

### Self-Assembled Multicompartment Liquid Crystalline Lipid Carriers for Protein, Peptide, and Nucleic Acid Drug Delivery

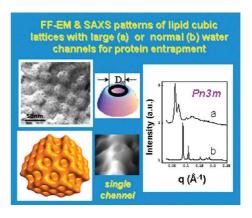
ANGELINA ANGELOVA,\*,† BORISLAV ANGELOV,‡,§,⊥
RADA MUTAFCHIEVA,¹ SYLVIANE LESIEUR,† AND
PATRICK COUVREUR†

<sup>†</sup>CNRS UMR8612 Physico-chimie-Pharmacotechnie-Biopharmacie, Univ Paris Sud, Châtenay-Malabry, F-92296 France, <sup>‡</sup>Department of Chemistry and iNANO, Aarhus University, Denmark, <sup>§</sup>Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Czech Republic, and <sup>⊥</sup>Institute of Biophysics, Bulgarian Academy of Sciences, BG-1113 Sofia, Bulgaria

RECEIVED ON SEPTEMBER 3, 2010

### CONSPECTUS

ipids and lipopolymers self-assembled into biocompatible nanoand mesostructured functional materials offer many potential applications in medicine and diagnostics. In this Account, we demonstrate how high-resolution structural investigations of bicontinuous cubic templates made from lyotropic thermosensitive liquid-crystalline (LC) materials have initiated the development of innovative lipidopolymeric self-assembled nanocarriers. Such structures have tunable nanochannel sizes, morphologies, and hierarchical inner organizations and provide potential vehicles for the predictable loading and release of therapeutic proteins, peptides, or nucleic acids. This Account shows that structural studies of swelling of bicontinuous cubic lipid/water phases are essential for overcoming the nanoscale constraints for encapsulation of large therapeutic molecules in multicompartment lipid carriers.



For the systems described here, we have employed time-resolved small-angle X-ray scattering (SAXS) and high-resolution freeze-fracture electronic microscopy (FF-EM) to study the morphology and the dynamic topological transitions of these nanostructured multicomponent amphiphilic assemblies. Quasi-elastic light scattering and circular dichroism spectroscopy can provide additional information at the nanoscale about the behavior of lipid/protein self-assemblies under conditions that approximate physiological hydration.

We wanted to generalize these findings to control the stability and the hydration of the water nanochannels in liquid-crystalline lipid nanovehicles and confine therapeutic biomolecules within these structures. Therefore we analyzed the influence of amphiphilic and soluble additives (e.g. poly(ethylene glycol)monooleate (MO-PEG), octyl glucoside (OG), proteins) on the nanochannels' size in a diamond (D)-type bicontinuous cubic phase of the lipid glycerol monooleate (MO). At body temperature, we can stabilize long-living swollen states, corresponding to a diamond cubic phase with large water channels. Time-resolved X-ray diffraction (XRD) scans allowed us to detect metastable intermediate and coexisting structures and monitor the temperature-induced phase sequences of mixed systems containing glycerol monooleate, a soluble protein macromolecule, and an interfacial curvature modulating agent. These observed states correspond to the stages of the growth of the nanofluidic channel network.

With the application of a thermal stimulus, the system becomes progressively more ordered into a double-diamond cubic lattice formed by a bicontinuous lipid membrane. High-resolution freeze-fracture electronic microscopy indicates that nanodomains are induced by the inclusion of proteins into nanopockets of the supramolecular cubosomic assemblies. These results contribute to the understanding of the structure and dynamics of functionalized self-assembled lipid nanosystems during stimuli-triggered LC phase transformations.

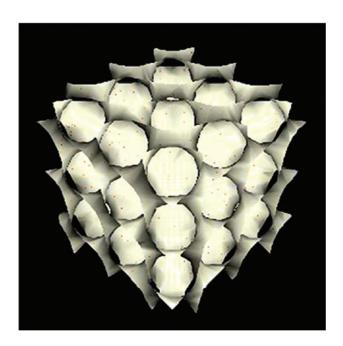
#### 1. Introduction

Current nanotechnology advancements in characterization of multicompartment lipidic nanocarriers and nanoporous templates with hierarchical architecture for encapsulation of biopharmaceuticals involve high-resolution structural studies of lyotropic thermosensitive LC assemblies that may accommodate therapeutic proteins, peptides, or nucleic acids. 1-17 The investigations, by means of time-resolved SAXS, of the dynamic topological transitions of selfassembled amphiphilic mixtures have allowed the discovery of lipid-based nanocarriers with tunable internal organizations and nanochannel sizes. 18-32 The phase states of nonlamellar lipids, amphiphilic mixtures with lipopolymers or lipophilic conjugates, such as peptide surfactants, squalenoyl (SQ) derivatives, and acylated and peptidolipidyl-modified cyclodextrins, have been studied toward the design of novel controlled drug delivery nanocarriers with enhanced performance. 33-53

