to convert the solvent (in most cases, water or buffer) into a solid-like, vitreous state (CUI *et al.*, 2007).

Figure 11 – Cryo-TEM example from a phytantriol cubosome. In this picture, it is possible to see the existence of a cubic particle with internal cubic crystalline structure.

2.3.3 Grid preparation for TEM and Cryo-EM

2.3.3.1 Negative stain EM

TEM imaging was realized at *Instituto de Biociências* (IB-USP) facility, using a Tecnai FEI G20 transmission electron microscope. Formvar coated copper grids with 200 mesh were used. Samples for imaging were prepared with low concentration solutions, ranging from 1 mg ml^{-1} to 2.5 mg ml^{-1} . Grids were prepared following these steps: deposition of 5 μl of each sample, followed by 5 μl of phosphotungstic acid, used as negative contrast; deposition of 5 μl of water to wash excess of acid (two times for each sample). A interval of 60 seconds was observed between each step. This imaging technique was used to verify the existence of particles in dispersion and to investigate aggregation. It is important to remark that, in order to characterize the internal morphology of the particles, cryogenic TEM is a more adequate technique. However, conventional TEM can provide important information regarding particle size and aggregation.

2.3.3.2 Cryo-EM

Cryo-EM measurements can bring details on the internal morphology of the system. Experiments were performed at LNNano – CNPEM in Campinas. The standard protocol for sample preparation is as follows: using a 300 mesh Lacey Carbon treated grid with a glow discharge of 15mA (for 10 seconds) in a controlled ambient within an automatic system (VibroBot Mark IV - FEI). 3 μ l of sample was added to the grids. A waiting time of 1 minute is observed before the blotting system dries the excess of the sample drop. Afterwards, the grid is rapidly put into liquid ethane in a nitrogen ambient, to form the vitreous ice in the sample. A waiting time of 30 seconds is done with the grid in the ethane ambient, then it is transposed to a liquid nitrogen ambient until it is brought to a JEM-2100 JEOL microscope at 80kV.

2.3.4 *Dynamic light scattering*

Dynamic light scattering is a technique widely applied in the characterization of colloidal systems (HASSAN; RANA; VERMA, 2015). In the field of nanoparticles used in pharmaceutical applications, DLS has become an important technique for physical characterization of these systems. Part of its popularization is credited to the availability of compact equipment with user-friendly interface (BHATTACHARJEE, 2016), providing good quality data. A typical experimental setup is shown on Figure 12. This method is based on the scattering of coherent light by particles diffusing in a solution in such a way that is described by the Brownian motion model (EISER, 2014). Particles that move under such conditions have their diffusion coefficient dependent on their sizes: small particles tend to have higher diffusion coefficient, while big particles have smaller values.

Particles in motion inside the sample scatter the incident laser beam, and the scattered light is detected by the detector (Fig. 12). Constructive and destructive interference occurs due to this motion and therefore the intensity of light detected varies over time. The correlation of intensity variations and fluctuations along time of these events is used to calculate the translational diffusion coefficient (D). By considering a monodisperse solution, it is possible to calculate the hydrodynamical radius (R_H) of the particles using the calculated value of D from experimental

Figure 12 – Typical DLS experimental setup (BHATTACHARJEE, 2016).

data, and using Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta R_H} \quad (2.5)$$

where k_B is the Boltzmann constant, T is the temperature of the solution in which the experiment is performed and η is the absolute viscosity of the solution. Since the calculation of D depends on the instrumental setup (BHATTACHARJEE, 2016), it is worth noting that DLS results heavily depend on the experimental and sample conditions. For instance, opaque samples provide low scattered intensity, since light is scattered multiple times before reaching the detector. This reason implies in the use of samples with low concentrations in which the solution is reasonably transparent to visible light. DLS data can also provide important information on polydispersity of the dispersion regarding the size of the particles. The polydispersity index (PDI) indicates if a sample is monodisperse ($PDI \leq 0.4$) or has more than one size population ($PDI > 0.4$). PDI values greater than 0.5 often indicate poor quality data.

DLS analysis was performed at Biophysics Laboratory located at Institute of Physics (USP) using a Malvern ZetaSizer Nano-ZS90 analyzer. Samples were diluted to concentrations of 0.5 mg ml^{-1} , 1.0 mg ml^{-1} and 2.5 mg ml^{-1} . The cuvette used was filled with 1 ml of each sample.

2.3.5 Zeta (ζ) potential

Zeta (ζ) potential is defined as the electric potential at the shear plane of a particle moving in a solution under the influence of an external electric field (KASZUBA *et al.*, 2010). The ions close to the particle form a fixed charged surface named Stern layer, as indicated in Figure 13. When a charged particle moves in a solution, counterions form a charged surface called diffuse layer (Fig. 13). Therefore, ζ -potential is measured at the slipping plane, located somewhere between the Stern layer and the diffuse layer. The diffuse layer composition varies depending on factors related to dispersion, such as pH, ionic strength and concentration.

