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Advances in structural design of lipid-based nanoparticle carriers for delivery of macromolecular drugs, phytochemicals and anti-tumor agents

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Abstract

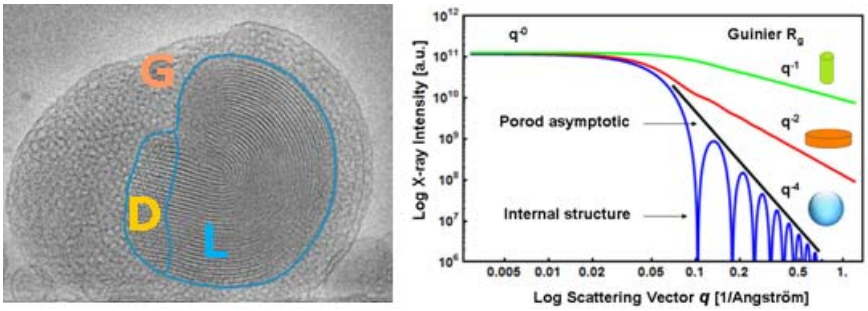
The present work highlights recent achievements in development of nanostructured dispersions and biocolloids for drug delivery applications. We emphasize the key role of small-angle X-ray scattering (BioSAXS) investigations for the nanomedicine design. A focus is given on controlled encapsulation of small molecular weight phytochemical drugs in lipid-based nanocarriers as well as on encapsulation of macromolecular siRNA, plasmid DNA, peptide and protein pharmaceuticals in nanostructured nanoparticles that may provide efficient intracellular delivery and triggered drug release. Selected examples of utilisation of the BioSAXS method for characterization of various types of liquid crystalline nanoorganizations (liposome, spongosome, cubosome, hexosome, and nanostructured lipid carriers) are discussed in view of the successful encapsulation and protection of phytochemicals and therapeutic biomolecules in the hydrophobic or the hydrophilic compartments of the nanocarriers. We conclude that the structural design of the nanoparticulate carriers is of crucial importance for the therapeutic outcome and the triggered drug release from biocolloids.

Key words: Liquid crystalline nanocarriers, drug delivery, BioSAXS, nanostructured lipid carriers, nanomedicines, self-assembled biomaterials.

Abbreviations:

BJO – *Brucea javanica* oil
CPP - critical packing parameter
GCL - Gemini cationic lipid
HPH - high-pressure homogenization
LLC – lyotropic liquid crystal
MLO – monolinolein
MO – monoolein
OA – oleic acid
PDI – polydispersity index
PEG - poly(ethyleneglycol)
siRNA - small interfering RNA
Small-angle X-ray scattering (SAXS

TOC graphics



Contents

1. Introduction

Lipid-based liquid crystalline nano-assemblies of lamellar and non-lamellar types present ongoing interest for development of biocolloidal nanocarriers and precision nanomedicines [1–31]. Biocolloidal self-assembled nanocarriers exhibit biocompatible properties low toxicity, and represent efficient delivery systems for drugs, amino acids, peptides, proteins and vitamins in various pharmaceutical, biomedical, and food applications [2,30]. A variety of soft matter nanostructures have been obtained (Fig. 1) through biomimetism [11,32,33], spontaneous [8-22] and directed assembly of amphiphilic building blocks [3,13,34], as well as through nanoarchitectonics principles [35,36]. Recent structural investigations of cubosomes, hexosomes, spongosomes, micellosomes, and liposomes will be summarized in this review from a drug delivery point of view. The inner liquid crystalline organizations and topologies of such nanocarriers have been proven as favourable for encapsulation of various types of therapeutic molecules (anti-cancer, anti-viral, anti-oxidant, antibiotic, *etc.*). Colloidal stability has been achieved through anchoring of poly(ethyleneglycol) chains on the surface of the nano-assemblies dispersed in excess aqueous medium [1-3,10,18,22,30,31]. The advances in the research on nanostructured biocolloids have enabled the design of mixed self-assembled systems for co-encapsulation of drugs with potential applications in combination therapies [10,15,37–49].

Figure 1

Tunable properties of the lipid nanocarriers may be imparted through variations of the amphiphilic composition [1-3,30,31]. Besides being safe and non-toxic, the lipid-based nanocarriers should also meet the requirements of (i) appropriate particle sizes and shapes, and (ii) cargo upload/release properties towards improved performance in drug delivery. Since the structural control is of primary importance for the mechanism of nanocarriers' assembly and loading, recent publications will be highlighted here in order to illustrate the key role of the small-angle X-ray scattering/BioSAXS investigations in novel nanomedicine design [50-74]. The incorporation of macromolecular therapeutics (siRNA, plasmid DNA, peptide and proteins) in liquid crystalline nanocarriers, studied by SAXS, presents strong current potential for therapeutic innovations.

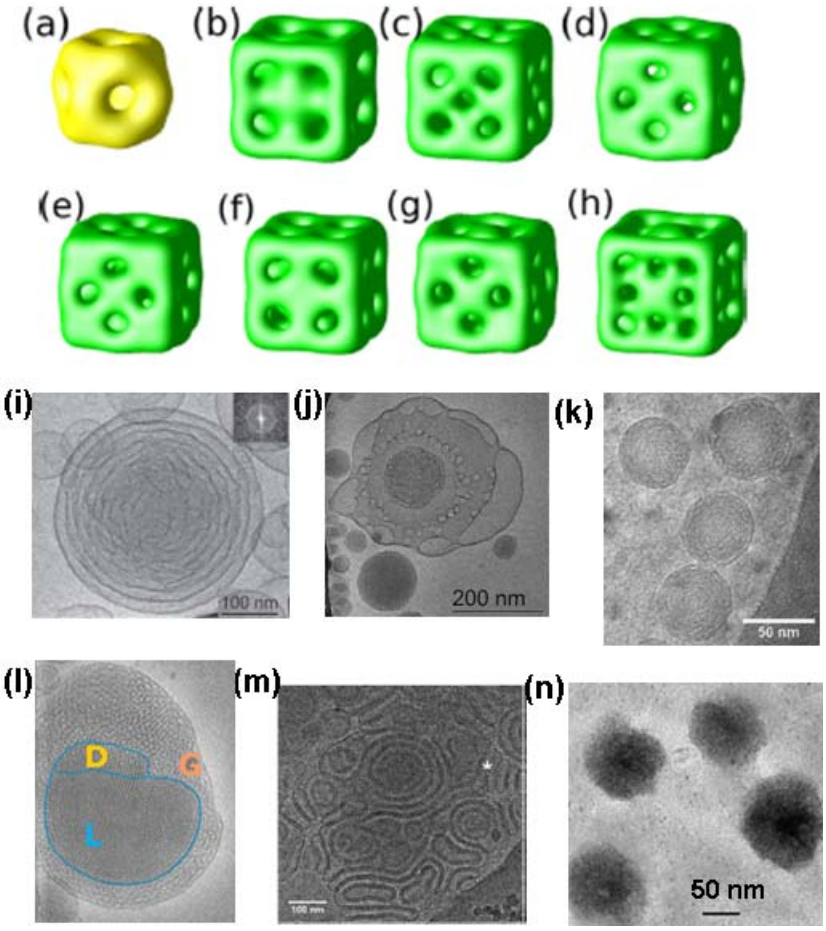


Fig 1. Cubosome nanoparticles of different geometries and topologies derived by simulations (a-h) [Reprinted with permission from [4]. Copyright (2015) American Chemical Society]. Electron microscopy images of self-assembled lipid-based nanocarriers for development of safe biocolloidal drug delivery systems: (i) cubosome with extra-large channels for encapsulation of hydrophilic macromolecules [6]; (j) spongosome loaded with an oil-type drug [10]; (k) hexosome for vaccine delivery [12], (l) multicompartment liquid crystalline particle loaded with a therapeutic protein [13], (m) siRNA-gemini surfactant/monoacylglycerol lipoplex nanoassemblies [14], and (n) dual drug-loaded (resveratrol and 5-fluorouracil) PEGylated nanoliposomes [15].

