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Tunable pH sensitive lipoplexes

H. Dhotel, M. Bessodes, N. Mignet

Université de Paris, CNRS, INSERM, UTCBS, Unité des Technologies Chimiques et Biologiques pour la Santé, Paris, F-75006, France.

Abstract

To provide long circulating nanoparticles able to carry a gene to tumour cells, we have designed anionic pegylated lipoplexes which are pH sensitive. The reduction of positive charges in nucleic acid carriers allows reducing the elimination rate, increasing circulation time in the blood, leading to improved tumour accumulation of lipid nanoparticles. Anionic pegylated lipoplexes have been prepared from the combined formulation of cationic lipoplexes and pegylated anionic liposomes. The neutralization of the particle surface charge as a function of the pH was monitored by light scattering in order to determine the ratio between anionic and cationic lipids that would give pH sensitive complexes. This ratio has been optimised to form particles sensitive to pH change in the range 5.5-6.5. Compaction of DNA into these newly formed anionic complexes was checked by DNA accessibility to picogreen. The transfection efficiency and pH sensitive property of these formulations was shown in vitro using bafilomycin, a vacuolar H⁺-ATPase inhibitor.

Keywords: Anionic lipoplexes, pegylated lipoplexes, pH-sensitive lipoplexes, gene delivery to tumor, anionic cholesterol.

1. Introduction

Non-viral nucleic acid delivery has made tremendous progresses these last 4 years thanks to the FDA approval of siRNA and mRNA both delivered by lipid nanoparticles (*1-4*). Despite these advances, systemic delivery of nucleic acid, in particular DNA, still suffers from limited endosomal escape and low circulation time resulting in low efficacy in delivering the gene at the target. Works on DNA delivery have involved different types of cationic lipids (*5, 6*) or polymers (*7, 8*), degradable cationic lipid/DNA complexes (*9, 10*) or polymer/DNA complexes (*11, 12*) and more recently ionisable lipids (*13, 14*) which made the difference, as regard to the previously studied nanocarriers, and became part of the recently accepted siRNA and mRNA drugs (*15*).

To improve nucleic acid delivery from endosomes, various strategies might be used, upon them triggered release by physical means (*16*), but also chemical triggers such as pH changes. In the present work, we have focused our studies on the pH sensitive strategy. This might involve the use of pH labile components into the formulation such as pH degradable lipid (*17, 18, 19*) or polymer (*20*). The pH sensitivity might also be provided by a relevant mixture of titrable anionic and cationic lipids in the formulation. Numerous works have been reported on such pH sensitive lipoplexes, mostly to increase gene delivery into the cells upon endosome pH drop (*21, 22, 23*). These are mostly cationic lipoplexes, as they allow a high nucleic acid compaction and an efficient cell uptake. For instance, in the LPDII particles, DNA/polylysine complexes were mixed with acid-sensitive CHEMS/DOPE/DOPE-Peg-folate liposomes to form acid-sensitive lipoplexes (*24*). The pH sensitivity is given by the CHEMS and DOPE lipids which are able to form a hexagonal phase at acidic pH, leading to a lipoplex destabilisation in the endosomal compartment (*25, 26*). This destabilisation might also be brought by fusogenic peptides (*27*). In our case, the idea is different. The concept is to form tunable pH sensitive lipoplexes (*28*), meaning that only the ratio between the anionic and the

cationic lipid would be responsible for the pH sensitivity, whatever the anionic and cationic lipid chosen.

Delivering nucleic acid to tumour using non-viral vectors involves two main requirements

- a long circulation time to obtain the highest possible vector accumulation in the tumour vascularisation; Since this requires poorly charged lipoplexes, in particular a limited amount of cationic charges (29), we have chosen to develop anionic lipoplexes
- a reversal of these anionic charges or an amplification of the cationic charges in the tumour environment upon pH drop, in order to obtain cationic lipoplexes that will efficiently enter the cells by binding to the anionic plasma membrane and deliver its DNA content.
- For this last step, acid sensitive PEG had been developed (30, 31, 32)
- a fusogenic lipid might also be incorporated into the lipoplex.

To reach these goals, we have designed tunable anionic pH sensitive lipoplexes. These complexes are anionic at physiological pH and pegylated to improve their circulation time, as compared to cationic lipoplexes (33). Moreover, they become cationic at pH under 6, in order to promote efficient tumour cell internalisation, since it is widely recognized that extracellular pH is acidic in ischemic tumour tissue (see Scheme 1).