In the specific case of charged nanoparticles used on pharmaceutical applications, ζ -potential analysis is crucial to determine the existence of cationic or anionic particles in solution. However, it is worth noting that, since various factors influence measurements as mentioned before, it is only possible to indicate the superficial charge of the particles through ζ -potential experiments (BHATTACHARJEE, 2016).

Figure 13 – Graphical representation of the ionic layers and the electric potential related to each region (KASZUBA *et al.*, 2010).

The measurement of ζ -potential can only be performed indirectly, by measuring the electrophoretic velocity (v_e) given in $\mu\text{m s}^{-1}$. Experimental setups are designed to measure the electrophoretic mobility (μ_e) defined as the ratio between the electrophoretic velocity and the magnitude of the applied electric field E given in V cm^{-1} :

$$\mu_e = \frac{v_e}{E} \quad (2.6)$$

Using Smoluchowski theoretical model to determine μ_e , Eq. (2.6) is modified and the resultant expression can be used to calculate experimental values of ζ -potential (BHATTACHARJEE, 2016):

$$\mu_e = \frac{\epsilon_r \epsilon_0 \zeta}{\eta} \quad (2.7)$$

Zeta (ζ) potential analysis was performed at Biophysics Laboratory located at Institute of Physics (USP) using a Malvern ZetaSizer Nano-ZS90 analyzer. Cubosomes prepared with PHY/F127 and $[C_{14}mim][Cl]$ were submitted to ζ -potential measurements.

RESULTS AND DISCUSSION

3.1 Sample preparation

Samples were composed by phytantriol, monoolein or Myverol 18-99K and non-ionic F-127 polymer and denoted as PHY-F127, GMO-F127 or MYV-F127. Phytantriol samples were prepared in water and PBS buffer (10 mM - pH 7.4). Monoolein and Myverol samples were prepared in water. All samples had the expected aspect: milky and opaque liquids with water-like viscosity. Similar aspects are reported in the literature by Akhlaghi *et al.* (2016) and Liu *et al.* (2013). Ionic liquid $[C_{14}mim][Cl]$ and TTAB were added after a precursor sample (lipid-polymer-water or lipid-water-buffer) was produced, respecting the weight/weight (cationic molecule:lipid) ratios: 0.05%, 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5%, 2% and 3%. Samples in water environment became more translucent for higher ratios of added $[C_{14}mim][Cl]$ or TTAB. When prepared in buffer, samples remained with the same aspect regardless the amount of $[C_{14}mim][Cl]$ or TTAB added.

3.2 Characterization of PHY-F127 cubosomes

3.2.1 Small Angle X-ray Scattering

3.2.1.1 Crystalline phase determination for blank cubosomes

(a)

(b)

Figure 14 – SAXS scattering data for blank PHY-F127 cubosomes in water. The diffraction pattern is noticeable and has relatively low noise. A Python script was used to identify the crystalline phase symmetry, indicating the presence of the $Pn3m$ cubic phase in these samples. Peaks were indexed with the respective reflection planes of the $Pn3m$ symmetry.

Diffraction patterns from SAXS data of PHY cubosomes were used to: (i) calculate the lattice parameter and (ii) identify the crystallographic spacegroup. The scattering data for PHY-F127 blank cubosomes — absence of IL or TTAB — is shown in Figure 14. In this case, the spacegroup was identified by relating each peak with the theoretical positions of a $Pn3m$ cubic structure (dotted vertical lines in Fig. 14a). Also, by labeling the position of each peak present in the diffraction pattern (Fig. 14b) as $q_1, q_2, q_3...$ and so on, four diffraction peaks were

used (see Table 4). In this case, calculating the ratios between peaks led to the detection of $Pn3m$ cubic structure, in accordance with the results of Akhlaghi *et al.* (2016). The ratios calculated were:

$$\frac{q_2}{q_1} = (1.223 \pm 0.033) \approx \frac{\sqrt{3}}{\sqrt{2}}$$

$$\frac{q_3}{q_1} = (1.410 \pm 0.049) \approx \frac{\sqrt{4}}{\sqrt{2}} = \sqrt{2}$$

$$\frac{q_4}{q_1} = (1.733 \pm 0.049) \approx \frac{\sqrt{6}}{\sqrt{2}} = \sqrt{3}$$

The results above were obtained with the computational routines described in Section 2.3.1.3. Briefly, a Gaussian fit was performed to each peak, in order to determine its central position, and ratios were automatically calculated. Figure 15 shows the program output with results.

Table 4 – Diffraction peaks for blank PHY-F127 cubosomes.

Peak	q (nm ⁻¹)
q_1	1.306 ± 0.033
q_2	1.597 ± 0.030
q_3	1.841 ± 0.054
q_4	2.264 ± 0.047

Figure 15 – Output from Python routine used to calculate the ratio between peaks in diffraction pattern. Four peaks were detected and ratios between each one and the first peak (q_1) were calculated.

