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Structural organization of proteocubosome carriers involving medium- and large-size proteins

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Proteocubosome assemblies present unique structural advantages for development of novel lipid-based carriers of proteins. With this objective, the mechanism of protein uptake as well as the protein location with respect to the nanochannel network structures created by bicontinuous lipid cubic lattices needs to be determined. Here, the three-dimensional (3D) organization of porous architectures obtained by self-assembly of monoolein (MO) in aqueous phase containing the protein transferrin, and the uptake of transferrin in preformed lipid cubic phase structures, is investigated by high-resolution synchrotron X-ray diffraction. Our structural analysis revealed that the incorporated transferrin induces a cubic (D_{Large}) → cubic (D_{Normal}) structural transition at temperatures around 35°C. We found that this structural transition is characterized by a sharp alteration of the size of the aqueous nanochannels (from 5.9 nm in D_{Large} at T = 20°C to 3.6 nm in D_{Normal} at 52°C) and the thickness of the lipid bilayer (from 3.1 nm at 20°C to 2.4 nm at 52°C) that constitutes the 3D lattice. In contrast, we established that non-loaded MO (hydrated in a saline buffer solution without protein) displays a lamellar-to-cubic (D_{Normal}) structural transition at temperatures above 21°C.

Key words: Nanoporous protein drug carrier – Proteocubosome – Diamond-type lipid cubic phase – Transferrin – X-ray diffraction.

Molecular therapeutics, proteomics and diagnostics require new generations of protein carriers and controlled release devices [1-8]. Nanostructured protein-carrier systems present strong ongoing interest for development of protein-delivery vehicles. Diverse 3D architectures and amphiphilic compositions could be versatile for this aim [9-11]. Among them, the bicontinuous lipid cubic nanostructures [12-28] represent some of the highest crystallographic symmetries possible.

Figure 1a shows a nanoporous structural element from a proteocubosome carrier with a diamond-type cubic lattice symmetry, which could be elaborated from a protein-embedding Q^{224} lipid cubic phase. The single-crystal structure of the Q^{224} bicontinuous lipid cubic phase involves intervening 3D aqueous nanochannel networks characterized by a huge surface area of the lipid/water interfaces per unit volume. This 3D organization, comprising lipid bilayers separated by periodic aqueous nanochannel systems, is stable in excess aqueous phase. It provides enhanced encapsulation efficiency for biomolecules and it favors slow release processes. The lipid cubic phases are biocompatible and hence non-toxic. Both water-soluble and membrane proteins can be incorporated in lipid cubic phase assemblies that provide a protective medium for biomolecules. The lipid/protein composition of the nanostructures could be modulated by the introduction of functional moieties for targeting of particular receptor sites.

Recently, by means of dynamic X-ray diffraction scans [21] we established that the detergent n-octylglucoside (OG) induces a new structural transition, cubic (D_{Large}) → cubic (D_{Normal}), in a 3D self-assembly system with the lipid monoolein (MO) hydrated in excess aqueous medium. The structural features of this transition present a potential for the development of novel protein-carrier lipid nanostructures. Upon the incorporation of OG, the aqueous nanochannels sizes (*Figure 1a*) increase in diameter from around 3 nm, in the D_{Normal} cubic lattice, up to 7 nm in the D_{Large} cubic structure.

The utilization of lipid cubic phase assemblies in protein delivery strategies will require detailed knowledge of the protein uptake and location mechanisms in the nanostructures generated. In the present work we investigate the structural mechanism of incorporation and uptake of transferrin, a relatively large globular protein (*Figure 1b*), in nanostructured carriers formed by monoolein, a representative non-lamellar lipid [15]. Transferrin is an iron binding protein [29] of ongoing interest as a ligand for tumor targeting [30], development of viral-like delivery systems [31], and for understanding of the cellular targeting, cellular uptake, and iron transport.

I. MATERIALS AND METHODS

1. Materials

Human halo-transferrin (iron-saturated) and 1-monooleoyl-rac-glycerol (MO) were purchased by Sigma. Inorganic salts (NaCl, NaH_2PO_4, Na_2HPO_4) were of Fluka Biochemica Microselect (> 99.5%) quality. The aqueous phosphate phase buffer was prepared with pH 7.

