DNA SUPRAMOLECULAR SELF ASSEMBLIES AS A BIOMIMETIC COMPLEX SYSTEM

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ABSTRACT

The structure of complexes made from DNA and suitable lipids (Lx), and designed for gene transfer was examined. Cryo Electron Microscopy, Small angle X-ray scattering and Dynamic Light Scattering showed that Lx form monodisperse and spherical multilamellar particles with a distinct concentric ring-like pattern. The same concentric and lamellar structure with different packing regimes was also observed when using linear dsDNA, ssDNA, oligodeoxynucleotides (ODN) and RNA. Lx ultrastructure is of highly ordered crystalline nature exhibiting lamellar and/or hexagonal phase. We have demonstrated structural similarities between this synthetic supramolecular auto-organization and that found in some viruses. Our data point towards the possible existence of a ubiquitous organization of genetic materials.

1. Introduction

Synthetic gene-transfer vectors have been subject to intense investigation since this strategy appears to be clinically safe. Potential methods of gene delivery that could be employed include DNA/polymer complexes [1] or DNA/cationic lipid complexes (Lipoplex, Lx, [2]) [3-6]. The genetic material to be delivered to target cells by these methods are plasmids. Plasmids (pDNA) are circular DNA which can be modified to contain a promoter and the gene coding for the protein of interest. Such plasmids can be expressed in the nucleus of transfected cells in a transient manner. In rare events, the plasmids may be integrated or partly integrated in the cell host genome and might therefore be stably expressed. Plasmids have a promising potential considering the fact that they may be applied in combination with a synthetic vector as carrier and that gene therapy by this means may be safe, durable, and used as drug-like therapy. Plasmid preparation is simple, quick, safe, and inexpensive representing important advantages over retroviral vector strategy. The successful use of this genetic tool for "in vivo" approaches to gene therapy will rely on the development of an efficient cell delivery system [7].

In general, the transport of nucleic acids (NA) is limited by their major biodegradability. pDNA in particular must be delivered totally intact to the nucleus of the target cell to enable expression of the transgene. The pDNA pathway after systemic administration involves many stages, each of which is a potential barrier to transgene expression. The following are the characteristics of an optimal synthetic transport system for gene transfer: (i), DNA compaction within micro particles of homogeneous size; (ii), protection of DNA against nucleases; (iii), transport of DNA in the target cell; (iv), intracellular separation of pDNA from synthetic vectors, and (v), nuclear penetration.

2. Lipoplexes as vectors for nucleic acids

DNA/cationic lipid complexes were first designed in 1987 [3]. Although they are numerous commercially available reagents of high ability in transfecting cells in *in vitro* cell culture models [3,4,8], only a few Lx system were successfully applied for *in vivo* gene transfer, especially following systemic administration [5,6,8].

We have contributed and accumulated knowledge in delivering DNA by using various synthetic vectors. Our first priority was at that time to obtain pharmaceutically suitable vectors regarding stability and reproducibility. We have designed and developed an efficient lipidic vector termed as DLS [6]. Intracellular distribution and uptake was studied in numerous cell culture models with various ODN types (modified or unmodified) [9-11]. Our observations suggested that complete release of the DNA from the endocytic vesicles can be achieved and support the notion of the complete or partial release of the DNA from the lipidic carrier [12].

We were the first to show that systemic administration of plasmid DNA led to widespread and longlasting reporter gene expression [6]. We further demonstrated increased DNA plasma half life and efficient uptake in blood cells following intravenous administration in mice [12]. The DLS system was developed for transgene expression [16,12] and applied in various experimental therapeutic model for gene transfer such as human MDR1 *in vivo* expression in mouse bone marrow progenitor cells [13], and glucocerobrosidase gene transfer [14].