3.2.1.2 Phytantriol cubosomes in the presence of $[C_{14}mim][Cl]$ and TTAB

The presence of $[C_{14}mim][Cl]$ or TTAB in cubosome samples was investigated regarding the crystalline structure. As previously mentioned in Section 3.2.1.1, the $Pn3m$ phase was found in PHY blank cubosome samples. However, the increase of IL in the series of samples shifts the diffraction patterns to smaller values of q , which can be directly related to an increase of the lattice parameter a , as reported in Fig. 16. Moreover, there is a decrease in the scattering intensity with higher quantities of IL in the samples in water (Fig. 16a), indicating that parts of the cubic structure have extinguished. It is worth noting that this phenomenon does not occur with the same intensity for samples prepared in buffer (Fig. 16b). A reasonable proposition is that the saline media might “preserve” the crystalline phase.

Figure 16 – Scattering data for PHY- $[C_{14}mim][Cl]$ samples (a) in water and (b) buffer. For all samples, $Pn3m$ crystalline symmetry was found. The presence of the crystalline cubic phase decreases with increment of $[C_{14}mim][Cl]$ and TTAB for water samples. Samples with 3% w/w (IL:PHY) in water have no diffraction peaks, indicating the absence of the crystalline phase.

The peaks detected allowed the calculations of the lattice parameter for PHY-F127

cubosomes in water using a Gaussian fit to the first peak of each diffraction pattern. An example of the output generated by the Python program is displayed in Figure 17. The lattice parameter results for samples prepared with IL in water and buffer are presented in Figure 18. Considering the nominal values for the lattice parameter, the result for blank cubosomes (0.00% w/w IL:PHY) is in agreement with values reported by Akhlaghi *et al.* (2016). Also, there is an steady increase of the lattice parameter with the increase of $[C_{14}mim][Cl]$ ratio in samples, fact that is equally indicated from the scattering data in Fig. 16, where the diffraction pattern moves to smaller values of the scattering vector q .

Figure 17 – Example of output from the Python routine used to calculate the lattice parameter for each diffraction pattern. The same script was used for all samples analyzed.

In order to investigate if the results above were particular to $[C_{14}mim][Cl]$, another cationic surfactant was used to prepare PHY-F127 cationic cubosomes. Samples were prepared with the same w/w ratios using TTAB, which has the same alkyl chain length as $[C_{14}mim][Cl]$. SAXS data is provided in Fig. 19. Although TTAB is a different molecule, scattering data for the PHY-TTAB systems were significantly similar to PHY- $[C_{14}mim][Cl]$ systems. In fact, even the same behavior in lattice parameter variation was identified, in such a way that the lattice parameter increased with higher w/w ratios of TTAB in the samples, as observed in Figure 20. Thus, the results observed by Liu *et al.* (2013) were not necessarily verified for the PHY-TTAB and PHY- $[C_{14}mim][Cl]$ systems.

Analyzing the results presented in Figures 18 and 20, there is evidence that the lattice parameter depends on the concentration of $[C_{14}mim][Cl]$ or TTAB, probably due to electrostatic

Figure 18 – Lattice parameter data calculated for PHY- $[C_{14}mim][Cl]$ systems. There is a visible increase in the lattice parameter related to the increase of $[C_{14}mim][Cl]$ in the samples. However, this increase is not as drastic as reported in the literature for other cationic molecules (LIU *et al.*, 2013). For 3% w/w, the diffraction pattern was present only in scattering data from the sample prepared in buffer.

interactions imposed by the presence of these cationic molecules. However, for the w/w ratios chosen between IL or TTAB and PHY, this dependence implies that the lattice parameter does not change drastically with the increase of IL or TTAB in the samples. In a comparison to a previous work by Liu *et al.* (2013), another cationic surfactant was used in PHY cubosomes formulation. In this case, the increase in concentration of didodecyldimethylammonium bromide (DDAB) have promoted significant variations in the lattice parameter and even phase transitions that were not observed in the $[C_{14}mim][Cl]$ or TTAB samples.

3.2.1.3 SAXS – Temperature variation

Considering possible drug delivery applications, temperature represents an important parameter to the stability of the carriers. The phase diagram of the PHY-water system (Fig. 1b) shows that there is a phase transition just above 40 °C, from cubic ($Pn3m$) to hexagonal. However, this transitions does not occur in such applications conditions, below 40 °C. Still, to perform a complete characterization of the system, it is important to identify other phase transitions that might not be present in the PHY-water phase diagram that might be related to the presence of

Figure 19 – Scattering data for PHY-TTAB samples prepared in (a) water and (b) buffer. For all samples, *Pn3m* crystalline symmetry was also found, as in the case of water. The crystalline cubic phase remains present in all samples with increment of TTAB, with a behavior similar to PHY- $[C_{14}mim][Cl]$ samples.

$[C_{14}mim][Cl]$. In this work, the concern was not to determine a new phase diagram, but try to recognize other crystalline phases at certain chosen temperatures, still considering the phase diagram of the PHY-water system. In this scenario, three temperatures were chosen, besides room temperature (22 °C – 25 °C): 40 °C, just below the phase transition from cubic to hexagonal in the PHY-water system; 50 °C, just above the transition aforementioned; and 65 °C, above the transition from hexagonal to reversed lamellar phase. Moreover, the w/w ratios between IL and PHY (in aqueous solution) analyzed were: 0.1%, 0.5%, 1.0% and 2.0%.

