EXPERIMENTAL REPORT

PROJECT at BM26 ref: SC 3357 June 04-08 (2012)

Title: Lipoplex structures formed by novel cationic gemini lipids with plasmid DNA or siRNA

Gene therapy is expected to lead to powerful new approaches for curing many diseases, a potential that is currently explored in worldwide clinical trials. The objective is to transport and transfer plasmid DNA (of several thousand base pairs), or small interfering RNA (of 19-25 base pairs) into the cell cytoplasm. Plasmid DNA (pDNA) is used to repair damage cellular DNA, while siRNA mediates the cleavage of complementary mRNA, leading to post-transcriptional gene silencing.

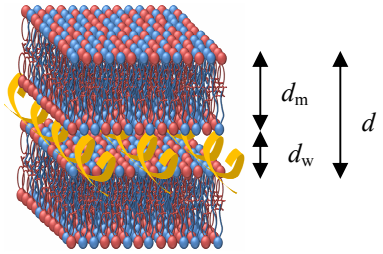
In the present project we planned to do a study of a wide variety of lipoplexes (cationic lipid + DNA or siRNA) covering a wide range of the mixed lipid composition (α) and at several lipid/plasmid DNA and lipid/siRNA effective charge ratios (ρ_{eff}), with the objective of analyzing the influence of these factors on the compaction process and on the lipoplex behaviour. The novel cationic gemini lipids (CLs) used in this proposal were synthesized by the Prof. S. Bhattacharya's group at the Dept. of Organic Chemistry, Indian Institute of Science, Bangalore (India). They are expected to improve the transfection of both, plasmid DNA and/or siRNA, with a decrease of the CLs cytotoxicity. To carry on this study, lipoplexes were previously characterized by means of several experiments: electrochemical as zeta potential, spectroscopic as fluorescence, and microscopic as cryo-TEM (at the Univ. Autonoma of Barcelona, Spain). In addition, a biochemical analysis (including transfection efficiency, cytotoxicity and confocal fluorescence microscopy) with *in vitro* cells, done in collaboration with the Indian Institute of Science, Bangalore (India), confirms their applicability in gene therapy when plasmid DNA is used to transfect to living cells. But scattering methods as through small angle X-ray scattering (SAXS), objective of the present project SC 3357, are also necessary to determine the lipoplexes structures. 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 compact pDNA or siRNA by forming strong lipoplexes with an easy release of the biopolymer into the cell.

Mixed liposomes, formed by cationic and zwitterionic helper lipids, are the most promising nonviral vectors in gene therapy, because they compact the anionic DNA or RNA biopolymers forming lipoplexes (CL-pDNA or CL-siRNA), attach to the anionic cell surface and transfer the biopolymer into the cell cytoplasm.[1-7] The efficiency of pDNA or siRNA 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 or lipid/RNA effective charge ratio (ρ_{eff}).[1-4,8,9]

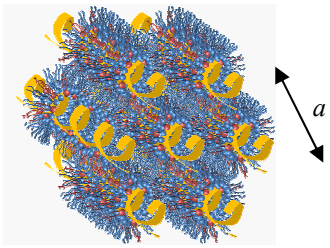
Nowadays, it is assumed that the main entry trail to the cells is the endocytosis, the interaction between positive charged lipoplexes and anionic membranes being strongly dependent on the lipoplex structure. In fact, lipoplexes with lamellar structure remain more stable being the pDNA or siRNA release 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 and cubic structures favor, after fusion, that pDNA or siRNA are easier released to the cytoplasm. For that reason, research groups involved in this area, are looking for hexagonal or cubic lipoplex structures with proved better efficiency in transfection than lamellar ones.

When the Bragg peaks on SAXS experiments show that lipoplexes form a lamellar structure, L_α, according to the Scheme below, lipoplexes can be represented as alternating layers of mixed lipids and DNA helixes where d is the sum of the thicknesses of the lipid bilayer, d_m, and the DNA aqueous layer, d_w. Accordingly, the Bragg peak on the diffractograms not corresponding to the lamellar structure arise from the DNA-DNA correlation, and its peak-centred at q_{DNA} permits to obtain the separation between DNA strands, d_{DNA}. Plots of the periodic distance of the lamellar structure, d, vs α and L/D, at the different charge ratios, ρ, should inform of the behaviour of d against these parameters. The values of the thickness of the DNA monolayer, d_w (= d - d_m), inform about the thickness where DNA is allowed in the structure found by SAXS and if it is appropriate to accommodate the monolayer of the hydrated DNA double helixes. On the other hand, when the Bragg peaks observed on SAXS diffractogram index

well on a 2*D* hexagonal lattice, H_{II}, similar to the Scheme, the spacing, *a*, of the cell unit can be directly determined from the diffraction pattern. In this hexagonal lattice, a monolayer of mixed lipids surrounds the DNA double helixes, the structure resembling inverted cylindrical micelles. A similar analysis can be done to the cationic lipid-siRNA structures (lamellar, hexagonal or cubic if found).



a) Lamellar structure (L_α)



b) Hexagonal structure (H_{II})

SAXS experiments have been done in solid samples, in equilibrium with buffered solution at physiological pH (=7.4), placed in sealed glass capillaries with an outside diameter of 1.5 mm. The sample to detector distance was selected to be appropriate to cover a *q* factor between 0.5 and 3.5 nm^{-1}. It means that the distance *d* (=$2\pi/q$) must range between 2 and 12 nm.