2. Sample preparation

Three types of lipid dispersions were prepared:

a) sample #1: MO was hydrated and dispersed in solution of 0.1 M NaCl in phosphate buffer NaH_2PO_4/Na_2HPO_4 (1:1, mol/mol);

b) sample #2: MO was hydrated and dispersed in excess aqueous phase (35 μl) containing the protein transferrin (500 μg/ml) in phosphate buffer. The incubation and vortexing cycles were conducted at 40°C for 3 h;

c) sample #3: a hydrated sample of self-assembled monoolein was preformed at 40°C in the absence of protein. The liquid

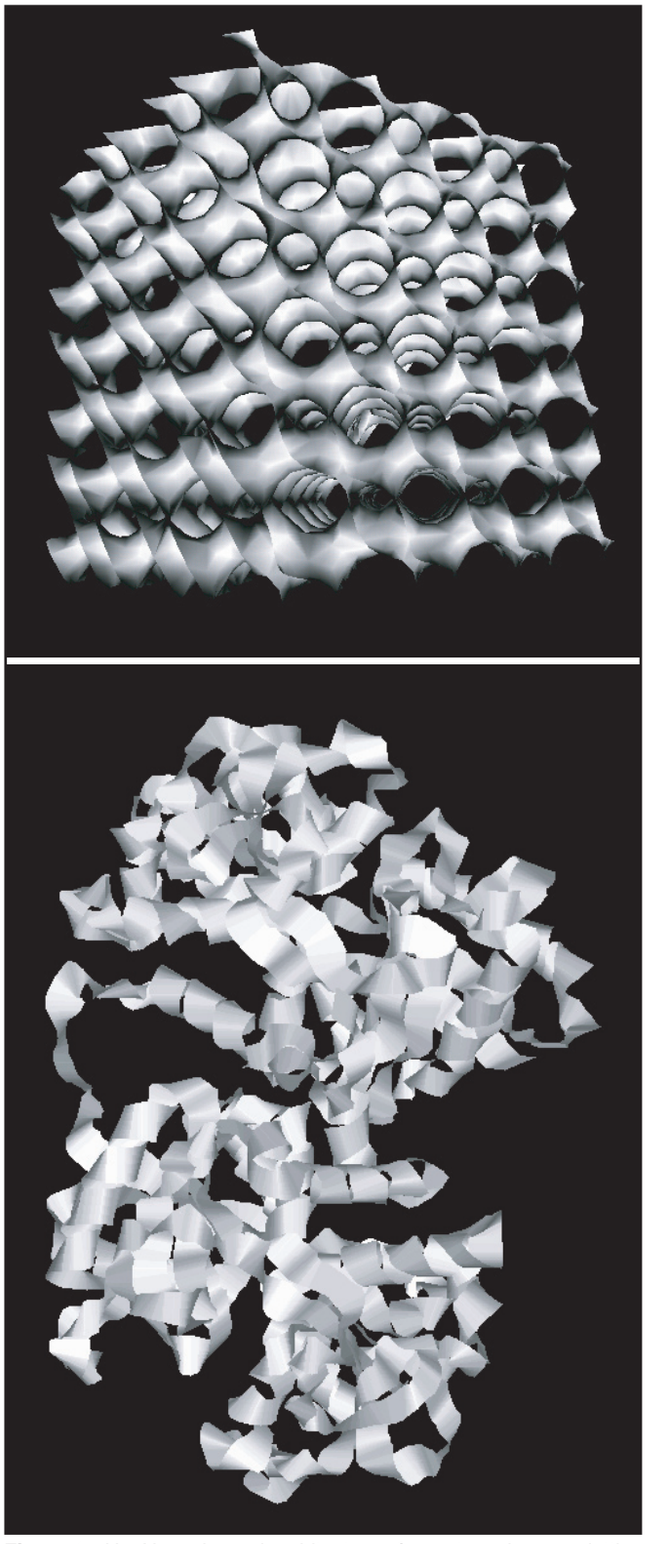

Figure 1 - Up: Nanochannel architecture of a proteocubosome lattice of the diamond-type single-crystal symmetry revealing the presence of open pores in the 3D structure. Down: Three-dimensional presentation of the protein transferrin (MW 75000 Da/mol; PDB file: 1JNF).