The results of these experiments revealed that at least until 50 °C the cubic *Pn3m* is maintained while the diffraction patterns shift to higher values of q , indicating a decrease of the lattice parameter a . However, at 65 °C the *Pn3m* phase has almost completely vanished from all four samples, and new diffraction peaks have appeared (see Fig. 22d and 22c). Since this

Figure 20 – Lattice parameter data calculated for PHY-TTAB systems. There is a visible increase in the lattice parameter related to the increase of TTAB in the samples. However, this increase is not as drastic as reported in the literature for other cationic molecules (LIU *et al.*, 2013).

Figure 21 – Scattering data at four different temperatures for blank PHY cubosomes (0.0% w/w IL:PHY). **RT**: room temperature.

new phase has not been identified, new experiments are necessary in order to try to reduce background signal noise and to determine the influence of $[C_{14}mim][Cl]$ in this transition. For now, it is possible to exclude the $Im3m$ ou $Ia3d$ cubic phases, due to wide separation between the

Figure 22 – Scattering data at four different temperatures for $[C_{14}mim][Cl]$ -PHY-water samples, with ratios indicated at the top-left of each graphic. It is possible to verify, specially in Fig. 22d that there is a new self-assembly phase that is still yet to be identified. **RT**: room temperature.

new diffraction peaks, uncharacteristic of those two crystal structures.

3.2.2 DLS data analysis

The analysis of dynamic light scattering data provided important information on the polydispersity and average particle size of each sample. DLS data for samples prepared with PHY-F127- $[C_{14}mim][Cl]$ system are shown in Figure 23. Data for samples prepared in water do not imply any noticeable tendency regarding particle size, at least until 1.5% of $[C_{14}mim][Cl]$ present in samples, with average sizes varying (not monotonically) between 300 nm and 350 nm, in accordance to the results reported by Akhlaghi *et al.* (2016). However, at 2% there is a significant increase in particle size. This fact can be related to the increase of the lattice parameter verified

in SAXS results (see Sec. 3.2.1) and to the increase in quantity of charged molecules in the sample. Considering polydispersity and diffusion coefficient results, DLS data provided values for the polydispersity index under 0.1, which can be related to fairly mono-disperse samples. However, particle size in PHY-TTAB samples does not have a noticeable increase in the same behavior as water samples. This fact can be related to preservation of the crystalline structure, as observed in SAXS data (Sec. 3.2.1.2).

Figure 23 – (a) DLS data for PHY-F127- $[C_{14}mim][Cl]$ samples in water and buffer. Despite the noticeable rise in particle size at 1.5% of $[C_{14}mim][Cl]$ in water, particle sizes mostly range between 300 nm and 350 nm. (b) Polydispersity data indicate that samples are fairly mono-disperse up to 2% w/w (IL:PHY).

Samples prepared with PHY cubosomes in the presence of TTAB had similar behavior when compared to $[C_{14}mim][Cl]$ -PHY systems, with sizes within the range between 300 nm and 350 nm up to 1.5% w/w (TTAB:PHY). TTAB samples also had polydispersity index values close to 0.1, indicating mono-disperse samples. Besides these similarities, particle size control is still to be achieved with the production protocols applied. Moreover, the increase in size indicated by DLS results might be influenced by the presence of positively charged molecules in the dispersion, resulting in close range interacting between cationic particles.

3.2.3 Zeta (ζ) potential analysis

DLS data combined with SAXS analysis, indicates that the systems investigated can provide particles within the expected range (300 nm – 350 nm) with cubic crystalline internal structure. However, it is still necessary to verify at what w/w ratios these samples are positively

Figure 24 – (a) DLS data for PHY-F127-TTAB samples in water and buffer. These samples had size and PDI results very similar to PHY- $[C_{14}mim][Cl]$ systems.

Figure 25 – Zeta potential results for PHY cubosomes with $[C_{14}mim][Cl]$ and TTAB. A steady increase is verified with the increase of cationic molecules concentration.

charged. Expectations were in the direction of an increase (towards positive values) in ζ -potential with higher concentrations of the ionic liquid or TTAB, due to their cationic nature. The same concentration used in DLS experiment was also used in ζ -potential analysis, again in an attempt to reduce effects of electrostatic interactions. The results for both systems are shown in Figure 25. Comparing the value of *zeta*-potential for blank PHY cubosomes (0% w/w) from Figure 25 with the results obtained by Akhlaghi *et al.* (2016), there is a difference of a few mV, but as

in the case of Liu *et al.* (2013) an increase is verified related to the increase of $[C_{14}mim][Cl]$ or TTAB concentration in solution. Samples with w/w ratio above 0.25% were clearly positively charged, as expected. The results from ζ -potential experiment also indicates that, within the range of 0.75% w/w to 1.5% w/w (IL to PHY), samples contained cationic nanoparticles with an fairly strict size range.

3.2.4 Cryo-EM of $[C_{14}mim][Cl]$ -PHY cubosomes

Figure 26 – Micrographs for blank PHY cubosomes in water showing particles with different shapes and sizes. However, the internal crystalline structure is visible in all particles.