2. Fabrication of lipid-based liquid crystalline nano-assemblies

The fabrication methods of lipid-based nanocarriers generally include hydration of mixed lipid films, extrusion, melting, sonication, homogenization and emulsification procedures [30,31,74-93]. The preparation protocols often involve premixing of structure-forming lipids

with amphiphilic stabilizers. Schematic diagrams representing the top-down approach for nanocarrier fabrication are shown in Fig. 2 (a-d).

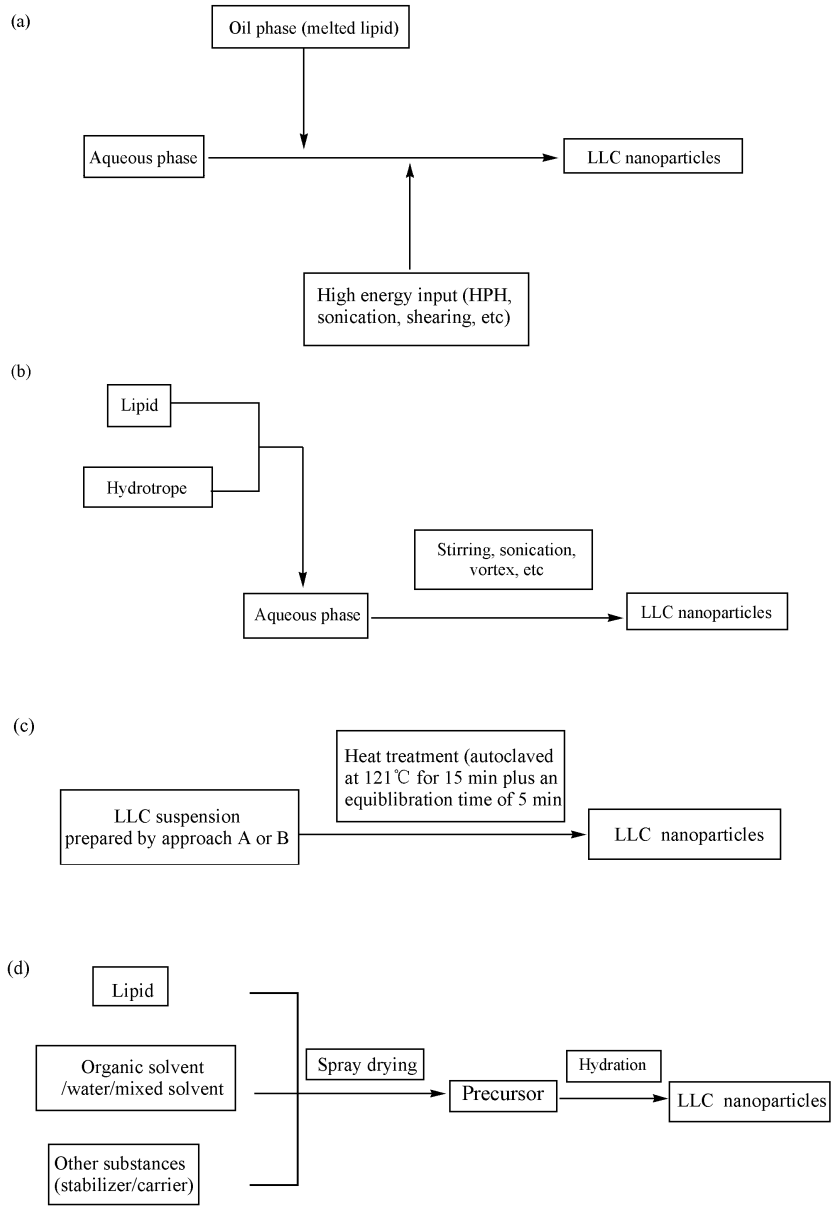


Fig 2. Schemes of fabrication protocols for dispersion and homogeneization (a-d) of lipid-based liquid crystalline nanocarriers.

Top-down approach for nanocarrier preparation. This approach has been broadly employed following the pioneering works of cubosome preparation using a lipid (oil) phase and an aqueous phase containing a dispersing agent [1-3,8-12,18,30,31]. Thus, lipids and water-insoluble drugs can be melted as the oil-phase component. The amphiphilic stabilizer and the hydrophilic drugs can be dissolved in the aqueous solution phase. The oil phase may

be dispersed into excess aqueous medium through high energy input such as high-pressure homogenization [HPH], microfluidization, sonication or shearing in order to form LLC nanoparticles. HPH has been the extensively used as a technique for preparation of lipid-based nanocarriers among ultrasonication and microfluidization.

Using SAXS for nanocarrier structural analysis, Mezzenga and colleagues have reported the preparation of lyotropic liquid crystalline (LLC) assemblies for controlled drug delivery [78]. The chosen lipids have been mixed in ethanol and the organic solvent has been entirely evaporated. Then, the mixtures have been dried under vacuum to form a dry lipid film. Mesophases of hydrated lipid mixtures have been obtained by repeated cycles of vortex mixing with aqueous phase and heating. The resulting nanocarriers have been pH-responsive and suitable for triggering of release of active ingredients and pharmaceutical molecules upon pH changes.

Nanocarrier dispersion along a dilution line in the phase diagram. The key factor in this approach is the employed hydrotrope, which may dissolve water-insoluble lipids and drugs to create liquid precursors for further dispersion into nanoparticles as well as to prevent the liquid crystal formation at high concentrations [2,30]. The dilution-based approach has several advantages: (a) low energy input is only required; (b) efficient generation of nanoparticles with inner LC organization at ambient temperature; (c) long-term particles stability is feasible; and (d) a negligible fraction of small vesicles present in the dispersed system.

Shearing. Besides the above approaches for lipid nanocarrier preparation, Glatter and colleagues have proposed a shearing method for rapid preparation of concentrated nanostructured dispersions, which have been characterized by SAXS [30]. Controlled particle sizes have been achieved using a laboratory built-up shearing device based on a Couette cell. Broader insights on liquid crystalline nanosystems formed through either high- or low-energy schemes can be found in published articles [2,18,30,31].

Bottom-up vs. top-down methods. Using SAXS, Boyd and colleagues have demonstrated the impact of the preparation method on the internal structure and morphology of nanoparticles derived from phytantriol-Pluronic®F127 assemblies as well as on the presence of vesicles populations coexisting with the dispersed cubosomes [31]. Bottom-up and top-down approaches have been employed for the preparation of phytantriol cubosomes stabilized by Pluronic®F127 (F127). Ethanol has been used for the preparation of the liquid precursors. Subsequently, it has been removed by means of a rotary evaporator. The top-down approach has used ultrasonication for nanoparticles dispersion. Cryo-TEM and physico-chemical data have shown that the cubosomes obtained by the top-down approach have small particle sizes (203 nm ± 4 nm) and a low PDI of 0.16. The lipid dispersion has contained only a small fraction of liposomes (≈7%) and has been stable up to at least 4 months. The structural results

have revealed that phytantriol cubosomes stabilized by Pluronic®F127 can be prepared *via* ultrasonication, which is an ideal candidate for industrial applications.