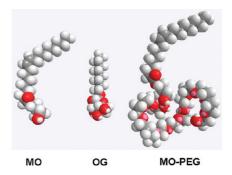
In this Account, we discuss structural studies of lipid and lipid—polymer self-assemblies in aqueous phase that characterized the nanochannel network organization of amphiphilic vehicles for entrapment of proteins and peptides, as well as of DNA. SAXS investigations established the mechanism of nanoconfinement of proteins in multicompartment lipid carriers, which is essential for slow release processes. Multicompartment phospholipid containers comprising a large outer vesicle membrane (0.1–1.0  $\mu$ m) with a liquid reservoir encapsulating a dispersion of small vesicles (20-500 nm), known as vesosomes, will not be discussed in this Account, which mostly focuses on bicontinuous selfassembled structures formed by monoglyceride lipids and on the control of the nanochannel sizes in cubosome vehicles. Actually, phospholipid nanoparticles entrapping therapeutic proteins in their aqueous reservoirs (liposomal nanomedicines) represent one of the most advanced classes of drug delivery systems.<sup>3</sup> Forefront work on long-circulating phospholipid nanoparticles for gene delivery has been reported with sterically stabilized (PEGylated) lipid/DNA lipoplex nanoparticles.<sup>8</sup> It is anticipated that the growing scientific interest in high-resolution structural investigations of particulate lipid carriers with inner multicompartment organizations will accelerate the foreseen clinical applications.

# 2. Self-Assembled Lipid Nanocarriers with a Multicompartment Cubosomic Architecture

Figure 1 shows the three-dimensional (3D) organization of a lipid/water assembly of a bicontinuous cubic structure characterized by the crystallographic space group *Pn3m*. The double-diamond (D-type) architecture reveals two cubic

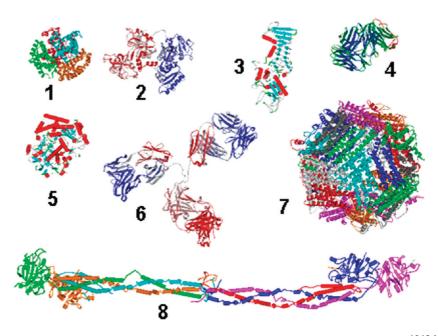


**FIGURE 1.** Three-dimensional nodal surface presentation of a lipid nanocarrier with multicompartment organization resulting from a bicontinuous double-diamond (*Pn3m*) cubic lattice structure.



**FIGURE 2.** Space-filling molecular models of glycerol monooleate (MO), octyl glucoside (OG), and poly(ethylene glycol)monooleate (MO-PEG).

networks of nanochannel compartments that are separated by lipid bilayer membrane compartments. The inherent multicompartment organization provides improved encapsulation efficiency for biomolecules (related to the high surface-to-volume ratio) and favors slow release processes. Small proteins could be accommodated and protected either in the aqueous channel compartments (water-soluble proteins) or in the lipid bilayer compartments (membrane proteins). The stability of the inverse bicontinuous cubic phase is governed by the curvature energy of the lipid monolayers, the stretching energy of the hydrocarbon chains, and the thermal fluctuations of the lipid bilayer. These energies are functions of the curvature, thickness, and rigidity of the lipid membrane. In general, multicompartment amphiphilic structures can be produced by self-assembly of (i) mixtures involving lipids with



**FIGURE 3.** Three-dimensional representations of protein molecules that can be encapsulated in lipid cubic phases: 10,13,48,49,54,58,61 (1) hemoglobin; (2) apo-transferrin; (3) serratiopeptidase; (4) Fab fragment of immunoglobulin; (5) glucose oxidase from *Aspergillus niger*; (6) immunoglobulin; (7) ferittin; (8) fibrinogen. The macromolecule sizes are comparable to or bigger than the nanochannel diameter in a MO normal cubic phase.