Imaging experiments were crucial to determine the existence of particles in dispersion. At first, samples prepared with the PHY-F127-water — absence of $[C_{14}mim][Cl]$ — were submitted cryo-EM. Micrographs revealed particles with several shapes, but distinguishable cubic internal structure. In fact, the presence of cubic shaped (see Fig. 26a) particles confirm results obtained by AKHLAGHI *et al.*. Using *ImageJ* software, it was possible to estimate the size of some

visible particles. Estimates varied between (256.1 ± 2.7) nm and (467.4 ± 2.4) nm. Also, some particles bigger and smaller than the previously mentioned were present in samples, as shown in Figure 27. These size estimates partly contradict DLS results, since there is indication of high polydispersity in the sample submitted to cryo-EM. However, in cryo-EM only a few particles are visible, unabling one to gather the information more consistent statistical relevance as in the case of DLS.

Figure 27 – Particles with different sizes and shapes in the same sample.

Some of these micrographs were submitted to crystallographic analysis, in order to obtain information about the internal cubic phase of the particles. After SAXS analysis, it was known that these particles had $Pn3m$ internal structure. However, FFT analysis can provide a comparative result to the lattice parameter calculated from X-ray scattering. Using the methods detailed previously, a lattice parameter of (6.97 ± 0.06) nm was found, which is in agreement to the results displayed in Section 3.2.1.1.

In accordance with Akhlaghi *et al.* (2016), not only particles with internal cubic crystalline particles were found in cryo-EM results. Figure 29 shows other structures that resemble vesicles (spherical particles with a shell formed by bilayer of molecules) formed by the self-assembly of an amphiphilic molecule. Regardless the presence of such structures in the samples analyzed, it is still to be determined the nature of these aggregates.

Figure 28 – FFT analysis for blank PHY cubosomes. Patterns correspondent to **(a)** particle in Fig. 26a, **(b)** particle in Fig. 26c and **(c)** particle in Fig. 26d. A lattice parameter of (6.97 ± 0.06) nm was calculated, in agreement to SAXS results.

Figure 29 – Along with **(a)** particles, what appear to be **(b)** vesicles were present in the same sample.

Samples prepared with the presence of $[C_{14}mim][Cl]$ in water were also submitted to cryo-EM. For this experiment, samples with 0.25% w/w (IL:PHY) were analyzed. Figure 30 shows particles with internal crystalline structure, in the same fashion as blank PHY cubosomes, and in accordance with the phase diagram presented by Liu *et al.* (2013), despite the fact that samples were prepared in PBS buffer in that work. Again, sizes and shapes are variable, and FFT analysis (Fig. 31) provided a calculated lattice parameter of (7.32 ± 0.20) nm. This result is slightly higher than the value calculated from SAXS data. There is an indication that the presence of $[C_{14}mim][Cl]$ increases polymorphism and polydispersity of the system, as previously indicated by DLS data.

Figure 30 – Micrographs for blank PHY cubosomes in water showing particles with different shapes and sizes. However, the internal crystalline structure is visible in all particles.

Figure 31 – FFT analysis for PHY cubosomes in the presence of $[C_{14}mim][Cl]$. Patterns correspondent to a cubic $Pn3m$ structure with lattice parameter of (7.32 ± 0.20) nm, slightly higher than the value calculated in SAXS analysis.

3.3 Characterization of GMO and MYV cubosomes

3.3.1 SAXS of GMO and MYV cubosomes

Regarding GMO-F127 samples, it was expected to find cubosomes with internal structure belonging to $Im\bar{3}m$ space group (YAGHMUR; GLATTER, 2009) following the results obtained by Spicer *et al.* (2001). Scattering curves for GMO cubosomes are shown on Figure 32. From full scattering data, it was observed that, for blank cubosomes prepared with the GMO-F127 system both with BU and TD protocols, three diffraction peaks were distinguished from the rest of the scattering curve (Fig. 33). The diffraction patterns for these samples are displayed on Figure 33. Indexing each as q_1 , q_2 and q_3 respectively – from the lowest to the highest value of q for the detected peaks – it was possible to use the program described in Section 2.3.1.3 to determine their positions and ratios, and thus indexing them to Miller indices related to the reflection planes from the reciprocal crystal lattice.

Figure 32 – Diffraction patterns for samples prepared with GMO-F127 system (BU and TD). From calculations, it was possible to assign Miller indices to each peak, corresponding to $Im\bar{3}m$ cubic structure.

Table 5 – Values for the scattering vector of each detected peak for blank GMO-F127 cubosomes (BU and TD). These data were used for the calculation of the lattice parameter for both samples.

Sample	q_1 (nm ⁻¹)	q_2 (nm ⁻¹)	q_3 (nm ⁻¹)	Lattice parameter (nm)
GMO(TD)	0.584 ± 0.021	0.833 ± 0.023	1.012 ± 0.022	15.18 ± 0.26
GMO(BU)	0.548 ± 0.021	0.773 ± 0.022	0.947 ± 0.022	16.24 ± 0.28

(a)

(b)

Figure 33 – SAXS scattering data for blank GMO-F127 cubosomes in water. The diffraction pattern is noticeable and has relatively low noise. A Python script was used to identify the crystalline phase symmetry, indicating the presence of the $Im\bar{3}m$ cubic phase in these samples. Peaks were indexed with the respective reflection planes of the $Im\bar{3}m$ symmetry.