Heat treatment may help decreasing the fraction of small particles corresponding to vesicles in the dispersion. Thus, cubic nanoparticles with a narrower particle size distribution and good colloidal stability have been obtained [30,31]. In general, heat treatment is inappropriate for systems loaded with fragile protein molecules and temperature-sensitive drugs.

3. Nanoscale characterization of lipidic nanoparticle dispersions by SAXS

Small-angle X-ray scattering (SAXS) has been established as an indispensable structural method for characterization of nanostructured lipid dispersions [50-97]. Figure 3 shows calculated scattering curves of aqueous dispersions containing nanoparticles of different shapes and sizes. The three *q*-wavevector regions (at very small, medium and increasing scattering angles) differentiate the nano-objects, present in the aqueous dispersion, according to their size, shape, and surface roughness associated with the form factor [70].

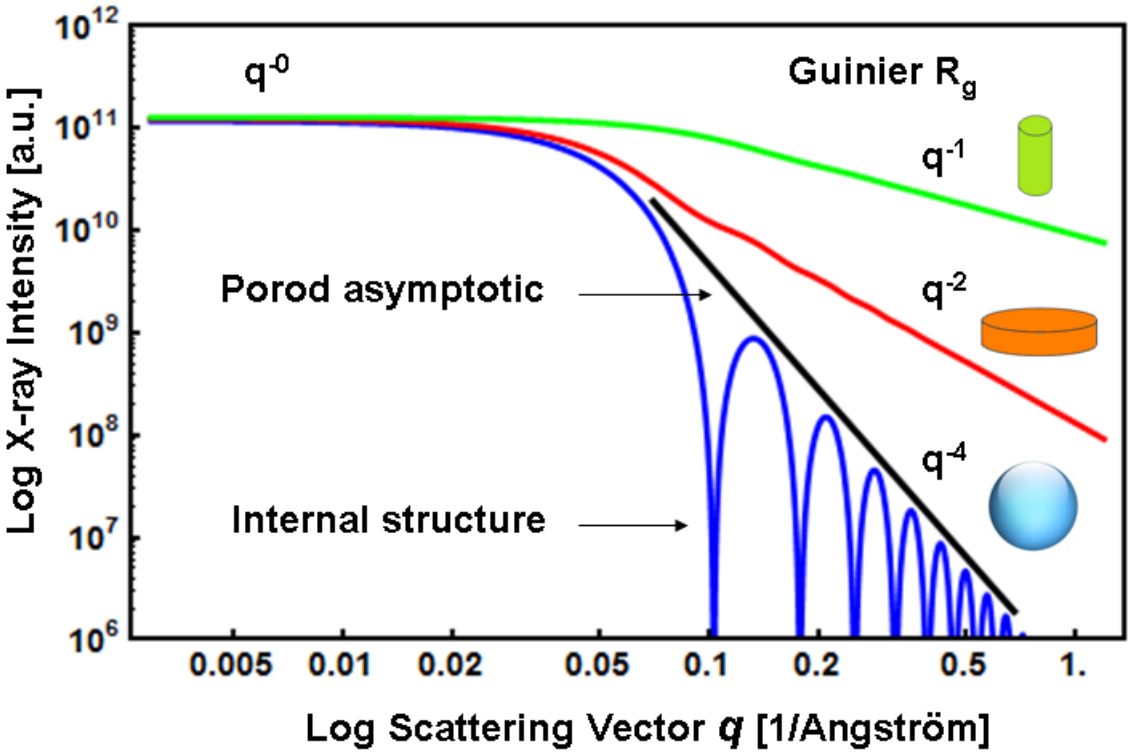


Fig 3. Calculated small-angle X-ray scattering (SAXS) curves in a broad *q*-vector range, which provides information about the size, the shape and the surface roughness of the scattering nano-objects present in the biocolloidal dispersion (adapted from Ref. [70]).

The inner organization of the liquid crystalline assemblies is reflected by the structure factor of the nanoparticles associated with typical Bragg peaks. The reader is referred to specialized articles in the SAXS field regarding the detailed formalism for fitting and interpretation of the SAXS data that may be acquired from liquid dispersions of lipid nanoparticles [54,67-70,96].

Nanoparticle shapes and inner organizations have been suggested as crucial for their controlled release properties and interaction with cells and biological fluids [2,60]. The stability of the lipid nanocarriers of liquid crystalline types in human plasma has been evaluated by SAXS analysis [1]. Figure 4 shows the time dependence of the SAXS patterns of hexosome and cubosome particles upon contact with plasma. The observed structural features constitute an essential outcome before the *in vivo* nanocarriers application as drug delivery systems.

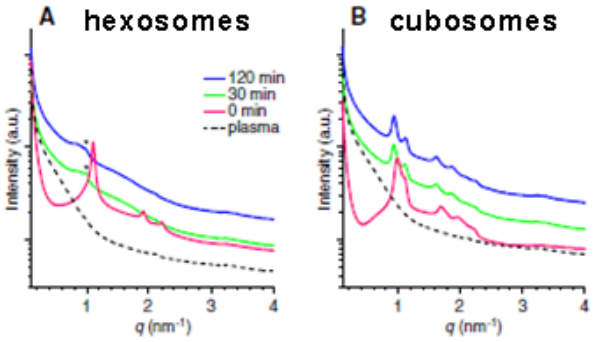


Fig 4. SAXS patterns employed for investigation of the stability of hexosome and cubosome nanocarriers in human plasma. Reprinted from Ref. [1] with permission from Elsevier. Copyright (2016) Elsevier Ltd.

SAXS characterization of stimuli-responsive self-assembled nanostructured lipid systems for drug delivery and diagnostics has been reported in several recent publications [74-87]. The structural arrangement and parameters of the responsive lipid systems have been varied through temperature changes, ionic type, pH, light and electromagnetic stimuli. Phase changes of the structural organizations have been detected by SAXS in response to the addition of metal ions to liquid dispersions of lipid nanoparticles as well through pH changes. In particular, a salt-induced phase transition from a lamellar to a bicontinuous cubic phase structure has been studied with cationic DDAB/phytantriol nanoparticles [76]. The L_{α}-H_{II} phase transition in the slf-assembled system has been monitored by SAXS [74-76]. This and several other studies have demonstrated that time-resolved synchrotron SAXS has became an important tool for investigation of rapid phase changes, curvature variations, and the

structural dynamics of self-assembled lipid systems of biomedical interest through *in situ* millisecond time-resolved experiments [13,81,84,87].

In parallel, SAXS has revealed light-triggered effects in host−guest lyotropic liquid crystalline mesophases of lipids designed as molecular switches for "on demand" release of substances [81]. A small amount of a judiciously synthesized lipid bearing an azobenzene photoactive unit has been incorporated in host liquid crystalline mesophases composed of monoolein (MO) and oleic acid (OA). It has been demonstrated by SAXS that the conformation changes in the azobenzene moiety of the guest lipid at the molecular level do not affect the overall inverted hexagonal packing arrangement of the host lipid mesophase. However, the azobenzene moiety of the light-responsive lipid component has changed the nature of the hydrophobic lipid bilayer and affected the lipid−water partition coefficient of the studied model drug, in addition to the controlled permeability of the lipid membrane. Therefore, this building block of the assembly may induce controlled release of molecules (drugs, dyes) upon UV irradiation [81].

On the other hand, pH-sensitive lipidic cubic phase matrices for drug release have been characterized by SAXS as well [79]. The self-assembled system has comprised a host MO lipid and designed lipidic additives, which have formed a structured pH-sensitive lipidic matrix in water medium. Both hydrophilic and hydrophobic drug upload and release have been studied. The effect of an encapsulated anticancer drug (doxorubicin) on the lattice parameter of the MO-based cubic assemblies has been determined in a broad temperature interval.

