EXPERIMENTAL REPORT

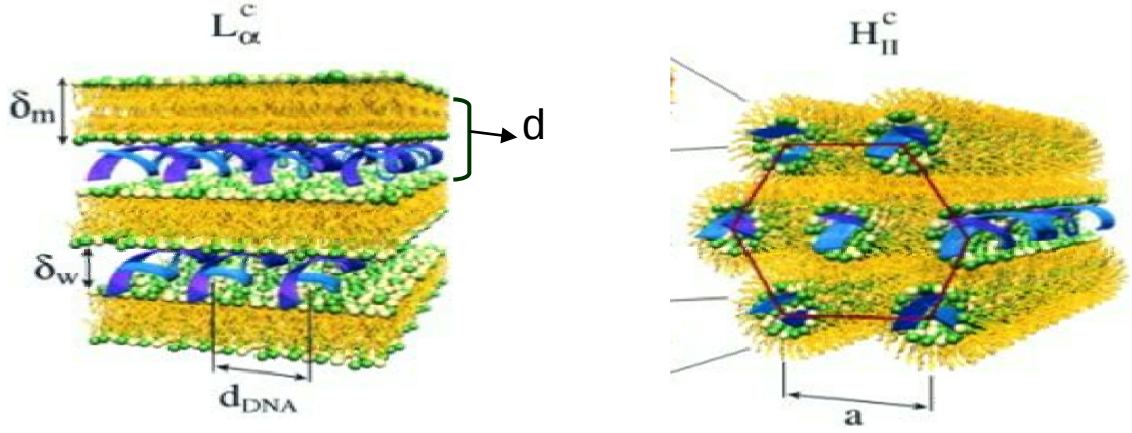
PROJECT at BM16 ref: 16-02-81

Determination of lipoplexes structures formed by mixed cationic lipids and DNA

Mixed liposomes, formed by cationic and zwitterionic helper lipids, are the most promising nonviral vectors in gene therapy, because they compact and condense DNA forming lipoplexes (cationic lipids + DNA).[1-3] The efficiency of DNA transfection using cationic lipids is improved by the presence of a zwitterionic helper lipid and it is highly dependent on the cationic and the helper lipid, the mixed lipid composition, α, and the lipid/DNA ratio, *CR*.[4,5] The electrostatic interaction between cationic liposomes and anionic DNA plays an important role in the lipoplex properties, which reinforces the interest on improving the knowledge of this interaction, because it shed light, about the formation process of lipoplexes, and on the transfection DNA mechanisms.

In the present experiment we have studied lipoplexes covering the whole range of the mixed lipid composition and at several lipid/DNA charge ratios, with the objective of analyzing the influence of these factors on the compaction process and the behaviour of the lipoplexes. To carry on this study lipoplexes were characterized in advance in our laboratory at the UCM-Madrid, by means of zeta potential and ethidium bromide fluorescence intercalation assay experiments together cryo-TEM experiments at the Univ. Autonoma of Barcelona. But, in order to analyze the structures formed by the lipoplexes, a set of small angle X-ray scattering (SAXS) at intermediate angle experiments is required, which has been the objective of the present project 16-02-81.

Cationic lipoplexes may form lamellar, hexagonal, or even cubic structures that interact with the cell membranes in a different way, thus resulting of relevance to conjugate the capacity of liposomes to condense and compact DNA by forming strong lipoplexes with an easy release of DNA into the cells. The effect of the lipoplex structure has been proved to be important in the early stage of the transfection.[3,6] Nowadays, it is assumed that the main entry trail to the cells is the endocytosis,[7] the interaction between positive charged lipoplexes and anionic membranes being strongly dependent on the lipoplex structure.[3] In fact, lipoplexes with lamellar structure remain more stable with no fusion occurring between the lipoplex and the vesicle being the DNA release relatively low. On the contrary, lipoplexes with hexagonal structure rapidly fused with the anionic endosomal vesicle, which provokes a loosing of the lipoplex condensed structure. So, the inverted lipoplex hexagonal structure inside endosome favors, after fusion, that DNA is easier released and expelled to the cytoplasm.[8] For that reason, research groups involved in this subjet, are looking for hexagonal or cubic lipoplex structures with proved better efficiency in transfection than lamellar ones.[6,9]



When the Bragg peaks on SAXS experiments show that lipoplexes form a lamellar structure, L_α, according to Scheme, the interlayer distance, d, directly related to the q factor ($d = 2\pi/q_{100}$), lipoplexes can be represented as alternating layers of mixed lipids and DNA helices where d is the sum of the thicknesses of the lipid bilayer, δ_m, and the DNA aqueous layer, δ_w. Accordingly, the Bragg peak on the diffractograms not corresponding to the lamellar structure arise from the DNA-DNA correlation, and its q_{DNA} factor permits to obtain the separation between DNA strands in the monolayer, d_{DNA}, ($= 2\pi/q_{DNA}$) (Scheme). Plots of the periodic distance of the lamellar structure, d, vs α and L/D, at the different charge ratios, CR, should inform of the behaviour of d against these parameters. The values of d are related to thickness of the mixed lipid bilayer, δ_m, and the thickness of the DNA monolayer, δ_w ($= d - \delta_m$), a parameter that inform of the thickness where DNA is allowed in the periodicity structure found by SAXS and if it is appropriate to accommodate the monolayer of the hydrated DNA helices.