Calculating the ratios between peaks of Table 5, for both samples, using the Python program (Fig. 34), we have the following results:

$$\begin{aligned} \text{GMO(TD):} \quad \frac{q_2}{q_1} &= (1.414 \pm 0.093) \approx \frac{\sqrt{4}}{\sqrt{2}} = \sqrt{2} & \frac{q_3}{q_1} &= (1.740 \pm 0.095) \approx \frac{\sqrt{6}}{\sqrt{2}} = \sqrt{3} \\ \text{GMO(BU):} \quad \frac{q_2}{q_1} &= (1.412 \pm 0.087) \approx \frac{\sqrt{4}}{\sqrt{2}} = \sqrt{2} & \frac{q_3}{q_1} &= (1.723 \pm 0.093) \approx \frac{\sqrt{6}}{\sqrt{2}} = \sqrt{3} \end{aligned}$$

These results indicate that the cubic structure for all blank cubosomes prepared with the GMO-F127 system, both from BU and TD protocols, is $Im\bar{3}m$, in accordance with results previously reported (BOGE *et al.*, 2016; CHONG *et al.*, 2011; DONG *et al.*, 2006). This

information is important because it confirms that $Im3m$ only existing for very high contents of water in GMO-water systems and in the presence of F127 (MAZZONI *et al.*, 2016). With the data from Table 5 and using Equation 2.3, it was possible to determine the lattice parameter for each GMO/F127 sample. The results are also shown in Table 5 and are in the same order of magnitude reported by Dong *et al.* (2006) (around 13 nm).

Figure 34 – Output of the Python program used to identify the $Im3m$ symmetry for blank GMO cubosomes.

3.3.1.1 Interaction between ionic liquid $[C_{14}mim][Cl]$ and GMO and MYV cubosomes

Samples with both GMO and MYV in the presence $[C_{14}mim][Cl]$ were analyzed. SAXS data (Figure 35) indicate that the increase of $[C_{14}mim][Cl]$ concentration in the sample has caused a displacement of the diffraction peaks towards larger values of q (Fig. 35a), indicating a variation in the unit cell lattice parameter as ionic liquid concentration increases. However, when determining the symmetry group, it was noticed that for all concentrations analyzed, it remained $Im3m$. For MYV cubosomes (Fig. 35b), a similar behavior was detected. However, the diffraction pattern has vanished for $[C_{14}mim][Cl]$ ratios above 0.25% w/w (IL:MYV). Figure 36 has a comparison of the lattice parameter behavior for GMO and MYV cubosomes in the presence of $[C_{14}mim][Cl]$. In the case of MYV cubosomes, there is no evident change caused by the increase of IL in the samples for samples until 0.25% w/w (IL:MYV), despite the fact that, for higher ratios, no diffraction peak was detected. On the other hand, for GMO cubosomes, the lattice parameter changes with the increase of IL in the samples, but not in the same way as PHY cubosomes.

Figure 35 – Scattering data for **(a)** GMO and **(b)** MYV cubosomes in the presence of $[C_{14}mim][Cl]$. Both systems have $Im3m$ symmetry. It is noticeable on both systems that the increase of $[C_{14}mim][Cl]$ in samples implies in the extinguishing of the cubic phase, hence the decrease of the diffraction pattern intensity. For MYV cubosomes with $[C_{14}mim][Cl]$, samples prepared with ratios above 0.25% w/w (IL:MYV) have no diffraction peaks.

3.3.2 EM imaging of GMO cubosomes

Samples GMO(TD) and GMO(BU) with blank cubosomes (Figure 37) was submitted to negative stain EM. Particles with apparent cubic shape were found (Figure 37b) and their average edge size was calculated with *ImageJ*[®] software, providing a result of (386 ± 20) nm. Several other works published cubosomes with cubic shape, including (TRAN *et al.*, 2015) for example. EM images for GMO(BU) revealed that the particles have aggregated in groups of a few units, as shown in Figure 37c. It seems natural to relate this situation with the visible aggregation of the sample.

Figure 36 – Lattice parameter for GMO and MYV samples with $[C_{14}mim][Cl]$ ionic liquid. Despite the different ratios analyzed, there is a clear difference in the lattice parameter values for MYV and GMO cubosomes. However, the behavior verified in PHY cubosomes — the increase of the lattice parameter with the increase of IL — is not verified with these data.

Figure 37 – Micrographs for GMO(BU) and GMO(TD). (a) shows various GMO(TD) particles, while (b) shows the magnification of one particle with cubic shape. (c) and (d) show GMO(BU) particles that have aggregated.

CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Conclusions

Nanoparticles designed for drug delivery applications are definitely part of a vast and interesting field of research. Cubosomes, in their turn, configure a really remarkable system, not only for its crystalline structure formed by the assembly of organic molecules, but also for the numerous possibilities for therapeutic applications. Physical characterization of these systems also stands as an interesting approach of biophysical and biochemical sciences applied to nanotechnology and nanomedicine. In that scenario, it is worth to investigate new possibilities for these systems. Considering the growth in gene therapy research, the use of “charged” cubosomes to load and deliver DNA and other drugs to unhealthy cells seems as an opportunity to use these particle for more audacious applications.

In this work, nanoparticles with internal cubic crystalline structure were produced successfully. It is worth mentioning that, from the studied and tested preparation protocols, the one used to produce phytantriol cubosomes stabilized with Pluronic F127 stood out as more reproducible. The addition of ionic liquid $[C_{14}mim][Cl]$ or cationic surfactant TTAB

to the formulation has shown to alter physicochemical properties of the dispersion for certain concentrations, but the main goal – produce cationic nanoparticles with cubic crystalline structure – was successfully achieved. In the case of the dispersions produced with monoolein, stabilized with Pluronic F127, samples definitely contained nanoparticles with cubic structure, but the production process still needs to be polished. The reason to remain working with both lipids (monoolein and phytantriol) is that each can be used to produce particles with different structural characteristics that may be suitable for different applications. Still, the fact that these particles are viable opens the possibility of performing tests to determine the efficiency of such systems in loading and delivering the intended molecules.

4.2 Future considerations

Despite the fact that an important part of the original project was achieved, the produced cubosomal dispersions need to be tested for very relevant characteristics from a pharmaceutical perspective. Tests of blank and loaded cubosomes toxicity are among the experiments that might provide important information about the viability of these systems. The use of specific cells and test procedures still need to be determined, based on previous experiments reported in the literature.

From the physical characterization point of view, other techniques such as AFM may be applied in order to investigate the binding (or existence) of charged macro molecules to the surface of the cationic nanoparticles. Preparation of loaded charged cubosomes with other hydrophobic drugs might also be performed, along with tests of loading and release efficiency. Regarding preparation protocols, PHY cubosomes production is already consistent. However, GMO protocols still need to be improved. The use of GMO is important in order to tailor not only the particles components, but also their crystalline structure, allowing different applications when compared to PHY cubosomes. A proposition for the continuity of this project is to better tailor the production protocol in order to produce cubosomes with the desired size and surface charge, enabling these nanoparticles to be powerful carriers of several drugs.

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COMPUTER SCRIPTS USED TO ANALYSE EXPERIMENTAL DATA

```
def saxs_plot(files, begin, end, line_width=1):
    for i in range(len(files)):
        data = np.genfromtxt(files[i], skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
        plt.plot(data['q'], data['I']-i*1000000, "-", lw=line_width, label=str(files[i])[3:7])
        plt.xlabel('q [nm-1])')
        plt.ylabel('Intensity [a.u.]')
        plt.yticks([])
        plt.savefig(str(files[0])+'_linear.png')

def saxs_plot_log_mod(files, begin, end, samples, sample_legend=False, line_width=1):
    for i in range(len(files)):
        data = np.genfromtxt(files[i], skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
        plt.plot(data['q'], np.log10(data['I'])-i, "-", lw=line_width, label=samples[i])
        plt.xlabel('q [nm-1])')
        plt.ylabel('log(I) [a.u.]')
        plt.yticks([])
        plt.savefig(str(files[0])+'_log.png')
    if sample_legend == True:
        plt.legend(frameon=False)

def saxs_plot_log(files, begin, end, line_width=1):
    for i in range(len(files)):
        data = np.genfromtxt(files[i], skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
        plt.plot(data['q'], np.log10(data['I'])-i, "-", lw=line_width, label=str(files[i])[3:7])
        plt.xlabel('q [nm-1])')
        plt.ylabel('log(I) [a.u.]')
        plt.yticks([])
        plt.savefig(str(files[0])+'_log.png')
```

Figure 38 – Python routine used to plot SAXS data.

```
def plot_peak_lines(file, begin, end, min_height, max_dist, graph, struc, offset=0, legend_loc=3):
    struc_name = str(struc)
    plt.plot([], label = struc_name, ls='-.', color='grey', lw=1)
    data_I = np.genfromtxt(file, skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
    peaks = detect_peaks(data_I['I'], mph = min_height, mpd = max_dist, show=graph)
    fractions = ratios[struc]
    if offset != 0:
        print('Peak lines function output: first detected manually set (nm-1) = ', offset)
        for j in fractions:
            plt.axvline(x=offset*j, ls='-.', color='grey', lw=1)
    else:
        print('Peak lines function output: first detected peak (nm-1) = ', data_I['q'][peaks[0]])
        for j in fractions:
            plt.axvline(x=data_I['q'][peaks[0]]*j, ls='-.', color='grey', lw=1)
    plt.legend(frameon = False, loc=legend_loc)
```