SAXS measurements (Fig. 5) have demonstrated that lyotropic liquid crystalline systems composed by monolinolein and linoleic acid (3 wt%) are responsive to pH variations and may reversibly switch their structure and physical properties [78]. A structural change from an inverted *Im3m* cubic phase to an inverted H_{II} hexagonal phase has been provoked by a pH jump from neutral (pH 7) to acidic (pH 2) medium, which has simulated the environmental conditions of intestine (37 °C and 150 mM ionic strength) and stomach, respectively. The established pH responsiveness has been attributed to the linoleic acid component (a weak acid with pKa≈5), which has been in a charged state at pH 7 and in a neutral protonated form at pH 2. The latter has imposed changes in the mean critical packing parameter (CPP) of the self-assembled liquid crystalline mixture. The studied system has been efficient for controlled release of a model hydrophilic drug phloroglucinol. UV-Vis spectroscopy studies of the drug diffusion and release at different pH values have indicated that the *Im3m* cubic phase releases 4 times faster at pH 7 in comparison to the release from the inverted hexagonal H_{II} phase at pH 2. Therefore, the performed SAXS analyses helped to establish an ideal candidate carrier system for oral administration of drugs and targeted delivery in intestine or colon tracts.

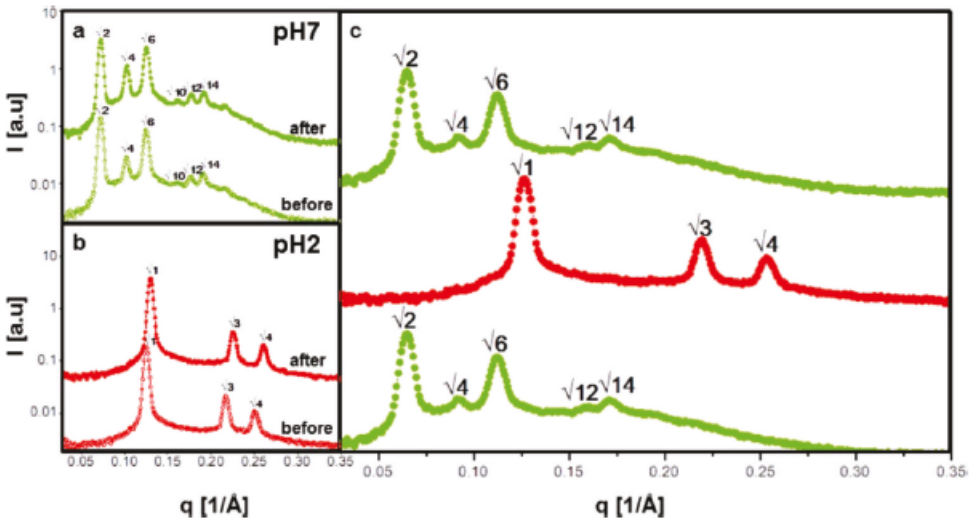


Fig 5. SAXS patterns of pH-responsive monolinolein/linoleic acid lyotropic liquid crystals. The bicontinuous cubic phase of the *Im3m* space group is characterized by a set of Bragg reflections spaced in the ratio √2, √4, √ 6, √10, √12, and √14 at pH 7. The inverted hexagonal phase displays reflections spaced at √1, √3, and √4 at pH 2. The observed pH-induced structural changes are reversible. Reprinted with permission from Ref. [78]. Copyright (2011) American Chemical Society.

A perforated bicontinuous cubic phase with pH responsive topological channel interconnectivity has been obtained by mixing weighed amounts of the MLO lipid and a detergent solution of the outer membrane protein F (OmpF). The samples have been prepared in sealed Pyrex glass tubes, which have been vortexed and heated up to 45 °C [80]. The formed homogenous mixtures have been allowed to cool down to 37 ° C in an incubator. The presence of the OmpF membrane protein in the bicontinuous cubic phase has provided a unique topological interconnectivity between the two networks of water channels in the 3D assembly, thus enabling active molecular gating. The pores of the perforated mesophase could be tuned by exploiting a pH-mediated pore-closing response mechanism. The SAXS method has been employed all along the preparation and characterization of the perforated cubic lipid/protein assembly [80].

The effects of the hydrostatic pressure on colloidally stabilized lipid nanoparticles with inner nonlamellar organization have been evidenced by SAXS as well [82]. The internal lipid structure has been systematically tuned between bicontinuous cubic (*Pn3m* and *Im3m*), micellar cubic (*Fd3m*), inverted hexagonal (H_{II}), and inverted micellar (L_2) phases depending on the lipid/oil ratio and the variation of the hydrostatic pressure (from an atmospheric value to 1200 bar and back to the atmospheric pressure). The effects of pressure on the lipid

nanoparticles have been determined by synchrotron radiation SAXS. They have demonstrated the robustness of the lipid nanoparticles as a function of the applied pressure and their suitability for various technological applications [82].

Other recent examples of time-resolved SAXS include determinations of the structural features upon temperature-triggered phase transitions in lipid assemblies (Fig. 6). The stability of the mixed lipid assemblies and their responses to the environmental conditions has been evaluated [77,84,89]. The thermal behavior observed in the SAXS investigations of Uhrikova *et al*. have revealed that the mixed lipid systems may show considerable structural diversity, coexistence of phases, and aggregated states [98-100]. High concentrations of Zn^{2+} ions have induced a macroscopic phase separation in the lipid mixtures.

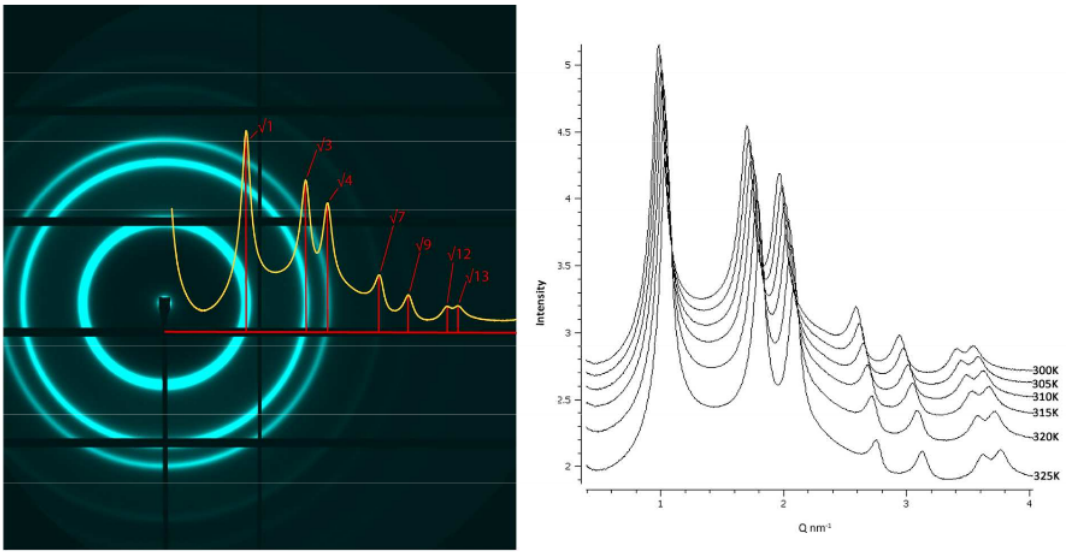


Fig 6. SAXS data demonstrating the temperature-induced variations of the lattice parameter of an inverse hexagonal phase of a mixed lipid system. Reproduced with permission from reference [77]. Copyright (2016) American Chemical Society.