crystalline phase was then incubated with a buffer solution of transferrin (200 μl, C_{TR} = 5.8 x 10^{-6} M) for 3 h at 40°C. After the vortexing cycles, the cubic structure was dialyzed three times using a buffer and equilibrated in excess of phosphate buffer solution (40 μl).

All dispersions contained 20 wt.% lipid phase in excess of aqueous solution containing or lacking the protein. The prepared samples were equilibrated and stored in a refrigerator at 4°C.

3. Synchrotron X-ray diffraction

For synchrotron X-ray diffraction measurements the samples were sealed in glass X-ray capillaries with an outer diameter 1.5 mm. Time-resolved X-ray scans were recorded with a heating rate 2°C/min from 1 to 100°C. The temperature of the sample holder was controlled by a Peltier element.

The synchrotron X-ray diffraction experiments were performed at beamline D24 of LURE (Orsay, France). Details of the experiments for investigating the amphiphilic structural phase behavior were the same as previously reported [8, 21]. The reciprocal X-ray spacings s were determined as $s = (2/\lambda) \sin\theta = 1/d = q/(2\pi)$ (where λ = 1.489 Å is the X-ray wavelength and 2θ is the scattering angle), and they were calibrated using tristearin (d_{001} = 4.497 nm) and silver behenate (d_{001} = 5.8378 nm) as standard samples.

II. RESULTS AND DISCUSSION

1. Structural phase sequence of the monooleoyl glycerol lipid upon its full hydration in NaCl-containing buffer solution

Figure 2 shows the time-resolved X-ray diffraction patterns of MO that is fully hydrated in excess 0.1 M NaCl phosphate buffer solution (sample #1). Our structural results indicate that the lipid MO forms in this salt environment, at temperatures between 1 and 20°C, a lamellar structure rather than a single bicontinuous cubic phase. The mechanism of formation of the lamellar phase in the MO/NaCl/buffer system is not studied in detail here as it is the lipid/protein supramolecular assembly into cubic-type nanostructures that presents the main interest of this work. Our sample preparation (#1) did not involve either an extended preincubation on ice or an incubation at sub-zero temperatures. Such procedures have been shown [27] to cause a phase transformation of the MO/water system into a lamellar

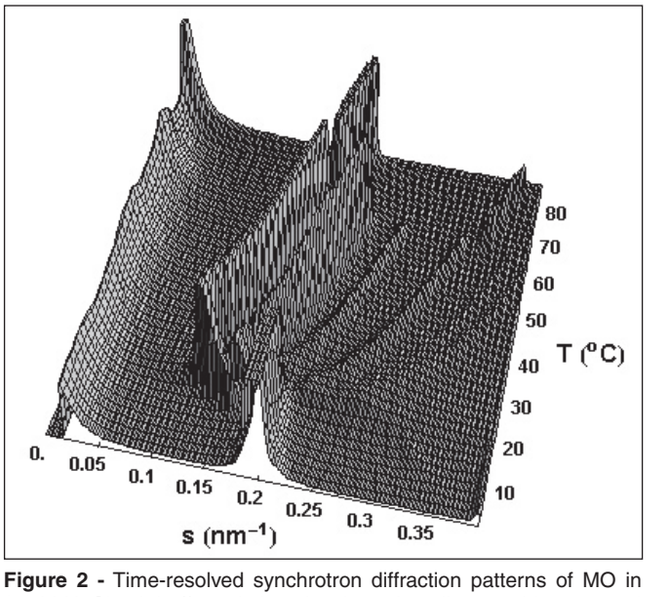

Figure 2 - Time-resolved synchrotron diffraction patterns of MO in 0.1 M NaCl salt buffer solution showing a lamellar-to-cubic structural transition.