On the other hand, when the Bragg peaks observed on SAXS diffractogram index very well on a $2D$ hexagonal lattice, H_{II}, similar to Scheme, the spacing, a, of the cell unit can be directly related to the q factor ($a = d_{DNA} = 4\pi/(3^{(1/2)}q_{10})$. In this hexagonal lattice, a monolayer of mixed lipids surrounds the DNA helices, the structure of the DNA-mixed lipids resembling inverted cylindrical micelles. These values indicate that in the hexagonal structure DNA helices are more separated that in the lamellar one.

SAXS experiments have been done from solid samples in equilibrium with buffered solution placed in sealed Hilgenberg glass capillaries with an outside diameter of 1.5 mm.

The samples studied in this project were formed by mixed liposomes containing: i) the calf tymus linear DNA of double strand; ii) a zwitterionic helper lipid (1,2-dioleoyl-sn-glycero-3-phosphoetanolamine (DOPE)) and, iii) a cationic lipid among of the different length, head polar, and charges as follows:

1) A gemini surfactant $C_{16}C_nC_{16}$, constituted by: two hydrophobic hexadecyl hydrocarbon chains, two ammonium cationic heads separated by a alkyl spacer, C_n, with n = 2, 3, 5, and 12.
2) A cationic lysine-derived lipid $C_6(N^{\alpha}LK)_2$, constituted by: two hydrophobic decyl hydrocarbon chains, two lysine cationic heads separated by a alkyl spacer, C_n, with n = 6.

The samples were prepared in our laboratory of the UCM-Madrid, at physiological pH (=7.4) covering the whole range of mixed lipid composition (cationic/(cationic+ neutral) lipids, and at 7 different cationic lipid/DNA charge ratios. Each capillary were measured twice. Five sets of SAXS experiments (around 90 capillaries for each cationic lipid/DOPE-DNA system) were done which means that around 450 diffractograms were collected in the 12 shifts disposed.

Since the experiment was completed on february 15, the results were analyzed deeply, founding in all the systems lamellar phases as the main structure, and in some systems at low molar composition of the cationic lysine-derived lipid, $C_6(N^{\alpha}LK)_2$, an hexagonal phase were also found. After the whole experimental study (zeta potential, Gelred Electrophoresis, agarose gel electrophoresis, cryo-TEM, and SAXS, experimental biological experiments have been done to complete the study. Results have confirmed the moderate to high transfection efficiency and the low toxicity of all cationic lipids used on the study. All the results have been organized to be published in three manuscripts. First of them, entitled "*Why is less Cationic Lipid Required to Prepare Lipoplexes from Plasmid DNA than Linear DNA in Gene Therapy?"* was sent end of May for publication in *The Journal of the American Chemical Society (JACS),* and the editor and two reviewers have considered to be acceptable for the journal after some modifications, already done, and submitted again for its final acceptation. Other two manuscripts are currently in progress and it is planned to send them, one to *JACS* and the other to *Soft Matter* journals.

References

(1) Felgner, P. L.; Heller, M. J.; Lehn, J. M.; Behr, J.-P.; Szoka, F. C. *Artificial Self-Assembling Systems for Gene Delivery*; American Chemical Society: Washington, DC, 1996.
(2) Lasic, D. D. *Liposomes in Gen Delivery*; CRC Press: Boca Raton, FL, 1997.
(3) Ewert, K.; Slack, N. L.; Ahmad, A.; Evans, H. M.; Lin, A. J.; Samuel, C. E.; Safinya, C. R. *Curr. Med. Chem.* **2004**, *11*, 133.
(4) Dias, R. S.; Lindman, B. *DNA Interaction with Polymers and Surfactants*; Wiley & Sons: Hoboken, 2008.
(5) Ahmad, A.; Evans, H. M.; Ewert, K.; George, C. X.; Samuel, C. E.; Safinya, C. R. *J. Gene Med.* **2005**, *7*, 739.
(6) Pozzi, D.; Caracciolo, G.; Caminiti, R.; De Sanctis, S. C.; Amenitsch, H.; Marchini, C.; Montani, M.; Amici, A. *ACS Applied Materials & Interfaces* **2009**, *1*, 2237.
(7) Lin, A. J.; Slack, N. L.; Ahmad, A.; George, C. X.; Samuel, C. E.; Safinya, C. R. *Biophys. J.* **2003**, *84*, 3307.
(8) Koltover, I.; Salditt, T.; Rädler, J. O.; Safinya, C. R. *Science* **1998**, *281*, 78.
(9) Bouxsein, N. F.; McAllister, C. S.; Ewert, K. K.; Samuel, C. E.; Safinya, C. R. *Biochemistry* **2007**, *46*, 4785.