ODN delivery by the DLS system was studied in various in vitro cell cultured models such as inhibition of HIV [9,10]. We recently reported the use of antisense ODN directed against VEGF RNA for treating AIDS

Kaposi's Sarcoma in *in vitro* and *in vivo* setting [11]. Significant activity (39% inhibition) was observed at

We are currently appliyng these vectors for the delivery of ON aimed at correcting splicing alteration and of siRNAs (small interfering RNA).

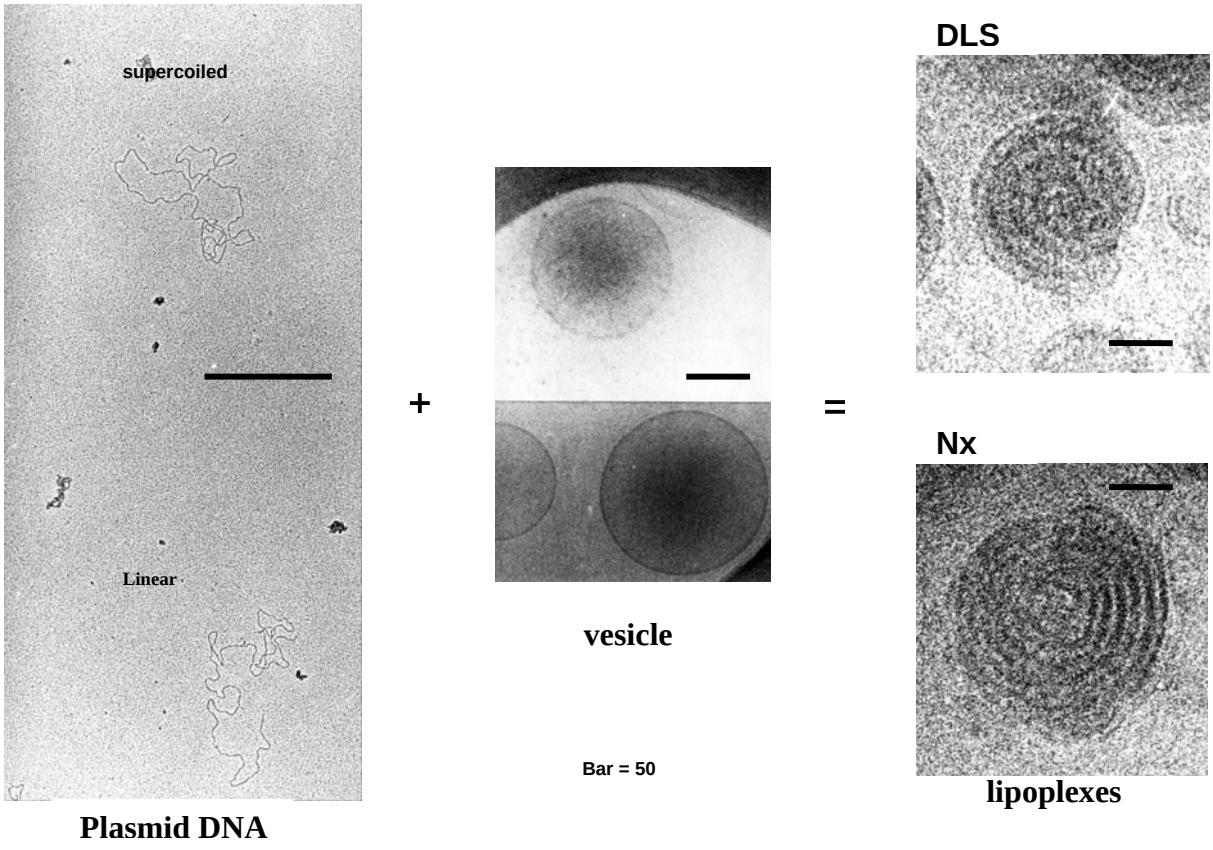


Figure 1: Cryo-EM observation of Lx formation

nanomolar range dose (0.010 µM). This result confirms those previously obtained in HIV culture models showing activity even at subnanomolar concentration. This level of ODN activity is unprecedented illustrating the potential of the DLS system for ODN delivery. Daily intratumoral administration of VEGF-ODN conduce to a marked change in tumor growth, in cell proliferation and in the number of mitotic figures as observed in thin tissue sections[11].