4. BioSAXS studies of liquid crystalline nanocarriers for nanoencapsulation of macromolecular drugs

Cationic lipid carriers have received broad attention towards the development of non-viral gene therapies. BioSAXS studies have been performed to characterize the nanoencapsulation of plasmid DNA (pDNA) and siRNA in nanocarriers [98-121]. In particular, surface-functionalized phospholipid vesicles for DNA encapsulation have been studied by small-angle scattering towards gene delivery applications [121]. The dehydration-rehydration method has been employed in the sample preparation protocol. The size of the plasmid DNA and its

quantity have been critical for the macromolecular encapsulation, which has turn out to vary in the range of 10-90%.

The role of the lipid type for the complexation of plasmid DNA macromolecules in nanocarriers has been emphasized [103,106,108,110,114]. Inverted hexagonal and cubic type lipid carriers, as well as multilamellar assemblies, have been highly efficient for DNA compaction and vectorization [103,106]. Owing to the large size of the plasmid DNA macromolecules and their high charge, dramatic structural phase transitions have been observed upon nanoencapsulation of pDNA in cationic lipid nanocarriers [3,104,108,114]. Figure 7 shows a structural transformation of monoolein-based cationic nanocarriers induced upon the formation of lipoplexes with a plasmid DNA encoding for the therapeutic protein brain-derived neurotrophic factor (BDNF). The strong electrostatic interaction with the cationic nanoparticles and the effect of the rigid plasmid macromolecule on the interfacial membrane curvature have contributed to the formation of ordered nanoassemblies of an inverted hexagonal packing as evidenced by SAXS [104].

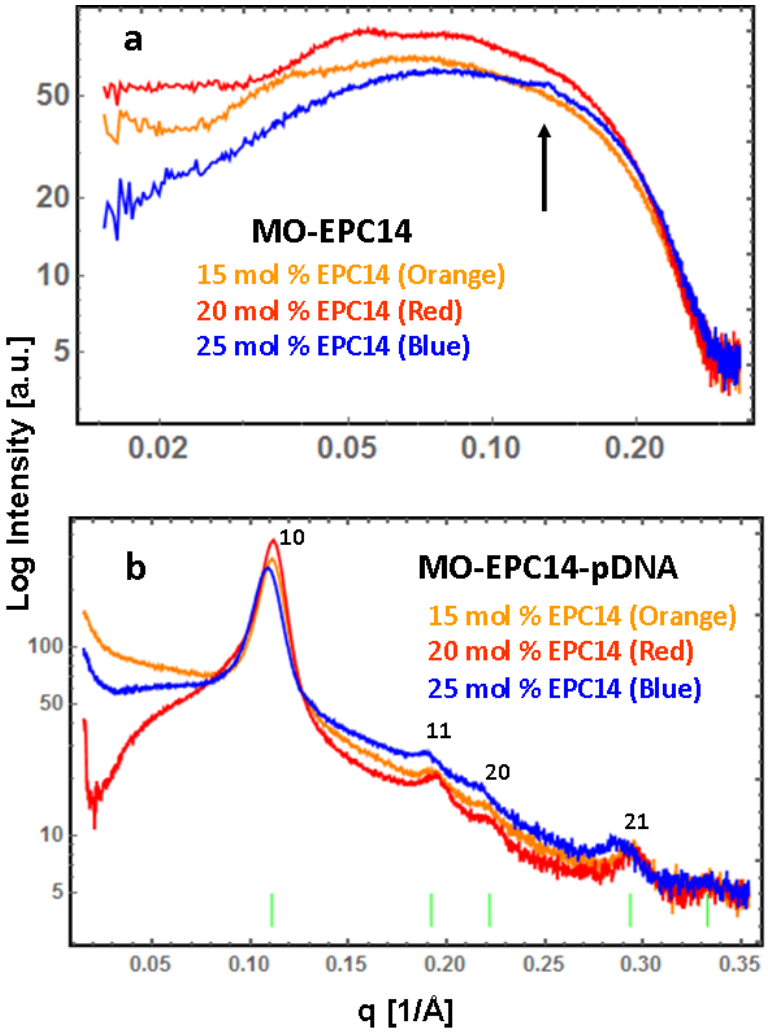


Fig 7. Synchrotron SAXS patterns of aqueous dispersions of self-assembled lipid MO/EPC14/DOPE-PEG_{2000} carriers at varying molar percentages of the cationic component (15, 20 and 25 mol% EPC14) before (a) and after complexation (b) with plasmid DNA (pBDNF). The Bragg peaks of an inverted hexagonal lattice are indexed in (b). Reprinted from Ref. [104] with permission from Elsevier. Copyright (2016) Elsevier Ltd.

Small interfering RNAs (siRNAs), composed of 21–23nt nucleotide duplexes, are key macromolecules that trigger gene-specific inhibition of target genes [101,102,105,107,109]. A number of challenges have hampered the efficient delivery of free siRNA into target cells, ***e.g.*** its high molecular weight, high anionic charge, hydrophilic nature and low stability. Therefore, siRNA necessitates delivery carriers, which should avoid the immune responses associated with the viral vectors. Among the nonviral carries for siRNA delivery (micelles, liposomes, polymers, and nanoparticles), cationic lipid-based carries have been the most extensively developed [101,102,105,107,109]. In this field, the SAXS method has been recognized as a fundamental technique to identify the internal structure of the spontaneously assembled nanocarrier systems and lipoplexes with siRNA.

Martínez-Negro *et al* have studied gemini cationic lipids ($(C_{16}I_m)_2(C_2H_4O)nC_2H_4$ with n = 1, 2, or 3 spacer groups) (GCL) in a mixed assembly with the biocompatible neutral lipid monooleoyl glycerol (MO) as a highly efficient carrier for siRNA delivery [14]. The performed SAXS investigation (Fig. 8) has shown the formation of three lyotropic liquid crystal phases, namely a bicontinuous gyroid cubic phase (*Ia3d*), a micellar cubic phase (*Pm3n*) and a lamellar Lα phase. These mesophases coexisted at certain molar fractions of the Gemini cationic lipids in the mixed lipid nanocarrier. At low Gemini cationic lipid content (high content of MO), two cubic phases have been present: one bicontinuous double gyroid cubic phase (*Ia3d*) and a micellar cubic phase belonging to the *Pm3n* crystallographic space group. When the GCL molar fraction increased, a phase transition from *Ia3d* and *Pm3n* cubic phases into a lamellar Lα phase has occurred. The obtained lipoplexes with siRNA have shown high silencing activity and a negligible cytotoxicity, which has validated their interest for *in vivo* knockdown studies.

