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Lactose-ornithine bolaamphiphiles for efficient gene delivery in vitro

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ABSTRACT

The development of new nonviral vectors characterized by high transfection efficiency and low cytotoxicity remains an important challenge in the field of gene delivery. Unsymmetrical bolaamphiphiles (bolas) appear as new emerging candidates for this application. In this work, new unsymmetrical bolas, bearing neutral lactonic acid and cationic ornithine residues at the two ends of a hydrophobic spacer, were synthesized and their properties were compared to analogues bearing a gluconic acid residue. The new bolas showed DNA binding and condensation at higher N/P ratios than their gluconic analogues, probably due to their larger neutral head group. Whereas the size of the complexes of the new bolas with DNA (bolaplexes) increased with N/P, as a result of charge neutralization, their formulations with DOPE at high N/P were of small size (ca. 200 nm). These DOPE formulations showed high transfection efficiency in different cell lines (HeLa, COS-7 and HepG2), close to that of jetPEI. Their cytotoxicity was relatively low, which allowed repetitive transfection in vitro. Fluorescence imaging showed that the bolaplexes bind rapidly to cell surface and internalize mainly through endocytosis. This work suggests a new type of efficient nonviral vectors based on bolaamphiphiles.

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1. Introduction

Gene therapy treats various diseases by insertion of functional genes into individual cells and tissues in order to replace the defective genes (Anderson, 1998; Cross and Burmester, 2006; Hoag, 2005). The success of gene therapy is largely dependent on the development of suitable vectors or vehicles for in vivo gene delivery (Lavigne and Gorecki, 2006; Williams and Baum, 2003). A number of viral and nonviral vectors have been developed for transfecting eukaryotic cells. Viral vectors are replication-defective viruses (retrovirus, adenovirus, etc.) with part of their coding sequences replaced by the therapeutic gene(s). They are highly efficient but can be pathogenic (Check, 2005a; Woods et al., 2006) and immunogenic (Check, 2005b), raising safety concerns. An additional drawback of viral vectors is the limited size of DNA they can deliver. In these respects, non-viral gene delivery systems are an attractive alternative (Demeneix et al., 2004; Lavigne and Gorecki, 2006; Li and Huang, 2007; Nishikawa and Huang, 2001). Currently, cationic polymers and lipids are the two most established types of non-viral vectors (Demeneix et al., 2004; Martin et al., 2005; Mastrobattista et al., 2006; Mintzer and Simanek, 2009; Zuber et al., 2003). Their complexes with DNA are called polyplexes and lipoplexes, respectively. Nonviral vectors are particularly suitable with respect to simplicity of use, large-scale production, ability to carry DNAs of large size, and lack of immune response.

One of the most explored types of nonviral vectors is cationic lipids. A variety of them has been developed for lipoplex-based gene delivery (Hirko et al., 2003; Martin et al., 2005; Safinya, 2001; Wasungu and Hoekstra, 2006). Lipids like DOTAP, DOGS, lipofectamine, etc., are typical examples of highly efficient agents for in vitro (cell culture) transfection. However, there are several key problems limiting applications of cationic lipids in gene delivery. At first, their complexes with DNA appear as infinite (usually sandwich-like) structures (Koltover et al., 1998; Safinya, 2001), so that the lipoplexes are relatively large and polydisperse. Therefore, they exist in variety of structures with single or multiple DNA molecules with size ranging from tens of nanometers up to microns. Second, the stability of the complexes is poor because the cationic lipids interact strongly with serum proteins, thus limiting their in vivo applications.

An innovative direction in the development of lipid-based nonviral vectors is the use of bipolar lipids, so-called bolaamphiphiles (bolas). These are analogues of lipids bearing two polar groups from the opposite sides of the hydrophobic chain(s) (Fuhrhop and Wang, 2004), so that they can form monolayer membranes, instead of bilayers formed by lipids (Fig. 1). A distinctive feature of bolas is the presence of membrane spanning alkyl chains, which are believed to be responsible for their enhanced physical stability

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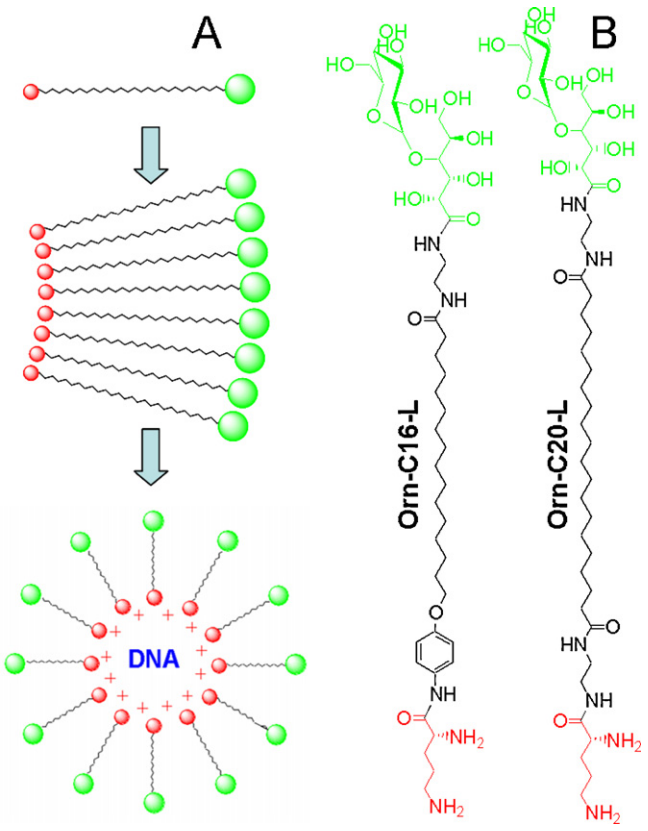