In order to enhance stability in serum following systemic administration we have designed the first globally anionic charged lipoplex termed as Neutraplex [15]. Neutraplex delivery system improved significantly the bioavailability and the ODN pharmacokinetics profile using whole body Positron Emission Tomography (PET) and an enzyme-based competitive hybridization assay [16]. The anionic vector appears as a promising delivery system for *in vivo* administration of therapeutic ODN.

3. Biophysical examination

3.1. Components before complex formation

In order to obtain stable, homogeneous complex assemblies, we decided to set a method by adding the two components in a stable and homogeneous form. The cationic lipid was integrated in Small Unilamellar Vesicles (SUV) and pDNA as solute in precise conditions.

Several lipids have been used in attempts to prepare liposome-like particles. One such lipid mixture is Lipofectin TM (Invitrogen Corp, Carlsbad, CA) which is formed with the cationic lipid DOTMA, N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride, and DOPE, dioleylphosphatidyl ethanolamine at a 1:1 molar ratio. The lipidic particles prepared with this formulation spontaneously interact with DNA through

the electrostatic interaction of the negative charges of the nucleic acids and the positive charges at the surface of the cationic lipidic particles. This DNA/liposome-like complex fuses with tissue culture cells and facilitates the delivery of functional DNA into the cells [3]. Cationic

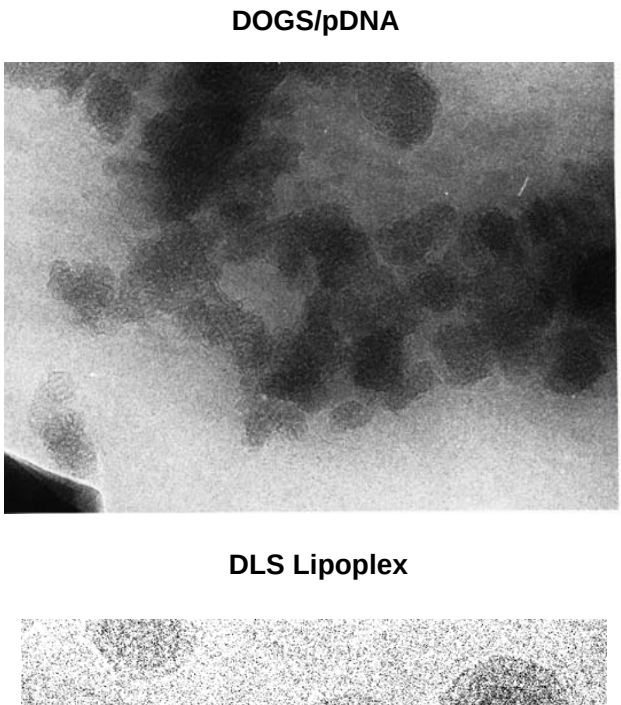


Figure 2: Cryo-EM observation of DOGS/pDNA and DLS lipoplex

lipid particles have been developed: Lipofectamine.TM. (Invitrogen Corp, Carlsbad, CA), composed of DOSPA, 2,3-dioleyloxy-N[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoracetate and DOPE at a 1:1 molar ratio. Lipofectace.TM. (Invitrogen Corp, Carlsbad, CA) composed of DDAB, dimethyidioctadecylammonium chloride and DOPE at a 1:1 molar ratio. DOTAP.TM. (Boehringer Mannheim, Ind.) is 1-2-dioleoyloxy-3 (trimethyl ammonia) propane. Behr et al. [17] have reported the use of a lipopolyamine (DOGS, Spermine-5-carboxy-glycinediotade-cylamide) to transfer DNA to cultured cells. Lipopolyamines are synthesized from a natural polyamine spermine chemically linked to a lipid. For example, DOGS is made from spermine and dioctadecylamidoglycine [17].