nonlamellar propensity,<sup>5,6,9,11–15,17,18,33,35–41,46–50</sup> (ii) mixtures of amphiphiles and hybrid lipid derivatives with nonlamellar properties, for instance, squalenoyl compounds,<sup>4,20</sup> or (iii) lipid/peptide self-assemblies forming inverted-phase LC nanostructures.<sup>51,52</sup>

Nonionic lipids, such as glycerol monooleate (MO) (Figure 2), are suitable for protein stabilization. 10-14,35-37,48-50,54,55 The self-assembled MO/water LC phases 9,11,38,54,57-61 represent a biocompatible, nanostructured lipid membrane medium with tunable hydration, which offers new opportunities for design of protein and peptide carriers with multicompartment architecture and controlled release properties. 10,16,19,26,55,57 The thermoreversible lamellar-to-cubic phase transitions of polar monoglyceride lipids have attracted huge research interest 5,6,9,12,13,15,17,18,38-40 in view of various applications including peptide and protein drug delivery.

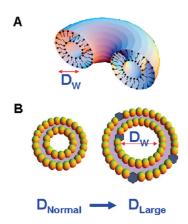
### 3. Biomolecular Uptake and Release Governed by the Hydration Level of Multicompartment Cubosomic Carriers

Studies of hydration and swelling of nonlamellar lipid phases, formed by glycerol monooleate, monolinolein, or phytantriol, <sup>35,38,56,57</sup> and in particular of their bicontinuous cubic assemblies constitute a fundamental step in the invention of nanocarriers with inner multicompartment organizations. The maximum water uptake is sensitive to temperature<sup>35,38</sup> and

determines the 3D cubic membrane surface-to-volume ratio that controls the drug loading and the slow release of protein and peptide drugs. 16,17,56

Pioneering studies on protein-containing MO/water systems have been conducted at low hydration levels corresponding to about 40 wt % aqueous phase and 60 wt % lipid. The protein nanoconfinement interfered with the structural impact of the macromolecules (Figure 3) on the self-assembly organization of the lipid, which competes with the proteins for hydration water. Under these conditions, bicontinuous cubic gyroid (*la3d*) or primitive cubic (*lm3m*) phases have formed in some of the partially hydrated systems. Str. Such phases have not formed in fully hydrated MO/water assemblies in the absence of proteins.

The diameter,  $D_{\rm w}$ , of the aqueous channels in a double diamond (Pn3m) lipid cubic lattice of a normal type ( $D_{\rm Normal}$ ) (Figure 4A) is usually in the range<sup>14,49,50</sup> of 2.5–3.5 nm. The  $D_{\rm w}$  value can be estimated after the determination<sup>53</sup> of the cubic lattice parameter, a, and the lipid bilayer thickness,  $L:D_{\rm w}=0.707a-L$ . Despite the fact that the nanochannel size in the cubic lattice<sup>38</sup> formed by the hydrated MO does not exceed the dimensions of medium and large proteins, like apo-ferittin<sup>10</sup> (474 kDa), transferrin<sup>13,48,49</sup> (75 kDa), immunoglobulin<sup>13,49</sup> (145 kDa), and fibrinogen<sup>49</sup> (420 kDa) (Figure 3), these proteins have been entrapped in self-assembled lipid cubic phases. Structural SAXS investigations have concluded that large enzyme macromolecules, like



**FIGURE 4.** (A) Cross section of a nanochannel compartment with a diameter  $D_{w}$ , surrounded by a lipid bilayer and (B) nanochannel swelling caused by the inclusion of an interfacial curvature modulator in the lipid bilayer compartment. The increase of the channel diameter ( $D_{w}$ ) upon the transition from a  $D_{Normal}$  to a  $D_{Large}$  cubic phase is associated with a decrease in the membrane curvature.

glucose oxidase and ceruloplasmin, cannot be located exclusively in the water channels of the nanoperiodic cubic templates. <sup>59,60</sup> Toward mimicking the physiological conditions for protein drug delivery, the research is being extended to fully hydrated cubic lipid systems for protein nanoconfinement. <sup>11,14,50,56,64–70</sup>