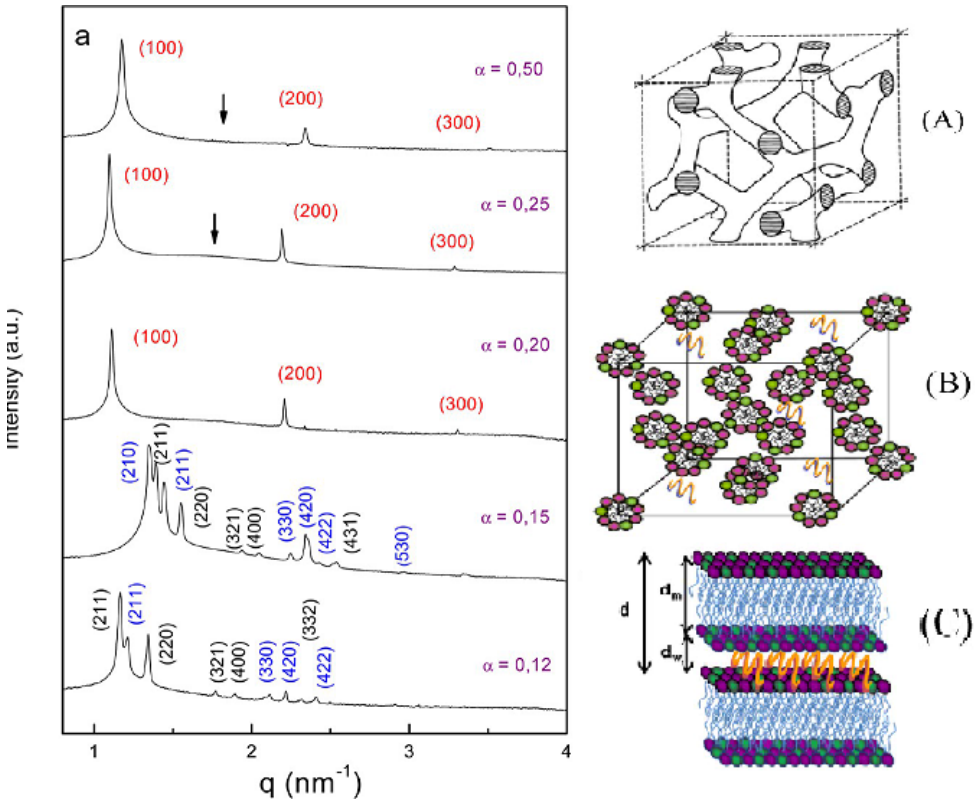


Fig 8. BioSAXS patterns characterizing the assembly of $(C_{16}I_m)_2(C_2H_4O)_3C_2H_4$/MO-siRNA lipoplexes at varying fractions of cationic Gemini lipids (α). Reproduced with permission from reference [14]. Copyright (2016) American Chemical Society. The right panels show schemes of (A) bicontinuous cubic gyroid (*Ia3d*), (B) micellar cubic (*Pm3n*), and (C) lamellar Lα phases.

Borgheti-Cardoso *et al* have designed an *in situ* gel system for encapsulation of siRNA through a detailed structural analysis of the phase behavior of mixtures of monoglycerides, oleylamine, propylene glycol and tris buffer [116]. The oleylamine has been chosen as a cationic lipid to confer a positive charge to the nanocarrier systems. The internal structure of the assemblies has been examined by SAXS. Gindy *et al* have loaded siRNA into lipid nanoparticles consisting of the cationic amphiphile CLinDMA, cholesterol, and a poly(ethylene glycol) conjugate with a dimyristoyl lipid anchor (PEG-DMG) [115]. The performed SAXS investigation has indicated that the lipid nanoparticles undergo a structural reorganization from initially disordered phases into well-ordered lamellar structures upon complexation with siRNA (Fig. 9).

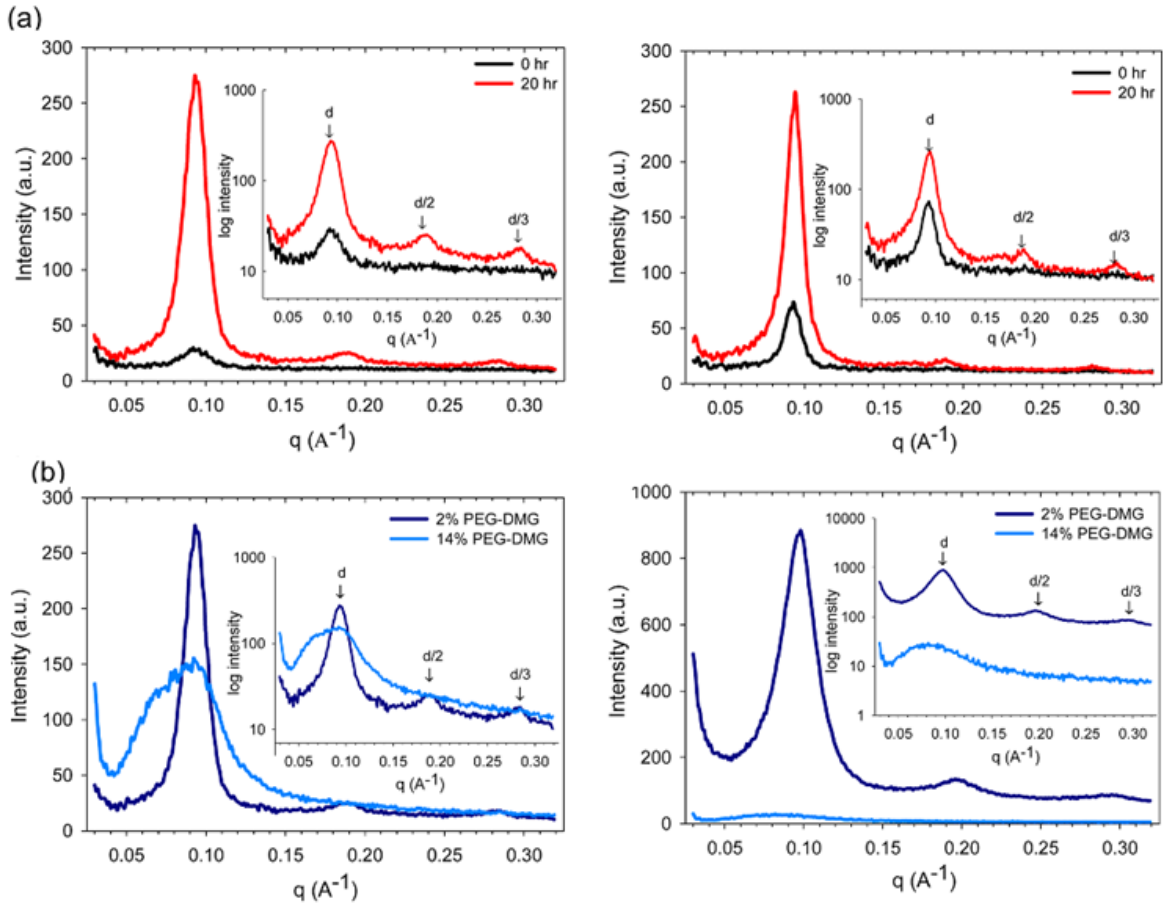


Fig 9. BioSAXS plots of supramolecular structure evolution of lipid nanoparticles for siRNA delivery. Reproduced with permission from [115]. Copyright (2014) American Chemical Society. Structural changes are shown as a function of the concentration of the PEG-DMG conjugate.

In addition to siRNA and plasmid DNA nanoencapsulation, BioSAXS studies have been broadly performed with liquid crystalline nanocarriers for peptide and protein macromolecular drug nanoencapsulation [122-151]. The structural effects of the guest peptide and protein molecules on the nanoorganization of the host lipid phases have been investigated in a number of studies towards optimisation of the stability of the drug delivery systems [122-151]. Charged peptide and protein molecules have exhibited strong influence on the lipid nanostructures as they may bind to the lipid/water interfaces and interfere with the hydration state and the lipid membrane curvature. Self-assembled lipid nanostructures have displayed very high upload capacity for charged proteins and peptides in their aqueous compartments and interfacial regions. Control over the encapsulation rate in cubosome type nanocarriers has been performed through variation of the amphiphilic compositions, which may tune the structural dimensions of the lipid cubic assemblies, the unit cell dimensions, and in particular the aqueous channel diameters in the nanostructure.