Figure 39 – Python routine used to identify the symmetry present in SAXS data

```

def gauss(x,a,x0,sigma):
    return a*np.exp(-(x-x0)**2/(2*sigma**2))

def calc_lat_param_err_gauss(files,begin,end,min_height,max_dist,inf_limit,sup_limit,graph,adj=False,pr=False):
    """ Function used to calculate lattice parameters of saxes cubosome data """
    x = np.linspace(0,5,300)
    vector_adj = []
    first_peak = []
    i = 0
    """ Read files """
    for fname in files:
        data_I = np.genfromtxt(fname, skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
        peaks = detect_peaks(data_I['I'], mph = min_height, mpd = max_dist, show=graph)
        """ Check when peaks are not detected """
        if len(peaks) != 0: """ routine for detected peaks """
            first_peak.append(data_I['q'][peaks[0]])
            x = data_I['q'][peaks[0]-inf_limit:peaks[0]+sup_limit] """ isolate peak data within range """
            y = data_I['I'][peaks[0]-inf_limit:peaks[0]+sup_limit] """ isolate peak intensity within range """
            """ Gaussian fit """
            mean = np.sum(x*y)/np.sum(y) """ calculate average """
            sigma = np.sqrt(np.abs(np.sum((x-mean)**2*y)/np.sum(y))) """ calculate standard deviation """
            if y != np.array([]):
                popt,pcov = curve_fit(gauss,x,y,p0=[max(y),mean,sigma]) """ gaussian fit function """
                fit = popt[1] """ fit output """
                stdev = popt[2] """ fit error """
                q_adj = unp.uarray(fit, stdev) """ create array with uncertainties """
                vector_adj.append(q_adj)
                if adj == True:
                    x_new = np.linspace(data_I['q'][peaks[0]]-0.01*inf_limit, data_I['q'][peaks[0]]+0.01*sup_limit, 100)
                    fig = plt.figure(i)
                    plt.plot(x, y, '.', x_new, gauss(x_new,*popt), '-')
                    plt.title(fname)
                    plt.xlabel('q [nm-1]')
                    plt.ylabel('Intensity [a.u.]')
                    plt.yticks([])
                    i+=1
            elif y == np.array([]):
                print(fname, '--> No detected peaks')
                print('-----')
                continue
            """ Plot fitted curve """
        elif len(peaks) == 0:
            """ Output (no peaks detected for current file) """
            print(fname, '--> No detected peaks')
            print('-----')
            continue
        params = 2*np.pi*np.sqrt(2)/(np.array(vector_adj))
        """ Output (print results) """
        if pr == True:
            print('Calculated lattice parameters:')
            print(params)
    return(params) """ calculated lattice parameters """

```

Figure 40 – Python routine used to fit each diffraction peak in SAXS data.

```

def find_cubic(files,begin,end,min_height,max_dist,inf_limit,sup_limit,graph,adj=False):
    """ Function used to calculate ratios and identify symmetry """
    x = np.linspace(0,5,300)
    vector_adj = []
    i = 0
    position = ['1st','2nd','3rd','4th','5th','6th','7th','8th','9th','10th']
    """ Read files """
    for fname in files:
        data_I = np.genfromtxt(fname, skip_header=begin, skip_footer=end, usecols=(0,1), names=['q','I'])
        peaks = detect_peaks(data_I['I'], mph = min_height, mpd = max_dist, show=graph)
        """ Check when peaks are not detected """
        if len(peaks) != 0: """ routine for detected peaks """
            for j in peaks:
                x = data_I['q'][j-inf_limit:j+sup_limit] """ isolate peak data within range """
                y = data_I['I'][j-inf_limit:j+sup_limit] """ isolate peak intensity within range """
                """ Gaussian fit """
                mean = np.sum(x*y)/np.sum(y) """ calculate average """
                sigma = np.sqrt(np.abs(np.sum((x-mean)**2*y)/np.sum(y))) """ calculate standard deviation """
                if y != np.array([]):
                    popt,pcov = curve_fit(gauss,x,y,p0=[max(y),mean,sigma]) """ gaussian fit function """
                    fit = popt[1] """ fit output """
                    stdev = popt[2] """ fit error """
                    q_adj = unp.uarray(fit, stdev) """ create array with uncertainties """
                    print(position[i]+' peak detected (nm-1) = ',q_adj) """ print detected peaks positions """
                    vector_adj.append(q_adj)
                    if adj == True:
                        x_new = np.linspace(data_I['q'][peaks[0]]-0.01*inf_limit, data_I['q'][peaks[0]]+0.01*sup_limit, 100)
                        fig = plt.figure(i)
                        plt.plot(x, y, '.', x_new, gauss(x_new,*popt), '-')
                        plt.title(fname)
                        plt.xlabel('q [nm-1]')
                        plt.ylabel('Intensity [a.u.]')
                        plt.yticks([])
                        i+=1
            elif y == np.array([]):
                print(fname, '--> No detected peaks')
                print('-----')
                continue
            """ Output (no peaks detected for current file) """
        elif len(peaks) == 0:
            print(fname, '--> No detected peaks')
            print('-----')
            continue
        vector_adj = np.array(vector_adj)
        ratio_results = []
        first = vector_adj[0]
        for k in vector_adj:
            ratio_results.append(k/first)
    return(np.array(ratio_results)) """ calculated ratios between peaks """

```

Figure 41 – Python routine used to calculate ratios between diffraction peaks.