DLS SUV are composed of DOGS and the neutral lipid DOPE. Briefly, they are formed following injection of water in excess to a ethanol solution of the lipids. Neutraplex SUV are composed as well of DOGS, DOPE, and a anionic phospholipid (cardiolipin) which interact with DNA under a meticulous formulation process allowing formation of globally negative charged particles [15,16]

Neutraplex SUV are carefully prepared to obtain a highly homogeneous population in regard to size (mean size: 198 nm, and width: 54 nm) as measured by dynamic light scattering size analysis and structure (>95% unilamellar vesicles) as observed by cryoEM (Figure 1). The bilayer measured 5.0 nm.

The genetic material to be delivered to target cells by these methods are plasmids. Plasmids are autonomous extra chromosomal circular DNA. They can be modified to contain a promoter and the gene coding for the protein of interest. Such plasmids can be expressed in the nucleus of transfected cells in a transient manner. In rare events, the plasmids may be integrated or partly integrated in the cell host genome and might therefore be stably expressed. Plasmids have a promising potential considering the fact that they may be applied in combination with a synthetic vector as carrier and that gene therapy by this means may be safe, durable, and used as drug-like therapy. Plasmid preparation is simple, quick, safe, and inexpensive representing important advantages over retroviral vector strategy. For the Lx preparation pDNA was purified in the supercoiled form (Figure 1) and free (<1%) of endotoxin (bacterial protein contaminating pDNA extraction following the recombinant production). pDNA is presented in a low salt solution (50mM NaCl) of pDNA.

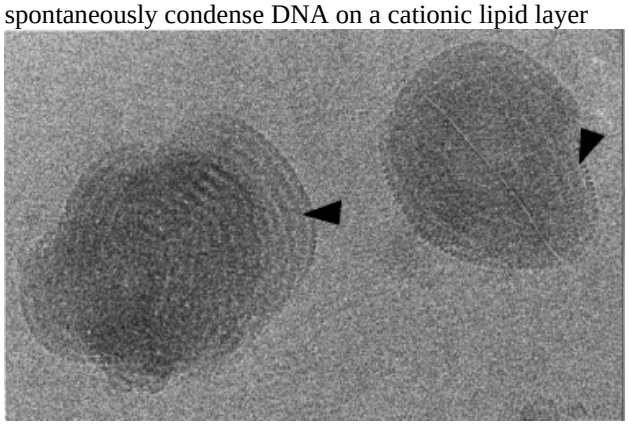
3.1. Lipoplexes

Lx preparation is spontaneously formed by adding DNA to cationic small unilamellar vesicles (SUV) [6,15].

The addition of pDNA to highly homogenous SUV, in precise experimental conditions, resulted in the formation of stable lipid-pDNA complexes. Complex formation of DNA with cationic lipids leads to the respective condensation of both entities by electrostatic interactions. As a consequence, control of the thermodynamic parameters of complex formation is crucial to obtain homogeneous and reproducible particles.

DLS (Figure 2) and Neutraplex (Figure 3) particles appear spheric, monodisperse, homogenous in size and

remarkably structured (mostly lamellar, rolled and condensed). In contrast to DOGS/pDNA complex particles do not exhibit concentric winding but rather aggregates of planar packed DNA chain (Figure 2). DOGS allows for the side-by-side alignment of DNA in a liquid crystalline phase (Figure 2A). DOGS spontaneously condense DNA on a cationic lipid layer