Multifunctional lipid-based nanoparticles have been prepared either by encapsulation or surface decoration of the carriers by peptide and protein fragments. The obtained nanoparticles have been successfully used for targeted delivery of nanomedicines both *in vitro* and *in vivo*. Phytantriol cubosomes have been successful for delivery of protein vaccines and the preparation of sustained release vaccine formulations. Nanoparticle-mediated growth factor delivery has shown promise for treatment of Alzheimer's disease models. Topical administration of therapeutic proteins by nanoparticles has been realized through the skin (stratum corneum), the nose, or the inner ear among other *in vivo* applications. Sustained release formulations have been realized with anti-tumor peptides, for instance in breast cancer therapy. Co-administration of tumor-penetrating peptides with cancer drugs, as well as the strategies of enzyme-responsive release, may provide new possibilities for development of future combination therapies. In most of the cases, SAXS is required for characterization of the peptide and protein macromolecular drug encapsulation in the nanostructured lipid systems. Generally, the self-assembled lipid nanomaterial responds by structural changes to the incorporation of the guest peptide and protein pharmaceuticals [122-151].

5. Self-assembled nanocarriers of phytochemical antioxidants and anti-tumor agents

Phytochemicals belong to classes of low molecular weight drugs with a wide range of molecular structures [152-173]. They frequently exhibit very low water solubility, which limits their *in vivo* administration. Nanosized liposomes, microemulsions and lipid nanoparticles have been employed to increase the bioavailability and to lower the toxicity of *Brucea javanica* oil (BJO) (Fig. 10), which shows antitumoral, antimalarial, and anti-inflammatory properties [152-155]. SAXS measurements have been performed to study the internal structures of lipid nanoparticles, with sizes below 250 nm, upon loading with BJO. The nanocarrier dispersions have been prepared *via* the high pressure homogenization method. The uptake and incorporation of drug molecules in the self-assembled nanostructures have been evidenced by SAXS. *Brucea javanica* encapsulation in nanocarriers has been highly efficient in improvement of the cytotoxicity of the formulation against cancer cells [152].

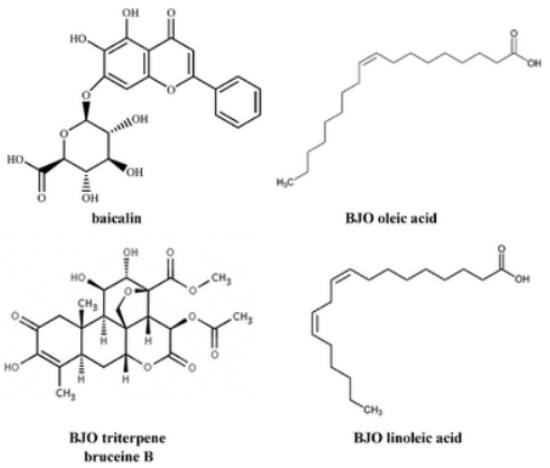


Fig 10. Chemical structures of phytochemical antioxidants and anti-tumor agents successfully encapsulated in nanocarriers. Reprinted from Ref. [10]. Copyright RSC (2015).

Increased bioavailability of baicalin (a multi-purpose drug) has been achieved through incorporation in liposome systems of different lipid/cholesterol content [156]. Long-circulating stealth liposomes with a targeted functionality have been prepared as well. The folate receptor-targeted formulations have been characterized by SAXS and Cryo-TEM, which revealed the formation of unilamellar vesicles. The folate-receptor-targeted liposomes loaded with baicalin, have shown higher cytotoxicity and cellular uptake in comparison to the non-targeted liposomes.

Phytochemical multi-drug encapsulation has also been of great interest for nanocarriers preparation and structural analysis. For example, baicalin and *Brucea javanica* oil have been simultaneously encapsulated in nanoparticles, which exhibited increased drug efficacy in anticancer treatment [10]. SAXS and Cryo-TEM studies have revealed the multicompartment, sponge-type nano-organization of the multidrug-loaded liquid crystalline carriers (Fig. 11).

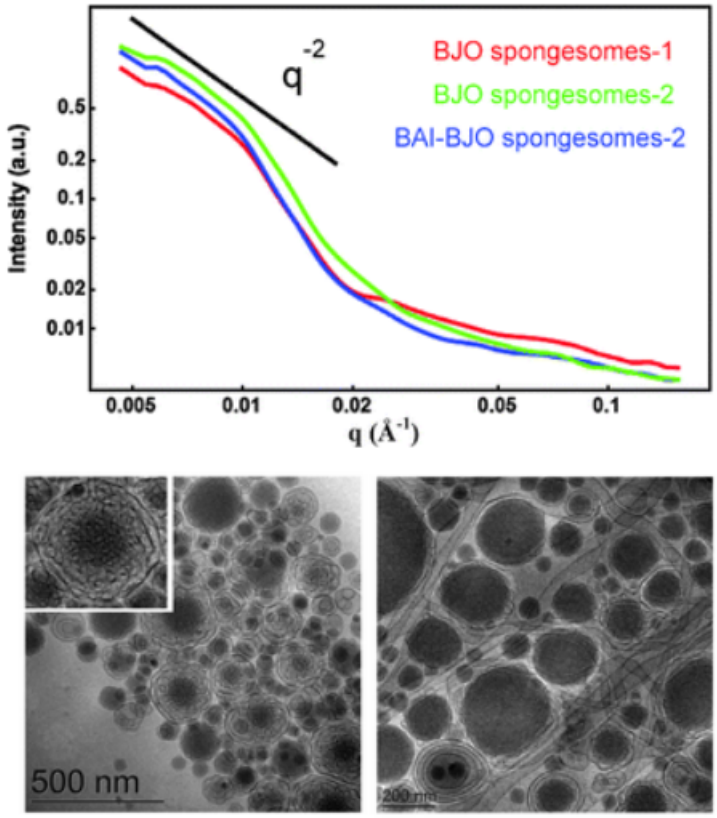


Fig 11. BioSAXS (top panel) and Cryo-TEM (bottom panel) characterization of sterically stabilized spongosomes for multidrug delivery of anticancer nanomedicines. Reproduced with permission from Ref. [10]. Copyright RSC (2015).

The low solubility of curcumin (extracted from *Curcuma longa*) has been a serious problem for its use in the development of anti-inflammatory, anti-cancer and neuroregenerative nanomedicines [158]. Various nanoparticulate types have been designed to solve this challenge [158,162-164]. Lipid cubic nanoparticles of sizes around 170 nm have been used as nanocarriers of curcumin. They have increased its bioavailability upon oral administration in rats of up to 400% as compared to free curcumin [163]. In addition, curcumin has been incorporated in monoolein-based aqueous dispersions of nanoparticles prepared with different mixtures with sodium cholate, sodium caseinate, bentonite and poloxamer [162]. Heterogeneous dispersions of unilamellar vesicles, cubosomes and sponge type phases have been established by structural analysis (X-ray diffraction and Cryo-TEM). It has been suggested that the encapsulated curcumin compound may increase the negative curvature of the polar/apolar interfaces of the lipid nanoassemblies and therefore may stabilize the cubic phase formation [162]. Lipid-based carriers with encapsulated curcumin have shown improved physical stability when stabilized by whey protein and modified starch. The nanocarriers have been bigger in size (~ 270 nm) and have displayed controlled shell

degradation during simulated digestion [164]. Curcumin nanocarriers stabilized by polysorbate have been smaller in size (120 nm) and have exhibited lower relative uptake at the intestinal digestion phase [164].