# 4. Cubic Phase Swelling via Stimuli-Induced Modulation of the Membrane Curvature or Thickness

The lipid/water bicontinuous cubic networks are responsive to external stimuli. Tuning of the aqueous nanochannel sizes (Figure 4) constitutes a means for control of their encapsulation capacity for biomolecules. In order to get insight into the mechanism of nanoconfinement of protein, peptides, or DNA in labyrinthine cubic network structures,  $^{10-15,45-50,57-59}$  we discuss the monoolein cubic phase swelling as related to the changes in the lipid membrane curvature. The spontaneous monolayer curvature of the monoglyceride lipid assemblies is negative. The critical packing parameter of the amphiphile MO, favoring nonlamellar structures, has been estimated HO, favoring nonlamellar structures, has been estimated to be  $\eta=1.07$ . Alterations in the shape of the monoolein molecules can be effected by pressure or temperature stimuli,  $^{38,58}$  which modify the interfacial curvature.

Swelling of the diamond-type bicontinuous cubic phase can be induced by additives, which promote the fine diminution of the membrane curvature or thickness. Examples of cubic lattice swelling have been reported with self-assembled mixtures of MO with charged lipids, charged lipid-like peptides and cationic and nonionic surfactants. <sup>9,12,46,47,62–70</sup> X-ray diffraction (XRD)

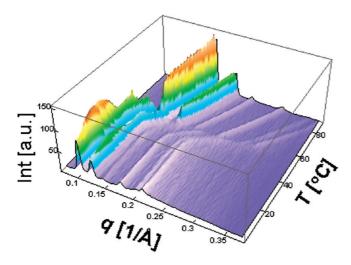
studies<sup>62,63</sup> have established that the addition of 10 wt % of the anionic lipid distearoylphosphatidylglycerol (DSPG) to the MO/water system leads to increased hydration, flattening of the bilayer curvature, and widening of the water channels in the diamond-type (Pn3m) cubic phase. The cationic surfactant lauroylcholine (LCh), having a peptide-like polar head, <sup>65</sup> and certain alkylated cationic and anionic peptides (lipid-like peptides), acting as peptide surfactants, <sup>9</sup> have displayed a capacity to modulate the bilayer curvature and the stability of the monoglyceride Pn3m cubic phase via interactions with the lipid membrane interface. <sup>9</sup> The water channel size,  $D_{\rm w}$  (Figure 4), in the MO cubic phase has been tuned via the anionic peptide surfactant concentration at low peptide content.

SAXS investigations have revealed that nonionic detergents such as octylglucoside (OG) and dodecyl maltoside (DM) exhibit a profound effect on the MO liquid-crystalline structure hydration. The monolayer curvature is reduced upon accommodation of such surfactant molecules at the apolar/polar interface and the aqueous nanochannels are enlarged (Figure 4B). This corresponds to a cubic phase swelling and a structural change to a less curved structure with larger water channels  $^{46}$  ( $D_{\rm w} \approx 7$  nm). The bicontinuous Pn3m cubic phase tolerates high concentrations of nonionic detergents before a phase transformation to a lamellar phase occurring at elevated surfactant content.  $^{12,47}$ 

Beyond the maximal hydration level ( $D_{\rm Large}$  phase) that can be attained with a diamond-type cubic phase with large water channels<sup>46</sup> upon augmenting the interfacial curvature modulator concentration,<sup>68–70</sup> the swelling can proceed through a phase transition to sponge phases.<sup>70</sup> The latter are lacking long-range order and their aqueous cave compartments could be up to three times larger than the nanochannels in a normal bicontinuous cubic phase. This can also serve for enhanced loading of biomolecules.

# 5. Encapsulation of Peptides, Proteins, or DNA in Inherently Multicompartment Lipid Phases

DNA encapsulation in lipid medium has been intensively investigated toward preparation of nonviral carriers of genetic drugs. TRD studies have revealed that self-assembled complexes of DNA with charged lipids could form inverted hexagonal or cubic LC phases. Owing to the rigidity and the large size of the DNA strains, it is impossible to accommodate DNA macromolecules (MW  $\approx 6\times10^6$  Da) in the nanochannels of a lipid cubic structure. As a result, the encapsulated DNA adopts a confined state. The transport properties of the cubic phase, being dependent on