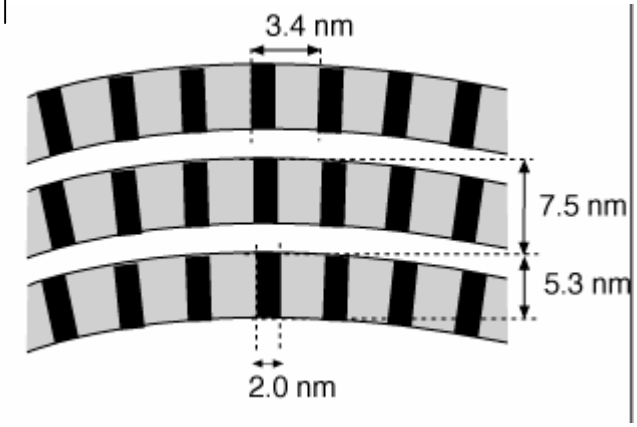


Figure 3: Analysis of the ultrastructure of Lx as determined by cryo-EM [15]

and result in the formation of nucleolipidic particles. This lipospermine-coated DNA shows some instability but high transfection efficiency [17]

We have extensively examined preparation of Lx for elucidating their ultrastructure. As shown in Fig. 3A, cryoEM examination of Neutraplex Lx prepared with a pDNA of 10,4 kbp reveals spherical particles exhibiting two kind of structures: mostly (>90% of total particles) a multilamellar organization of spherulite-like pattern and rarely (<10%) a punctate pattern.

Based on the cryoEM images, the thickness of the striated layers has a mean value of 5.3 nm with a periodicity of 7.5 nm. The outer striated layer has a thickness of 4.1 nm. Perpendicular to these layers, faint striations can be seen mainly on the edge and also inside the particles. These striations are 2.0 nm thick with a periodicity of 3.4 nm (Figure 3). The particles with punctate array [15] are confined by an outer shell. This outer shell bears the same thin striations than those found on multilamellar structures. When these striations can be detected in a cryoEM image they are observed in most of the Lx particles. In addition, the punctate structures are occasionally associated with these multilamellar structures.

Analysis by SAXS of our Lx sample (Figure 3) revealed three diffraction peaks: one indicating a repetitive spacing of $2\pi/0.094=6.9+/-1$ nm and the second and fourth order indicating the multi-layer structure ($2\pi/0.18$) [15]. The relative width of X-ray diffraction suggested highly ordered spherulite-like particles made of at least a dozen layers. Thus SAXS clearly confirmed the multilamellar structure of Lx and the periodicity observed with cryoEM [15].

The lamellar symmetry in Lx structure has been previously demonstrated by means of Small Angle X-ray Scattering (SAXS) [18,19]. Radler et al [18] and Lasic et al [19] showed the multilamellar nature of Lx and a periodicity of 6.5 nm. We could not reliably detect a peak corresponding to the DNA sandwiched between lipid bilayers, as previously observed [18,19]. This could be due to the beam intensity that did not allow for the observation of the weak DNA-DNA correlation peak; or it could also suggest that the DNA chains are not arranged in a parallel array between lipidic layers as assumed by these authors.

This concentric and lamellar structure with different packing regimes was also observed by cryoEM when using linear dsDNA, ssDNA, RNA and oligodeoxynucleotides (Figure 4) [15] .We can obtain a similar structural morphology in Lx formulated with all the nucleic acids tested despite of highly different structures and sizes as with pDNA (circular supercoiled DNA of 10.4 kbp, PM~6,870,600) and ODN (linear single strand DNA of 30 bases, PM=9900). SAXS analysis confirmed the crystalline phase nature of the complexes. However, there are some discrepancies in the details of these structures especially in the periodicity number order (data not shown).