2. Materials

2.1. Abbreviations of the lipids used

PEG: Polyethylene glycol; DOPE: Dioleoylphosphatidylethanolamine; CHEMS: Cholesteryl hemisuccinate; DPPC: Dipalmytoylphosphatidylcholine. The names of the different lipids were generated with AutoNom 2000 software which is based on IUPAC rules. The cationic lipid whose name according to the nomenclature is 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecylcarbamoyl methyl-acetamide or RPR209120 that we called

DMAPAP was previously described in the supporting information of Thompson et al (34). We can provide this lipid. The tetracarboxylated derivative, [(2-{Cholesteryloxy-carbonyl-[2-(bis-carboxymethyl-carbamoyloxy)-ethyl]-amino}-ethoxycarbonyl)-carboxymethyl-amino]-acetic acid, that we named CCTC, is described in Mignet et al (35).

2.2. Chemicals and DNA provided or synthesised

L- α -Dioleoyl Phosphatidylethanolamine (DOPE), and 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC) were purchased from Avanti Polar Lipids. The chol-PEG₁₁₀ was obtained in one step from the reaction of cholesteryl chloroformate and α -amino- ω -methoxy-PEG. The luciferase encoding gene was obtained as reported (33). Picogreen[®] was purchased from Molecular Probes, USA, Bafilomycin from Sigma, France, the BCA kit from Pierce and the luciferase kit from Promega.

2.3. Equipment

Anionic liposome was prepared by the film method on a rotary evaporator Heidolph, VWR, equipped with a vacuubrand CVC2 to control the pressure. Sonication was performed on sonicator branson 1210. Size and zeta potentials measurements were performed on a Zeta Sizer NanoSeries from Malvern Instruments equipped with a MPT2 autotitrator. Fluorescence was measured on a multilabel plate reader Wallac Victor2 1420 Multilabel Counter, Perkin Elmer, France, equipped with excitation and emission filters (350 \pm 10 nm, 450 \pm 10 nm).

2.4. Buffer preparation

1. Prepare the tris(hydroxymethyl)aminoethane-maleate 50mM, pH 7:

Prepare a solution of tris acid maleate 50 mM by mixing 6 g of tris(hydroxymethyl)aminomethane and 5.8 g of maleic acid in 1 L H₂O. Prepare a 50 mM

NaOH solution. Mix the 50 mL of the tris acid maleate solution and 48 mL of NaOH solution. Verify and adjust the pH by adding one of the tris acid maleate or NaOH solutions.

2. Prepare the solution of 5% glucose by mixing 5 g in 100 mL H₂O

3. Prepare the tris-maleate/glucose buffer: Mix the tris-maleate and the glucose solutions volume to volume to obtain the tris-maleate 25 mM, glucose 2.5 %

4. Prepare the Hépès 40 mM, and a solution of 20 % glucose (20 g in 100 mL H₂O). Mix the Hépès and the glucose solutions volume to volume to obtain the Hépès 20 mM, glucose 10 %

3. Methods

Obtaining anionic self-associated lipoplexes is not obvious, since a competition occurs between the anionic charge of the particles and the anionic charges of the nucleic acid. The easiest way to proceed is to obtain cationic lipoplexes core by mixing cationic lipid and DNA or mRNA. Then an anionic pegylated liposome is added to the mixture to form an anionic pegylated lipoplex (Scheme 1). The PEG lipid was used to avoid aggregation that would occur by mixing the cationic lipoplexes and the anionic liposome, and will also be useful to limit protein interaction upon systemic injection (35). The cationic lipid (34) we used bears two primary amines, one secondary and one tertiary amine, we designed original negatively charged cholesterol bearing four carboxylate moieties (33) in order to limit the amount of cholesterol in the liposomes.

Thanks to the presence of the carboxylate moieties, the anionic particle charge should be reversed to a cationic one at a determined appropriate pH. To reach this goal, the ratio between the two lipids has to be optimized, taking into accounts the DNA negative charges. The optimal ratio was determined by zeta potential measurements combined with titration experiments, as will be described (Data obtained represented Figure 1). Light scattering was also used to insure, that the particle size remained in the right size range (around 100-200

nm), which is fundamental to maintain the particle circulating into the blood stream (6). To evaluate if plasmid DNA was well confined into the structure formed, picogreenTM was used and the fluorescence associated to free DNA or compacted DNA was measured. Compacted DNA does not allow picogreenTM to intercalate into the DNA base pair and gives a low fluorescence level. The intracellular pH sensitivity of the anionic pegylated lipoplexes was shown in vitro using bafilomycin, an ATPase inhibitor, which reduces endosome acidification (Figure 2).