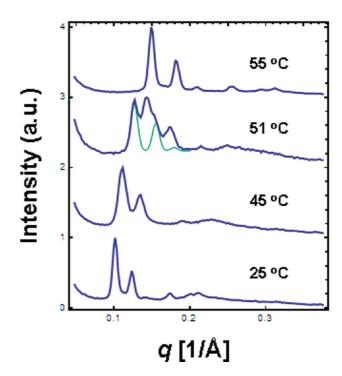


**FIGURE 5.** Time-resolved synchrotron radiation small-angle X-ray diffraction patterns (scan rate 2 °C/min) of a fully hydrated self-assembled MO/OG/transferrin mixture (amphiphiles molar ratio MO/OG 95/5 mol/mol; protein concentration 4 mg/mL; aqueous phase 0.1 M NaCl,  $10^{-2}$  M phosphate buffer, pH 7) recorded as a temperature dependence of the scattering intensities vs scattering vector  $\mathbf{q}$  (Å $^{-1}$ ). The peaks set with characteristic ratios,  $\sqrt{2}/\sqrt{3}/\sqrt{4}/\sqrt{6}/\sqrt{8}/\sqrt{9}/\sqrt{10}$ , of the q-values of the scattering maxima exists at all temperatures and identifies the (110), (111), (200), (211), (220), (221), and (310) reflections of a D-type bicontinuous cubic lattice of the Pn3m space group.

the nanochannel diameter, do not permit diffusion of entrapped large DNA molecules along the cubic network architecture. <sup>10</sup>

The incorporation of globular proteins in lipid cubic phases could also be hampered as a function of the size of the water channels or as a result of interactions that can modify the structure of the cubic phase and of the protein. 12,17,48–50,55,56 Several works have stressed that the incorporation of proteins or peptides into a normal diamond-type (*Pn3m*) cubic phase could induce its transformation to other lyotropic phase states. 10,37,49,55,57,59,60

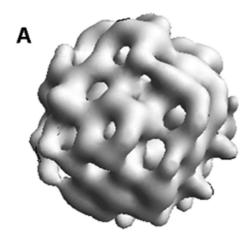
Our investigations, performed under full hydration of the lipid, show that the protein effect  $^{13,48,49}$  on the cubic lattice swelling is less significant. SAXS studies revealed that transferrin, a medium-size protein, changes the nanochannel hydration and swelling (the  $D_{\rm w}$  size) without modification of the cubic crystallographic space group. Figure 5 shows time-resolved SAXS data recorded with a MO/OG/transferrin/buffer system during a temperature scan from 1 to 95 °C. Neither lamellar phases nor an inverted hexagonal (H\_{II}) phase are established in the investigated broad temperature interval. The supramolecular organization of the protein carrier is entirely dominated by double diamond (D-type) bicontinuous cubic structures stable up to at least 95 °C.

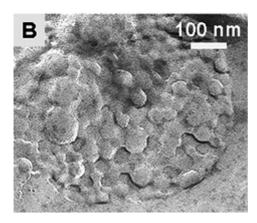


**FIGURE 6.** Selected SAXS frames from the dynamic temperature scan (Figure 5) with pattern acquisition at temperatures T=25, 45, 51, and 55 °C. The lattice parameters of the swollen cubic structures (Pn3m periodicity) are a=14.0 nm at T=25 °C and a=12.72 nm at T=45 °C. At T=51 °C, intermediate states are established in the phase coexistence region with swollen and normal-type cubic lattices for which  $a_{D_{\rm Large}}$  cubic =11.15 nm (L=3.30 nm) and  $a_{D_{\rm normal}}$  cubic =9.9 nm (L=2.92 nm). The green curve plotted at T=51 °C indicates the fitted peaks intensities for the  $D_{\rm Large}$  swollen cubic structure. A less hydrated, single cubic phase of the  $D_{\rm Normal}$  type prevails at T=55 °C with a lattice parameter  $a_{D_{\rm normal}}$  cubic =9.5 nm.

Figure 6 presents selected SAXS frames from the performed heating scan. They demonstrate the key alterations in the LC structure induced by the thermal stimulus. The maximal value of the cubic lattice parameter, determined at  $25\,^{\circ}$ C, is  $a = 14.0\,$ nm and corresponds to a channel diameter  $D_{\rm w} = 7.03\,$ nm. The dehydration provoked at temperatures above  $45\,^{\circ}$ C leads to the progressive transformation of the cubic  $D_{\rm Large}$  phase into a  $D_{\rm Normal}$  cubic structure. At temperature  $51\,^{\circ}$ C, a coexistence of a water-swollen phase, with large water channels ( $D_{\rm Large}$ ), and a normal diamond ( $D_{\rm Normal}$ ) cubic phase is established (Figure 6).