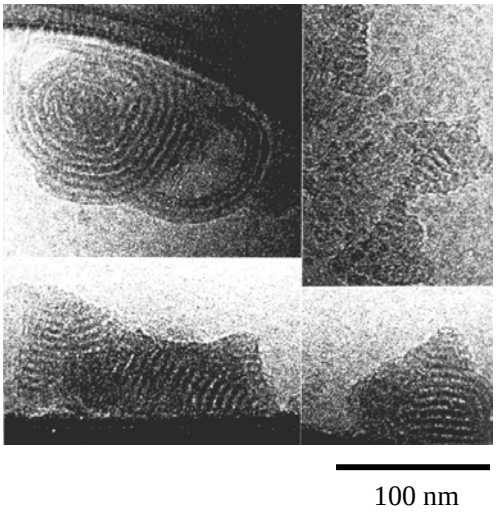


Figure 4: Cryo-EM analysis of Lx formed with oligodeoxynucleotides [15]

Dynamic light scattering examination [20] indicated a pDNA/Lx mean size of 254 nm (width: 108 nm) and exposed a monomodal population with a polydispersity of 0.193 [15]. As presented in Table 1, Lx formed with other DNA types, presented the same particles characteristics. Mean size may significantly vary but not to a high extent (184 to 254 nm). Polydispersity values are < 0.2 and attest for every preparation of the monodisperse nature of the colloidal suspension (Table 1).

Table 1. Particle Size analysis by dynamic light scattering

Nucleic acid	Size (polydispersity)
Circular double strand DNA	254 nm (0.193)
Linear double strand DNA	221 nm (0.048)
Long linear double strand DNA	224 nm (0.198)
Linear single strand DNA	261 nm (0.113)
RNA	188 nm (0.172)
Oligodeoxynucleotides	184 nm (0.045)

Long linear double strand DNA is the T4 phage DNA (169,372 base pairs). Linear single strand DNA (M13rmp8, 7,229 bases) is the DNA from M13 bacteriophage derivatives. Linear double strand DNA (M13mp8; 7229 base pairs) is the intracellular replicative form of the M13mp8 phage produced by chronic infection in E. Coli (Sigma ,St Quentin, France). RNA (ribonucleotide) is the 18S and 28S ribosomal RNA from calf liver (2000 and 5300 bases). ODN is a 27 bases oligodeoxynucleotides. (Xba I, Promega, Wis.).

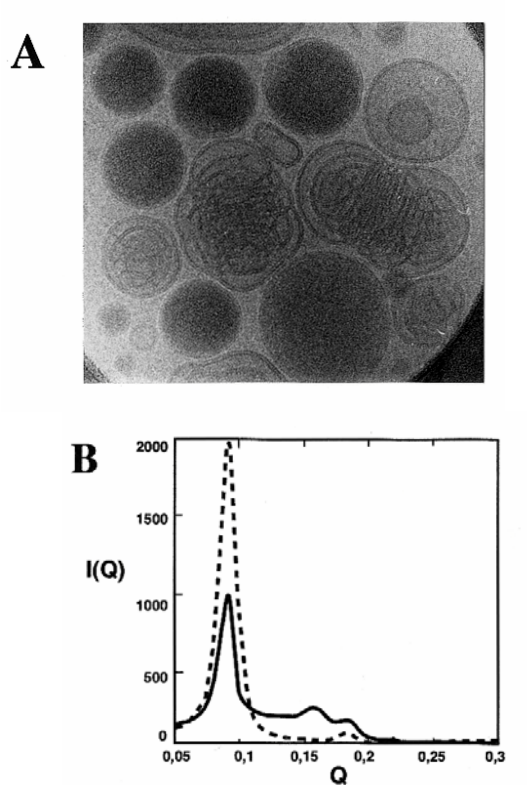


Figure 5: Cryo-EM and SAXS analysis of Lx formed with T4 phage virus DNA [15]

CryoEM examination of T4 phage DNA packed either in T4 capsides or in lipidic particles showed similar patterns (Figure 5 and 6). SAXS suggested an hexagonal phase in Lx-T4 DNA. Most of T4 DNA/Lx particles exhibit a high majority of punctate array (Figure 5). Conversely, spherulite/concentric motif are the majority specie in Lx-pDNA cryoEM images corroborating with the lamellarity detected by SAXS. As punctate and multilamellar structure were associated in some Lx particles it might be possible that both liquid cristalline and hexagonal coexist in the same particle. T4 DNA is more than 16 times longer than the pDNA used in this study. Our results indicate that both lamellar and hexagonal phases may coexist in the same Lx preparation or particle and that transition between both phases may depend upon equilibrium influenced by type and length of the DNA used.