3.1. Preparation of cationic liposomes

1. Dissolve separately the lipids DMAPAP (10 μ mol, 10 mg) and DOPE (10 μ mol, 7.3 mg) in chloroform (500 μ L each). Take care that the lipids are well dissolved separately before mixing them (*see Note 1*).
2. Mix them into a round bottom flask (10 mL) (*see Note 2*)
3. Put the flask at the evaporary evaporator to remove the solvent in a pressure controlled manner. First reduce the pressure from 1000 to 200 mbar in approximately 15 minutes with a middle rotation speed. When the drop forms, increase the rotation speed at its maximum level to drag the drop into the film. Then, reduce the pressure from 200 to 5 mbar in 30 minutes and leave the film under reduced pressure for an additional hour. (*see Note 3*).
4. The film being dry, add 1 mL (to afford a final concentration of 20 mM) milliQ filtered (0,22 μ m) H₂O and leave the flask under gentle rotation overnight at room temperature (*see Note 4*).
5. Mix gently the mixture on a vortex if the film is not fully detached from the wall. Sonicate the particles during 5 minutes to afford a rather homogenous size distribution of approximately 150-200 nm.

6. Control the size by dynamic light scattering (*see Note 5*). For measurements on a nanoZS (Malvern Instruments), dilute 5 μL of the particles obtained in a 500 μL cuve, start the measure in the automatic mode.

3.2. Preparation of cationic lipoplexes

Lipoplexes were prepared in tris-maleate 25 mM, glucose 2.5 % with a charge ratio cationic lipid/anionic lipid = 6, which corresponds to a ratio total lipid to DNA = 12.

1. Dilute the DMAPAP/DOPE suspension initially at 20 mM to 5 mM in H_2O .
2. Dilute 6 μg pDNA in 100 μl tris-maleate 25 mM, glucose 2.5 %
2. Dilute 15 μL of the 5 mM DMAPAP/DOPE suspension in 100 μL tris-maleate 25 mM, glucose 2.5 %
3. Add the plasmid DNA to the cationic liposome dropwise in few seconds with constant vortexing (*see Note 6*)
4. Leave the sample 1 h at room temperature to incubate before using it or adding it to the anionic liposomes.

3.3. Preparation of anionic pegylated liposomes

The anionic liposomes were prepared by the film method as described for the cationic liposome in 3.1.

1. Dissolve separately the lipids DPPC (5 μmol , 3.7 mg), CCTC (15 μmol , 7.4 mg) and Chol-PEG₁₁₀ (0.5 μmol , 2.5 mg) in chloroform (200 μL , 600 μL and 200 μL respectively). Take care that the lipids are well dissolved separately before mixing them (*see Note 1*).
2. Mix them into a round bottom flask (10 mL) and evaporate the chloroform under reduced pressure as described in 3.1.3 (*see Note 2 and 3*).

3. The film being dry, add 1 mL (to afford a final concentration of 20.5 mM) milliQ filtered (0,22 μm) H₂O and leave the flask under gentle rotation overnight at room temperature.
4. Mix gently the mixture on a vortex if the film is not fully detached from the wall.
5. Filter the particles successively on 0.45 and 0.22 μm polyethylsulfonate filters.
6. Control the size by dynamic light scattering (*see Note 5*). For measurements on a nanoZS (Malvern Instruments), dilute 5 μL of the particles obtained in a 500 μL cuve, start the measure in the automatic mode.

3.4. Preparation of anionic pegylated lipoplexes

The preformed cationic lipoplexes were added to the anionic liposomes according to the charge lipid ratio DMAPAP/CCTC (\pm) = 1.3.

1. Prepare a suspension of the anionic liposomes at 0.55 mM from the 20.5 mM suspension prepared in 3.3.
2. Dilute 100 μL of this anionic liposome suspension in Hepes 20 mM, glucose 10 %
3. Add the preformed lipoplexes described in 3.2 to the suspension of the anionic liposomes.