The swollen cubic phase states, with channels larger than the protein size, are stable also at body temperature (see Supporting Information for details). A small quantity of OG (5 mol %) appears to be efficient for enlargement of the aqueous compartments in self-assembled MO/OG cubic nanostructures through a smooth curvature change and increased hydration of the lipid lattice  $(D_{\rm w}>6~{\rm nm})$ .



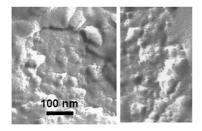


**FIGURE 7.** (A) Nodal surface modeling of a cubosome containing nanopockets for entrapment of protein molecules in the 3D cubic assembly. (B) Freeze-fracture electron microscopy image of a protein-loaded MO particle displaying cubosomal inner organization.

### 6. Mechanism of Encapsulation of Biomacromolecules in Functionalized Cubic Phases via Induction of Pocket Defects or Sponge-Like Disorder

Considering that the hydrodynamic diameter of globular proteins (Figure 3) is approaching or exceeds the size of the aqueous nanochannel diameter in the cubosome carrier (Figure 4), a question is raised about the location of the entrapped proteins. The SAXS results revealed that the structural organization of proteocubosome assemblies, characterized by a swollen D-type cubic lattice, remains in the same crystallographic space group upon encapsulation of a protein (Figures 5 and 6). Because the protein macromolecules are not able to locate inside the aqueous channels of the normal diamond cubic lattice ( $D_{\rm w} \approx 3$  nm), they might be encapsulated in cubosomal pockets of the supramolecular architecture.

An element of a proteocubosome carrier was modeled using mathematical algorithms as described in refs 11 and

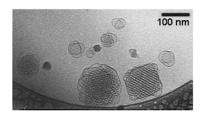


**FIGURE 8.** Freeze-fracture electron microscopy image of nanocubosomes obtained from MO/MO-PEG/protein/water cubic phase. The fracture section appears nonuniform and reveals nanodomains and nanopockets in the weakly ordered multicompartment organization of the PEGylated proteocubosome carrier.

50. Geometrically, the cubic bilayer membrane can fold like an infinite periodic minimal surface. However, the lipid bilayer cannot be infinite in real soft-matter nanoparticles obtained by fragmentation of self-assembled amphiphilic cubic phases. The cubic structure has bounding surfaces in space. Figure 7A shows the 3D organization of a cubosome constituted by a weakly ordered D-type cubic membrane intertwined with large aqueous channels. The presence of nanopockets, which are randomly distributed in the template of cubic symmetry, should facilitate the entrapment of proteins in the cubosome particle. The encapsulation of biomolecules is sensitive to the structural asymmetry. Indeed, the diamond-type cubic particle involves two networks of aqueous channels.11 Upon contact with the medium containing dissolved biomolecules, one of the nanochannel networks is open, while the other is closed. Such an asymmetry may have a role in the protein entrapment through the pockets in the cubic structure.

Protein-loaded cubosomes have been obtained upon spontaneous lipid/protein assembly with proteins of amphiphilic character. Freeze-fracture electron microscopy investigations established the hierarchical structural organizations of the proteocubosomes (Figures 7B and 8). The lipid/protein nanoassemblies appear to be built-up by cubosomal nanodroplets (Figure 7B). They lack a perfect single crystal organization. The entrapped proteins might be confined in the created nanopocket defects.

Figure 8 presents nanodomain patterns in a section of a PEGylated MO/protein cubic phase. It exhibits random orientations of multicompartment domains of a cubic symmetry. The FF-EM imaging confirms the concept of "nanopockets" for inclusion of biomolecules. The proteins may locate at the interfaces between the nanodroplet fragmented cubosome particles or associate with the surface of the interconnected cubosomal entities. <sup>49,50</sup> Our study indicates that functionalization by 1 mol % colipid (MO-PEG2000)



**FIGURE 9.** Cryo-TEM image of glycerol monooleate/Poloxamer 403 (92/8 w/w) cubosome particles produced by sonication (Adapted from ref 19).

does not modify the cubic symmetry, established by XRD, but it has an effect on the local nanodomain topology involving states of lower crystalline order ("sponge-like" local disorder).