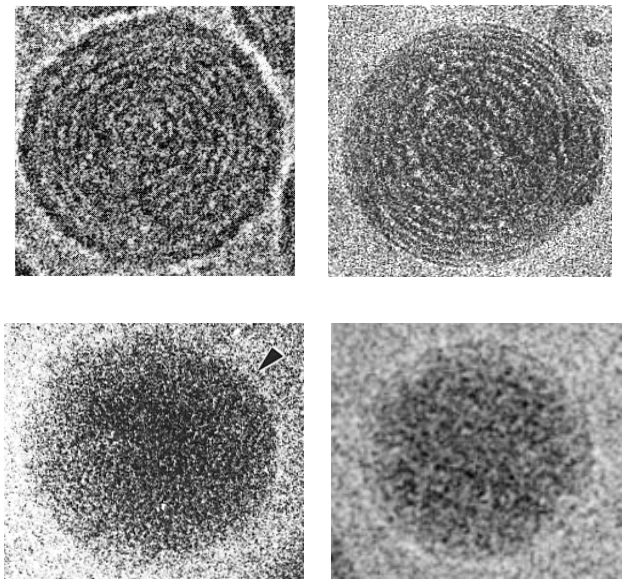
Lx is a multicomponent system governed by a combination of interactions caused by charge neutralization [22]. At critical concentrations and charge densities, liposome-induced DNA collapse and DNA-dependent liposome fusion are initiated[28]. We suggest that this phenomena induce thermodynamic forces towards compaction of the DNA macromolecule in the complex through a concentric winding where DNA is adsorbed on to the cationic head groups of the lipid bilayers.

4. Discussion and perspectives

In light of these observations we suggest that those Lx supramoleculaire assemblies are usefull tool to basic research for studying : (i), DNA condensation/decondensation phenomena in cell; (ii), DNA prebiotic assembling; and for appied research regarding development of DNA synthetic vectors.

Lx are developed for therapeutic gene transfer and requirements regarding homogeneity and reproducibility are high when considering clinical use. Elucidating their ultrastructure and the mechanisms involved will provide crucial informations towards better controlling manufacturing procedure.

Lx specific supramolecular organization is the result of thermodynamic forces, which cause compaction to occur through concentric winding of DNA in a liquid crystalline phase. Lipid fusion and cooperative DNA collapse processes are initiated at critical concentrations and charge densities [22]. Those parameters must be clearly delineated to define an optimized preparation. In addition, the more the supramolecular assemblies are homogeneous in liquid crystal structure, the more stable and efficient for gene transfer they are (data not shown). Results suggest that transition between lamellar and hexagonal phase in Lx particles may exist. To date, we do not know whether particles of one of this phase leads to a better stability or gene transfer efficacy. This specific question is under investigation in our laboratory.



Phage virus particles Neutraplex particles

Figure 6: Biomimetism of Lx particles and transition between multilamellar (upper images) and hexagonal (bottom images) phases

In the Lx formed here neutralization of DNA charges is due to the presence of cationic spermidine containing lipids (DOGS) . The side-by-side alignment of DNA in a liquid crystalline phase may be further promoted by spermine [23]. Tight supercoiling structures can be obtained *in vivo* by polycationic molecules or proteins such as spermine, spermidine, histones or histone-like proteins [20,24].

Polyamine spermine or spermidine are antique and ubiquitous compounds which stabilize the DNA double helix *in vivo* and which, consequently, were intensively studied as a valuable model system for studying DNA organization in biological structures [25].