3.5. Titration experiments

1. Prepare the HCl 0.1M buffer and fix it to the titrator (*see Note 7*).
2. Rinse the autotitrator cables with H₂O. Prime the HCl buffer and rinse again.
3. Dilute the anionic pegylated lipoplexes prepared in 3.4 in 10 mL H₂O and fix the flask at the autotitrator, including the pH electrode.
4. Enter the protocol: zeta potential measurements with an initial pH point taken at the pH of the solution, in this case pH 8.25 and points taken every 0.5 pH change until pH 3.7 is reached. Stir between each measure to insure the solution is homogeneous. Electrophoretic

mobility is converted automatically to the ζ potential according to the Smoluchowski equation by the system.

3.6. DNA complexation checked by Fluorescence

1. Prepare the picogreen[®] solution as described by the provider (1/200 in tris-EDTA buffer).
2. Load into a 96-well plate free DNA or complexed DNA (40 ng) in triplicates
3. Add 200 μ L of the picogreen solution (3.6.1) to each well filled with DNA and three more to obtain the picogreen background level.
4. Read the emission at 450 nm under an excitation at 350 nm on a multiplate reader able to measure fluorescence.
5. For the calculation, calculate the mean and the standard error on each triplicates. Remove the picogreen background from the sample data. Calculate the percentage of fluorescence of each sample by dividing the sample data by the value of the free DNA taken as 100% fluorescence.

3.7. In vitro experiments

1. B16 murine cells were grown into DMEM supplemented with L-glutamin (29.2 mg/mL), penicillin (50 units/mL), streptomycin (50 units/mL), and 10% foetal bovine serum.
2. The day before the experiment, seed B16 cells into 24-well culture plates at a density of 50,000 cells per well and incubate at 37 °C, under 5 % CO₂ for 24 h.
3. One hour before transfection, wash the cells with fresh medium with or without bafilomycin.
4. Add 100 μ L of cationic or anionic lipoplexes containing 0.5 μ g DNA onto each well in triplicate, and incubate the plates at 37 °C for 6 h in the presence of 5 % CO₂, then replace by fresh medium for 18 hours.

5. Wash the cells twice with PBS and treat with 200 μL of a passive lysis buffer (Promega). After 15 min, centrifuge the cells for 5 min at 12,000 tr/min.
6. Add 10 μL of the supernatant and 10 μL of iodoacetamide to a 96-well plate, and incubate at 37 °C for 1 h. Quantify the protein content with the BCA protein assay KIT (PIERCE) and report to BSA taken as a reference curve (*see Note 8*).
7. Quantify the luciferase activity using a commercial kit Luciferase assay system (PROMEGA): Load 10 μL of the lysed cells into a 96-well plate and place it into the lecture plate reader. Load the luciferase substrate to the injector. 50 μL of the luciferase substrate is injected via an injector, and the absorbance is read immediately at 563 nm on a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer).
8. For the calculation, background of the untreated cells, taken as negative controls, was removed from the sample data. The relative counts obtained for luciferase quantification were divided by the protein content in each well to normalise the results per μg of protein. The cationic formulation was taken as the positive reference formulation.

4. Notes

1. Solubility of the lipids should be checked with intensive care since presence of insoluble entities will appear in the film and reduce particle homogeneity after hydration
2. The ratio between the volume to be reduced (or the amount of lipids) and the round bottom flask is important since the film should occupy as much flask wall as possible. From the surface of the flask occupied by the film will depend the number of layers in the liposomes.
3. Make sure that the film is not crackled by a too rapid pressure reduction. If so, dissolve again the lipids in 1 mL CHCl_3 and start again part 3. It is always preferable to obtain an homogeneous film along the flask wall, it will provide more homogeneous liposome size after the hydration step.

4. Evaporation and hydration time are usually reported as shorter, but we have found that taking time to do these steps are required to form homogeneous liposome sizes.
5. All buffers and water used should be filtered on 0.22 μ m filters since any dust might interfere with light scattering experiments.
6. In order to maintain an excess of cationic charges and hence avoid precipitation by going through a charge ratio (+/-) equal to 1, we add DNA on the cationic lipid and not the opposite order
7. All buffers should be degazed prior titration to avoid any volume error.
8. The concentration range of BSA should be done for each experiment since the values are fully dependent on the incubation time. It should be performed in the same buffer as the buffer used for the cells, in this case passive lysis buffer.