# 7. Aqueous Dispersions of Multicompartment Liquid Crystalline Particles

The fragmentation and dispersion of lipid cubic phases into LC nanoparticles with inner structure (Figure 9) has often been done by the copolymer Pluronic F127 or Poloxamer 407 displaying detergent properties. 19,22–24,26,27 The mechanism of fragmentation of the bicontinuous cubic membranes has not been fully elucidated. The established procedures for cubosome production employ powerful energy techniques such as high shear homogenization by microfluidization, ultrasonication, and high-temperature treatment in an autoclave. 24

Nanoparticles with well-ordered internal cubic structure and periodic bilayer inner organization have been prepared upon heat treatment to a temperature that is above the cloud point of the stabilizing copolymer.<sup>24</sup> At ambient temperatures, the dispersed systems predominantly consist of coexisting cubic and vesicular particles. Cryo-TEM investigations have demonstrated<sup>24</sup> that the majority population of the nanoparticles has vesicle morphologies, which can fuse into nonequilibrium aggregates. The heat treatment at 125 °C induces the transformation of the bilayer vesicles into cubic nanostructures. The resulting MO/Pluronic F127 and MO/oleic acid/Pluronic F127 nanoparticles with cubic Pn3m or Im3m inner symmetries remain stable for several months at room temperature.<sup>24</sup> The heat treatment causes a notable increase in the nanoparticle size due to the vesicle fusion into cubosome objects, which have dense internal bilayer organization.

The method of high shear homogenization, followed by heat treatment at 125  $^{\circ}$ C in an autoclave, does not appear adapted for the purposes of encapsulation of fragile peptide and protein biomolecules. The latter cannot support

powerful agitation and shearing cycles, as well as high-temperature heating. An alternative method<sup>22</sup> involves the preparation of cubosomes along a dilution line from the phase diagram of the monoglyceride/ethanol/water mixture. With this approach, the precipitation of nanoparticles with cubic and vesicular morphologies is induced via dilution in a lack of high-energy input. This method may be suitable for preparation of peptide-functionalized cubosomes in the cases when the peptide conformation will not be influenced by the presence of organic solvent (typically between 7% and 8% w/w ethanol). The methodology requires only a small amount of amphiphilic copolymer (1% Poloxamer 407) for dispersion of the LC mixture.

In a few SAXS reports, the inner structure of the dispersed monoglyceride nanoparticles has been the same as that of the bulk LC phase. 18 At various temperatures, the internal organization of monolinolein (MLO)/Pluronic F127 dispersions was compared with that of nondispersed MLO/water phases. 18 Identical phase behavior has been established for nanostructured lipid dispersions (obtained by an optimized shearing procedure) and the corresponding bulk LC phases. The SAXS analysis has yielded nearly the same structural lattice parameters for the bulk phases and the dispersed states. Based on these data, it has been concluded that the hydrated MLO adopts thermodynamic equilibrium LC organizations. Despite these interesting results, protein or DNA encapsulation, preserving the inner LC structure of the lipid nanoparticles, has not been reported yet for such hierarchically organized nanostructured fluids.

# 8. Biocompatibility of Sterically Stabilized Liquid Crystalline Lipid Carriers

Recent works<sup>28–32</sup> focus on polymeric functionalities for the steric stabilization of the multicompartment lipid nanocarriers and determination of their biocompatibility. To solve the problem of cytotoxicity,<sup>28</sup> polysaccharides and other amphiphilic polymers have been proposed as emulsifiers and stabilizers for cubosomes. Cubic NPs have been stabilized by PEG copolymers with lipid-mimetic hydrophobic anchors,<sup>29</sup> but data regarding their cytotoxicity are still not available. Cubosome particles have also been stabilized or functionalized with hydrophobically modified starch,<sup>30</sup> hydrophobically modified ethyl hydroxyethylcellulose (HMEHEC),<sup>31</sup> or hydroxypropyl methylcellulose acetate succinate (HPMCAS).<sup>32</sup>

Cryo-TEM micrographs have shown that the LC dispersions obtained with the emulsifier HMEHEC contain coexisting vesicles, cubosomes, and mixed multicompartment

nanoobjects.<sup>31</sup> On storage, the lipid vesicles in the MO/ HMEHEC dispersions undergo topological transformations to multicompartment nanoparticles.<sup>31</sup> Depending on the storage conditions, various coexisting mixed objects have been observed in the MO/HPMCAS dispersions as well.<sup>32</sup> Vesicles have transformed into multicompartment objects, comprised of joint cubosome and vesicle compartments, or into nanoparticles with inner cubic lattice organization.<sup>29,31</sup> These results demonstrate the importance of the investigations of the structural organization of the lipid nanocarriers toward achievement of nanostructures with enhanced encapsulation capacity for biomolecules.