Lipid-based water-in-oil microemulisions and micelles have been successfully applied as carriers of natural antioxidants (gallic acid, p-hydroxybenzoic acid, protocatechuic acid and tyrosol) [159]. Structural studies have shown that depending on the emulsifier charge (cationic or anionic/non-ionic type) the interaction with the antioxidants may lead to a shape transition from spherical micelles to rod-like micellar assemblies. The encapsulation of polyphenol antioxidants in multilamellar vesicles has been quantified through high encapsulation rate, namely ~ 16% of antioxidant has remained encapsulated even after 30 days of storage in aqueous phase [161].

Synergistic effects of the antioxidants rutin and vitamin E have been achieved through optimisation of the composition of self-nanoemulsifying drug delivery systems containing oil, surfactant and co-surfactants. The optimised formulation in terms of globule size, self-emulsification time, transmittance and cumulative percentage of drug release, has resulted in ~2 fold increase in the relative oral bioavailability and antioxidant activity in *in vivo* and *in vitro* tests [161]. Nanoemulsions of larger droplet sizes (~ 100 nm) prepared by a spontaneous emulsification method, followed by high-pressure homogenization, formed a system suitable for solubilization of resveratrol as antioxidant and vitamin E added to the oil phase. The formulation has shown a potential to permeate through the nasal mucosa and to serve as a proper carrier of antioxidant molecules to the blood brain barrier [160]. Incorporation of phenylethyl resorcinol in cubic nanostructured lipid carriers has led to increased accumulation in the skin [159].

The solubility of quercetin, which exhibits higher antioxidant activity compared to rutin, has been increased up to 5% by using lipid mixtures of soy phosphatidylcholine and glyceroldioleate with small addition of ethanol in the encapsulation matrix [165]. X-ray scattering has shown the appearance of different non-lamellar bulk liquid crystal matrices under hydration. Depending on the lipid composition, the loading of quercetin has had no effect on the parameter of the H_{II} phase (at low soy phosphatidylcholine content). It has slightly increased the unit cell dimension of the cubic phase and shifted the system towards a solution of reverse micelles at higher temperatures (at high soy phosphatidylcholine content). The effect of quercetin has been explained by its location in the lipid hydrocarbon chain region. Using P80 as a amphiphilic stabilizer, it has been possible to prepare nanoparticle dispersions with tunable size from 80 to 210 nm at varying lipid compositions and concentrations of quercetin [165].

Optimisation of cubic phase nanocarriers for oral administration has been of strong interest [63]. The oral bioavailability of simvastatin and amphotericin B (poor water-soluble drugs) has been significantly enhanced with cubic nanoparticles (150-300 nm size) by ~ 2-

fold as compared to the crystal powders [59]. SAXS measurements have shown the stability of doxorubicin-loaded phytantriol-based cubic liquid crystalline nanoparticles in simulated gastrointestinal fluids. Moreover, higher cell cytotoxicity of the nanocarriers loaded with an antitumor agent (~ 16-fold) against MCF-7 cell lines has been recorded in comparison to the free drug [143].

Controlled release of donepezil (a drug used for Alzheimer disease treatment) has been achieved using hexagonal phase carriers formed by a monoglyceride lipid and OA in water as a precursor of a lyotropic lipid crystalline mesophase for intramucosal administration [61]. SAXS and Cryo-TEM have been used to determine the amphiphilic composition (phytantriol and oleic acid) enabling the vesicle to hexosome nanocarrier transition at different pH values (between pH 6.7 and 7.7). This pH-induced phase transition from stable vesicles to dispersed inversed hexagonal nanoparticles (hexosomes) enables the adsorption at the mucosal surface. Therefore, it can be used in targeted drug delivery to the buccal mucosa with some advantages over the oral delivery to the gastrointestinal tract [61].

Confocal laser scanning microscopy has confirmed the ability of fluoro-labeled cubosomes (~ 270 nm) to penetrate through the whole human abdominal skin layers [62]. Nanodispersions composed of monoolein and oleic acid have been characterized by SAXS as a monodisperse system (~150 nm) of hexagonal liquid-crystalline phase particles, which have been chosen as nanocarriers to improve the topical retention of the photosensitizer protoporphyrin IX *via* skin penetration [26]. Skin penetration studies have confirmed that the chosen formulations increases the skin uptake ~ 10 (*in vitro*) and ~20-fold (*in vivo*) as compared to the uptake of control formulations without skin irritation [26].

Percutaneous administration of indomethacin (a model anti-inflammatory drug) encapsulated in monooleine/poloxamer dispersions has shown a sustained drug release. The X-ray diffraction study of the bicontinuous cubic phase of the *Im3m* (Q^{229}) symmetry has suggested that this structure may be responsible for a lower flux with respect to the analogous formulation containing the free drug in aqueous phase and to a control formulation based on a carbomer gel [53]. Cubosomal nanoparticles containing the hydrophilic anticancer drug 5-fluorouracil have been tested for targeting of cancer cells in liver (rat model). Nanoparticles (100 nm) administration has increased the concentration of the drug in the liver (nearly 5-fold) as compared to that of a free drug solution [15]. The nanotechnology for simultaneous use of anticancer drugs in a combination against different cancer cells, using nanoliposomes and other nanocarriers, is under development [166-173].

In all these structural studies, special attention has been paid to the interaction between the phytochemical or the anti-cancer drug molecules and the components of the lipid matrix. For instance, SAXS results have indicated that loading of the anaesthetic bupivacaine in nanostructures of glycerol monooleate may induce a phase transition from inverted-type bicontinuous cubic phase to an inverted-type hexagonal structure ($Pn3m \rightarrow H_{II}$) [65]. The

addition of medium-chain triglycerides (a mixture of caprylic and capric acid) into the lipid matrix has led to further influence of bupivacaine on the phase sequence and a transformation to an inverted type microemulsion (L_2). Therefore, SAXS and X-ray diffraction investigations appear to be needed for the design and controlled preparation of every self-assembled drug delivery system of interest.

6. Conclusion

Lipid-based nanoparticle carriers have gained increasing recent interest in biomedical and nanotechnological applications. Depending on the packing symmetries and densities of the lipid bilayer building blocks, various vesicular and multicompartment liquid crystalline nano-objects involving cubosomes, spongosomes, onion-like liposomes, or hierarchical supramolecular membranous nanoarchitectures, have been prepared and studied in terms of inner organization and structural complexity. The obtained nanostructures have displayed high solubilization and encapsulation capacities for hydrophilic, lipophilic and amphiphilic guest molecules, as well as the ability to protect the active molecules against hydrolysis or oxidation. Both the nanoparticles shapes and internal structures are essential for the drug delivery process and the interaction with the living cells and target sites.

Loading of small phytochemical molecules generally does not interfere with the sizes of the aqueous channel in cubic lipid assemblies, but may essentially modify the overall phase state and behaviour of the nanocarriers at high drug upload. The monoolein-based cubic phase nanocarriers are characterized by bigger aqueous channels with regard to the phytantriol-based cubic phase systems, and therefore have been more often used as matrices for encapsulation of macromolecular drugs (for instance proteins). BioSAXS studies of cationic lipid carriers for oligonucleotide, siRNA and plasmid DNA nanoencapsulation have revealed significant structural changes and topological shape transitions upon upload of the therapeutic macromolecules. In perspective, such structural investigations should be performed also with the novel class of CRISPR/Cas 9 therapeutics as macromolecular drugs for genome editing in pathological states.

Structural SAXS studies on encapsulation of small- and large-molecular weight therapeutic substances in lipid-based nanocarriers have markedly contributed to the success of the structure-based nanomedicine design. Moreover, lipid-based nanodelivery systems provide potential for development of novel combination therapies, for which poorly soluble phytochemical ingredients of pharmaceutical interest may be co-encapsulated with active macromolecular drugs in controlled-release formulations.

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