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References

1. Kulkarni, J.A., Witzigmann, D., Chen, S., Cullis, P.R., Van der Meel, R. (2019) Lipid Nanoparticle Technology for Clinical Translation of siRNA Therapeutics. *Acc Chem Res.* **52**, 2435-2444.
2. Kulkarni, J.A., Witzigmann, D., Thomson, S.B., Chen, S., Leavitt, B.R., Pieter R. Cullis, P.R., Van der Meel, R. (2021) The current landscape of nucleic acid therapeutics *Nature Nanotechnology* **16**, 630-643.
3. Buschmann, M.D., Carrasco, M.J., Alishetty, S., Paige, P., Alameh, M.G., Weissman, D. (2021) Nanomaterial Delivery Systems for mRNA Vaccines, *Vaccines* **9**, 65-95.

4. Ftouh, M., Kalboussi, N., Abid, N., Sfar, S., Mignet, N., Bahloul B. (2021) Contribution of Nanotechnologies to Vaccine Development and Drug Delivery against Respiratory Viruses *PPAR Res.* 6741290.
5. Bessodes, M., Dhotel, H., Mignet, N. (2019) Lipids for Nucleic Acid Delivery: Cationic or Neutral Lipoplexes, Synthesis, and Particle Formation. In: Ogris M., Sami H. (eds) *Nanotechnology for Nucleic Acid Delivery. Methods in Molecular Biology*, vol 1943. Humana Press, New York, NY.
6. Tranchant, I., Thompson, B., Nicolazzi, C., Mignet, N., Scherman, D. (2004) Physicochemical optimization of plasmid delivery by cationic lipids. *J. Gene Med.*, **Suppl. 1**, S24-S35.
7. Drean, M., Debuigne, A., Goncalves, C., Jérôme, C., Midoux, P., Rieger, J., Guégan, P., (2017) Use of Primary and Secondary Polyvinylamines for Efficient Gene Transfection, *Biomacromolecules*, **18**, 440-451.
8. David N. Nguyen, Jordan J. Green, Juliana M. Chan, Robert Langer, Daniel G. Anderson (2009) Polymeric Materials for Gene Delivery and DNA Vaccination, *Advanced Materials* 21, 847-867.
9. Byk, G., Wetzter, B., Frederic, M., Dubertret, C., Pitard, B., Jaslin, G., Scherman, D. (2000) Reduction-sensitive lipopolyamines as a novel nonviral gene delivery system for modulated release of DNA with improved transgene expression. *J. Med. Chem.* **43**, 4377-4387.
10. Mignet, N., Byk, G., Wetzter, B., Scherman, D., (2003) DNA Complexes with Reducible Cationic Lipid for Gene Transfer, *Methods in Enzymology*, Academic Press, **373**, 357-369.
11. Lutén, J., Van Nostrum, C., De Smedt, S., Hennink, W. (2008) Biodegradable polymers as non-viral carriers for plasmid DNA delivery. *J. Contr. Release* **126**, 97-110.

12. Heller, P., Hobernik, D., Lächelt, U., Schinnerer, M., Weber, B., Schmidt, M., Wagner, E., Bros, M., Barz, M. (2017) Combining reactive triblock copolymers with functional cross-linkers: A versatile pathway to disulfide stabilized-polyplex libraries and their application as pDNA vaccines, *J. Controlled Release*, **258**, 146-160.
13. Semple, S. C. et al. Rational design of cationic lipids for siRNA delivery (2010) *Nature Biotechnol.* **28**, 172–176.
14. Han, X., Zhang, H., Butowska, K., Swingle, K. L., Alameh, M. G., Weissman, D., & Mitchell, M. J. (2021) An ionizable lipid toolbox for RNA delivery. *Nature communications*, **12**, 7233.
15. Suzuki, Y., Ishihara, H. (2021) Difference in the lipid nanoparticle technology employed in three approved siRNA(Patisiran), and mRNA (COVID-19 vaccine) drugs *Drugs Metabol. Pharmacokinetics* **41**, 100424.
16. Do, H.D., Martin, B., Doan, B.T., Corvis, Y., Mignet, N. (2019). Advances on non-invasive physically triggered nucleic acid delivery from nanocarriers, *Adv. Drug. Deliv. Reviews* 138, 3-17.
17. Garinot, M., Masson, C., Mignet, N., Bessodes, M., Scherman, D. (2007) Synthesis and advantages of acid-labile formulations for lipoplexes. *Liposome Technology*, 3rd edition, chapter 8 (vol 1), G. Gregoriadis Ed., pp 139-163.
18. Martin, B., Sainlos, M., Aissaoui, A., Oudrhiri, N., Hauchecorne, M., Vigneron, JP., Lehn, JM., Lehn, P. (2005) The design of cationic lipids for gene delivery. *Curr. Pharm. Des.* 3, 375-394.
19. Roux, E., Stomp, R., Giasson, S., Pezolet, M., Moreau, P., Leroux, JC. (2002) Steric stabilization of liposomes by pH-responsive *N*-isopropylacrylamide copolymer. *J. Pharm. Sci.* **91**, 1795-1802.