The samples projected to study in the SC 3357 experiment were lipoplexes containing pEGFP-C3 plasmid DNA amplified in our laboratory, or commercial siRNA (with target sequence CUUACGCUGAGUACUUCGA), and mixed liposomes composed by two lipids:

i) A novel synthesized dicationic lipid constituted by a gemini surfactant ($C_{16}A^+R_nA^+C_{16}$) containing two hexadecyl hydrocarbon chains, jointed to two cationic heads (A^+) separated by a spacer (R_n). The cationic heads (A^+) have been: **a1)** two cationic imidazol groups or, **a2)** two cationic ammonium groups, while the spacer (R_n) has been: **b1)** an alkyl chain length, $(CH_2)_n$, with n = 2, 3, 5 and 12) or, **b2)** an ethoxyethylene series, $(CH_2CH_2O)_n$, with n = 1, 2 and 3.
ii) A commercial zwitterionic helper lipid (1,2-dioleoyl-sn-glycero-3-phosphoetanolamine (DOPE)).

Several sets of SAXS experiments for each cationic lipid/DOPE-pDNA or cationic lipid/DOPE-siRNA system, prepared in our laboratory of the UCM-Madrid, were planned to run covering 4 charge ratios (ρ_{eff} = 1.5, 2, 2.5, 3, and 5) and, at 4 mixed lipid compositions (α = 0.2, 0.4, 0.5, and 0.7). It drives to 20 capillars for each lipoplex system.

We planned to study lipoplexes with 14 cationic lipids which means around 560 capillars in total (280 for each gene agent plasmid DNA and siRNA). Due to this high number of capillars, we asked to apply for 15 shifts, but according to the BM26 recommendations we applied for 12 shifts. Finally, the evaluation committee accepted our proposal but with only 9 shifts. For this reason, during the experiment, the two researchers only could measure SAXS for half of the prepared capillaries (300), those corresponding to the cationic lipid/DOPE-pDNA lipoplexes. Thus, capillars containing samples of cationic lipid/DOPE-siRNA lipoplexes remain to be measured at ESRF in the coming application (along 2013) together other systems containing different cationic compounds.

Since the experiment was completed on June 8, the results have been analyzed deeply, founding in all the systems (and at most of the studied effective charge ratios, ρ_{eff}) one or two coexisting lamellar phases as the main structures, and in some systems, at low molar composition, one coexisting hexagonal phase was also found. After the whole biophysical experimental study (zeta potential, GelRed electrophoresis, agarose gel electrophoresis, fluorescence anisotropy, cryo-TEM, and SAXS) was completed, biological experiments (transfection, cell viability/cytotoxicity and confocal fluorescence microscopy) were done to complete the whole study. Results obtained with SAXS have been compared to that previously done by our group or elsewhere in order to get conclusions relating the founded structures to an optimum transfection efficiency and lower cytotoxicity of these lipoplexes formed by novel synthesized cationic gemini lipids and the plasmid DNA biopolymer. The results are going to be organized for publishing in three manuscripts (first one is under progress) in journals of high impact factor, as usually does our group whose contribution in this subject has been recognized by the research community through several recent publications.[5,10-12] The present proposal is part of the Project "*Compaction of DNA/siRNA with novel gemini lipids: transfection formulations for use in gene therapy*", supported by the Educational & Research National Departments of India (order nº DST/INT/SPAIN/P8/2009) and Spain (order nº ACI2009-0867) with a 4 years duration (2010-2013).

References

(1) 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.

(2) Bouxsein, N. F.; McAllister, C. S.; Ewert, K. K.; Samuel, C. E.; Safinya, C. R. *Biochemistry* **2007**, *46*, 4785.

(3) Pozzi, D.; Caracciolo, G.; Caminiti, R.; De Sanctis, S. C.; Amenitsch, H.; Marchini, C.; Montani, M.; Amici, A. *Appl. Mat. Interfaces* **2009**, *1*, 2237.

(4) Marchini, C.; Montani, M.; Amici, A.; Amenitsch, H.; Marianecci, C.; Pozzi, D.; Caracciolo, G. *Langmuir* **2009**, *25*, 3013.

(5) Muñoz-Ubeda, M.; Rodriguez-Pulido, A.; Nogales, A.; Martin-Molina, A.; Aicart, E.; Junquera, E. *Biomacromolecules* **2010**, *11*, 3332.

(6) Dias, R. S.; Lindman, B. *DNA Interaction with Polymers and Surfactants*; Wiley & Sons: Hoboken, 2008.

(7) Damen, M.; Aarbiou, J.; van Dongen, S. F. M.; Buijs-Offerman, R. M.; Spijkers, P. P.; van den Heuvel, M.; Kvashnina, K.; Nolte, R. J. M.; Scholte, B. J.; Feiters, M. C. *J. Control. Rel.* **2010**, *145*, 33.

(8) Ahmad, A.; Evans, H. M.; Ewert, K.; George, C. X.; Samuel, C. E.; Safinya, C. R. *J. Gene Med.* **2005**, *7*, 739.

(9) Leal, C.; Bouxsein, N. F.; Ewert, K. K.; Safinya, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 16841.

(10) Muñoz-Ubeda, M.; Rodriguez-Pulido, A.; Nogales, A.; Llorca, O.; Quesada-Perez, M.; Martin-Molina, A.; Aicart, E.; Junquera, E. *Soft Matter* **2011**, 5991.

(11) Muñoz-Úbeda, M.; Barrán-Berdón, A. L.; Mishra, S. K.; Aicart-Ramos, C.; Sierra, M. B.; Biswas, J.; Kondaiah, P.; Junquera, E.; Bhattacharya, S.; Emilio Aicart, E. *J. Amer. Chem. Soc.* **2011**, *133*, 18014.

(12) Barran-Berdon, A. L.; Muñoz-Ubeda, M.; Aicart-Ramos, C.; Perez, L.; Infante, M. R.; Castro-Hartmann, P.; Martin-Molina, A.; Aicart, E.; Junquera, E., *Soft Matter* **2012**, *8*, 7368. (Front cover of the issue 28)