### 9. Conclusion and Perspectives

This Account shows that structural studies of swelling of bicontinuous cubic lipid/water phases are essential for overcoming the nanoscale constraints for encapsulation of large therapeutic molecules in network-type lipid carriers. SAXS scans permit monitoring of how the diameters of the aqueous nanochannel compartments in cubosome structures can be tuned by external stimuli and membrane curvature modulating agents. They reveal that coexisting nanochannel structures and intermediate states can be typical for multicomponent amphiphilic mixtures subjected to thermal treatment. The encapsulation of small proteins occurs without perturbation of the cubosome structure. The entrapment of proteins with sizes bigger than the water channel diameters occurs via a "nanopocket defects" mechanism and spontaneous nanocubosome generation in the interior of the cubic lipid/protein assembly. Questions that require further investigations should consider the role of the structural asymmetry of the double diamond-type nanochannel network for the protein loading and release, the conformation of the entrapped proteins, the mechanism of fragmentation and steric stabilization of the cubosomes by biocompatible polymers, their functionalization for targeted delivery of therapeutic proteins, and interaction with cellular environment.

The authors thank Drs. C. Bourgaux, G. LeBas, V. Garamus, R. Willumeit, S. Funari, and B. Papahadjopoulos-Sternberg (NanoAnalytical Laboratory, San Francisco, CA) for continuous cooperation. This contribution is dedicated to the memory of Dr. Michel Ollivon.

**Supporting Information.** Addendums to Sections 3, 4, 5, 7, and 8 and a detailed presentation of the SAXS results. This material is available free of charge via the Internet at http://pubs.acs.org.

#### **BIOGRAPHICAL INFORMATION**

**Angelina Angelova** received a Ph.D. degree in Physical Chemistry from the Bulgarian Academy of Sciences where she was appointed a Research Associate in 1992. After postdoctoral studies at Max-Plank-Institute for Colloids and Interfaces (Berlin) and EMBL (Hamburg), she moved to IMEC (Belgium) in 2000 as a Senior Scientist. Currently, she works as a CNRS Research Scientist at the department "Physical Chemistry of Polyphase Systems", Paris-Sud University. Her research interests are in multiphase supramolecular assemblies.

**Borislav Angelov** received a Ph.D. degree in Biophysics from the Bulgarian Academy of Sciences where he was appointed a Research Associate in 2002. He was a fellow of the Marie Curie host site Laboratoire de Physique de Solides (LPS Orsay), Paris-Sud University. After a postdoctoral stay at the GKSS Research Centre (Geesthacht, Germany), he continued SAXS investigations at the Institute of Macromolecular Chemistry (Prague) and Aarhus University (Denmark).

**Rada Mutafchieva** has a Ph.D. degree in Biophysics from the Bulgarian Academy of Sciences, where she works as a Research Associate in the field of peptides and biological membranes. She was a visiting scientist at CNRS (France), Bielefeld University (Germany), and several other institutions.

**Sylviane Lesieur** received Ph.D. and Habilitation degrees in Physical Sciences from Université Pierre et Marie Curie (Paris VI). She is a Research Director at CNRS and a Head of the Department "Physical Chemistry of Polyphase Systems" at UMR8612. Her research interests include lipid nanocarriers for diagnostics and therapy and studies of interactions of membranes with supramolecular nanoassemblies of lipids.

**Pr. Patrick Couvreur** received a Ph.D. degree in Pharmaceutical Sciences at Université Catholique de Louvain (Belgium). He is currently Full Professor of Pharmacy at the Paris-Sud University and holder of a Chair "Innovation Technologique" (2009-2010) at the prestigious Collège de France. He was appointed a Senior Member of the "Institut Universitaire de France" (IUF). His research has led to the funding of two start-up companies (Bioalliance Pharma and Medsqual).

#### **FOOTNOTES**

\*Corresponding author. E-mail: Angelina.Angelova@u-psud.fr.

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