20. Kim, Y., Park, J., Lee, M., Kim, Y., Park, T., Kim, S. (2005) Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *J. Control. Release.* **103**, 209-219.
21. Shi, G., Guo, W., Stephenson, S., Lee, R. (2002) Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. *J. Control. Release* **80**, 309-319.
22. Guo, X., Szoka, F.C. (2003) Chemical approaches to triggerable lipid vesicles for drug and gene delivery. *Acc. Chem. Res.* **36**, 335-341.
23. Sato, Y. (2021) Development of Lipid Nanoparticles for the Delivery of Macromolecules Based on the Molecular Design of pH-Sensitive Cationic Lipids, *Chem. Pharm. Bull.* **69**, 1141–1159.
24. Lee, R., Huang, L. (1996) Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J. Biol. Chem.* **271**, 8481-8487.
25. Hafez, I., Cullis, P. (2000) Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. *Biochim. Biophys. Acta* **1463**, 107-114.
26. Koynova, R., Wang, L., Tarahovsky, Y., MacDonald, R. (2005) Lipid phase control of DNA delivery. *Bioconj. Chem.* **16**, 1335-1339.
27. Hafez I., Ansell, S., Cullis, P. (2000) Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids. *Biophys. J.* **79**, 1438-1446.
28. Simões, S., Slepushkin, V., Gaspar, R., de Lima, M., Düzgüneş, N. (1998) Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides. *Gene Ther.* **7**, 955-964.
29. Zalipsky, S., Brandeis, E., Newman, M.S., Woodle, M.C. (1994) Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine. *FEBS Lett.* **353**, 71-74.

30. Masson, C., Garinot, M., Mignet, N., Wetzler, B., Mailhe, P., Scherman, D., Bessodes, M. (2004) pH sensitive PEG lipids containing orthoester linkers: new potential tools for nonviral gene delivery. *J. Control. Release* **99**, 423-434.
31. Choi, J., MacKay, J., Szoka, F. (2003) Low-pH-Sensitive PEG-Stabilized Plasmid-Lipid Nanoparticles: Preparation and Characterization. *Bioconjugate Chem.*, **14**, 420,
32. Kale, A., Torchilin, V. (2007) Enhanced transfection of tumor cells in vivo using "Smart" pH-sensitive TAT-modified pegylated liposomes. *J. drug target* **15**, 537-548.
33. Mignet, N, Richard, C., Seguin, J., Largeau, C., Bessodes, M., Scherman, D. (2008) Anionic pH-sensitive pegylated lipoplexes to deliver DNA to the tumors. *Int. J. Pharm.* **361**, 194-201.
34. Thompson, B., Mignet, N., Hofland, H., Lamons, D., Seguin, J., Nicolazzi, C., de la Figuera, N., Kuen, R., Meng, Y., Scherman, D., Bessodes, M. (2005) Neutral post-grafted colloidal particles for gene delivery. *Bioconj. Chem.* **16**, 608-614.
35. Mignet, N, Cadet, M., Bessodes, M., Scherman, D. (2007) Incorporation of PEG lipid into Lipoplexes: On-line Incorporation Assessment, and Pharmacokinetics Advantages, *Liposome Technology*, 3rd edition, chapter **16** (vol 2), G. Gregoriadis Ed., pp 273-292.

Figure captions

Scheme 1: Schematised representation of the sequential process used for the formation of anionic lipoplexes and charge reversibility upon reduction of the pH. It does not take into account the structure of the complexes neither the position of DNA into the particle lipidic bilayer.

Figure 1: Zeta potential of the particles as a function of the pH. The pH was initially measured at pH 8.25 and decreased by addition of a solution of HCl at 0.1 M, in order to obtain steps with a pH decrease of 0.5. A measure of the electrophoretic mobility and dynamic diameter was measured every variation of 0.5 in pH after the pH was stabilised. The measures were taken at room temperature.

Figure 2 : Transfection of the cationic and anionic formulatoins using a plasmid encoding for the luciferase reporter gene in presence and absence of bafilomycin. The Y axis represents the level of luciferase expression per protein (μg), mean values + SD. The background level of the untreated cells was removed for each samples.