DNA such as pDNA used in this study is recombinant, thus purely “natural”. Consequently, DNA/cationic lipids complexes might be considered as hemi-synthetic or hemi-natural systems. It was established that DNA has a identical reactivity along his molecule chain. Ghirlando et al [26] indicated that DNA segments as short as 20 bp were able to condense in micellar aggregates and, in general, condensation of DNA by multivalent cations seems due to mechanisms independent from the length of the individual DNA molecules [27]. Thus, use of short chain or sequence might be representative in some conditions to the micro-(de)condensation occurring during replication, transcription or splicing.

In the nucleus DNA is wound up onto successive nucleosome core particles, forming a "beads on a string" complex. It has been an open question whether nuclear structure is affected by these same packing considerations. In a technically challenging work, Livolant and Leforestier [28] employed a combination of optical microscopy and freeze-fracture electron microscopy to show that nucleosome core particles form complex self-assembled structures.

Our results confirm that DNA condensation in liquid crystal complexes was insensitive to DNA size when using SUV, as previously found when using micelles [26,29]. As a consequence, elucidation of the ultrastructure of the Lx formed here might provide information in the DNA condensation/decondensation phenomena in cell.

The cryoEM images of punctate Lx particles formed with T4 DNA that we observe are strinkingly similar to the images of complete tail-deletion mutant of T4 [30] (Figure 4) and T7 [31] phages. T4 phage mutants produce a normal mature head but no tail, which enables a better observation of DNA organization [33,34]. These T4-related phage particles showed a spherical shape and an average diameter of 80 nm[33]. Recent investigations performed by using tailless mutants, indicated DNA packing domains in viral particles. It is noteworthy that T7 tail-deletion mutants exhibit in cryoEM images a concentric ring motif as well as a punctate motif as observed for Lx-pDNA [31].

Cerritelli et al[30] observed T7 tail-deletion mutants by CryoEM which revealed a concentric ring motif

" along the axis via which DNA is packaged and a punctated pattern in side views ". They suggest that T7 DNA is spooled around this axis in coaxial shells in a quasi-crystalline packing predicting the viability of such a model for other phage heads.

Interestingly, other report demonstrated thatT5 phage appear condensed in lamellar and concentric winding form when released in proteoliposomes following virus attachment [32]. DNA of bacteria under stress self organize in crystalline phase by forming complexes with cationic peptides [33]. Three-dimensional liquid-crystalline arrangements of biomolecules have been known since the pioneering work of Bouligand [34,35]. Self organization leads to the possible phase transition in live cells.

Furthermore, organization of such nucleotidic supramolecular assemblies is relevant for prebiotic chemistry [36]. As postulated by Lipowsky [37], cellular life might begin with a membranar vesicle containing just the right mixture of polymers. In light of the numerous observations made on DNA packaging in a natural setting or by various organic or inorganic condensing agents, our data corroborate the notion that a parallel between natural and synthetic DNA compaction can be drawn. We demonstrated for the first time a structural similarity between a synthetic supramolecular organization and viruses. Synthetic gene delivery particles must obey to reversible DNA condensation just as viruses do. Ultrastructure of Lx appears as a complex system and could constitute a valuable model system for studying nucleotidic organization in biological structures.

We strongly believe that the Lx complexes correspond to a ubiquitous supramolecular self organization of DNA and could be considered as an emergent complex system [38]. Self-organization in general, refers to the various mechanisms by which pattern, structure and order emerge spontaneously in complex systems.

Self-organization is a process in which pattern emerges at the global (collective) level by means of interactions among components of the system at the individual level. [39]. What makes a system self-organized is that the collective patterns and structures arise without: (i), the guidance of well-informed leaders; (ii), any set of predetermined blueprints; (iii), recipes or templates to explicitly specify the pattern [39]. Instead, structure is as an emergent property of the dynamic interactions among components in the system.

Self-organization appears to be an important mechanism useful for explaining pattern and structure in physical, chemical and biological systems. Lx appear as one DNA supramolecular self-organization model and elucidation of their ultrastructure is under investigation.

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