Fig. 1. Schematic presentation of bolaamphiphile, its hypothetic assembly into asymmetric monolayer membranes and formation of DNA complexes, so-called bolaplexes (A). Red and green parts represent cationic and neutral head groups, respectively. Chemical structures of the new bolas (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Damste et al., 2002; Fuhrhop and Wang, 2004). In nature, they are present in archaebacteria and ensure the integrity of the bacterial membrane at high temperature (90 °C) and low pH (1–1.5) (Derosa et al., 1986; Stetter, 1996). Importantly, bolas bearing both positively charged and neutral head groups could generate asymmetric membranes (in form of vesicles or nanotubes) having positively charged inner and neutral outer surfaces. These asymmetric membranes could function as a shell, where the inner shell surface binds the DNA molecule (Fig. 1), while the outer membrane surface is utilized for efficient exposure of the biological signal for specific targeting. Successful application of bolas for gene (Brunelle et al., 2009; Denoyelle et al., 2006; Eaton et al., 2000; Fabio et al., 2003; Gaucheron et al., 2001) and peptide (Parmentier et al., 2011) delivery has been shown only recently. For gene delivery, the most promising bolas bear a sugar (gluconic or lactonic) residue on one side and a mono- or oligo-cationic ammonium-based group on the other (Brunelle et al., 2009; Gaucheron et al., 2001). However, the development of bolaamphiphiles as vectors for gene delivery is still in its infancy and requires a systematic work in the field. Recently, we have developed unsymmetrical bolas bearing gluconic groups on one side and dicationic ornithine on the other (Jain et al., 2010). These bolas showed a strong tendency to self-assemble at very low concentration and bind DNA, forming particles that increase in size with increase of N/P ratios. Their transfection was detected in the presence of chloroquine or DOPE helping lipid, though the efficiency was relatively low. In the present work, we substituted the gluconic head-group by the lactonic one. A number of studies showed that the lactose residue is able to improve the transfection efficiency of different vectors, due to receptor-mediated endocytosis (Jiang et al., 2008; Morille et al., 2009) and other mechanisms (Fajac et al., 1999; Remy et al., 1995). Thus, the lactonic group could improve the transfection efficiency of our first generation bolas. In addition, as this polar group is much larger than the gluconic group, it could additionally improve the bola solubility in water and thus prevent aggregation of their DNA complexes. In the present work, we synthesized two bolas bearing lactonic groups on one side and ornithine groups on the other side (Orn-C16-L and Orn-C20-L, Fig. 1). Though bolas alone showed relatively weak transfection efficiency, the efficiency was improved remarkably in the presence of DOPE and was comparable to that of commercially available vectors. In this formulation, the obtained bolaplexes were relatively monodisperse, with small particle size (200 nm), which constitutes an important feature of prospective vectors for future in vivo applications. The high transfection efficiency of the present bolaamphiphiles suggests them as new type of non-viral vectors.

2. Materials and methods

2.1. Synthesis of bolaamphiphiles

All chemicals and solvents for synthesis were purchased from Sigma-Aldrich. Mass spectra were measured using Mass Spectrometer Mariner System 5155. LC-MASS was performed on Agilent, 1956 B/MSD. ^{1}H NMR spectra were recorded on Bruker 300 MHz spectrometer.

{4-tert-Butoxycarbonylamino-4-[4-(15-{2-[2,3,5,6-tetrahydroxy-4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-hexanoylamino]-ethylcabamoyl}-pentadecyloxy)-phenylcarbamoyl]-butyl}-carbamic acid tert-butyl ester (**1**). Lactonolactone (0.24 mmol, 0.08 g) was added to solution of *(1-{4-[15-(2-Amino-ethylcarbamoyl)-pentadecyloxy]-phenylcarbamoyl}-4-tert-butoxy-carbonylamino-butyl)-carbamic acid tert-butyl ester* (Jain et al., 2010) (0.14 mmol, 0.1 g) in 20 ml of methanol. Then, DIEA (1.4 mmol, 0.24 ml) was added and the reaction mixture was stirred at 70 °C for about 24 h. Solvents were evaporated in vacuo and water was poured into the reaction flask. The product **1** (0.12 g, 81%) was extracted with butanol and the solvent was evaporated in vacuo. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.43 (d, 2H), 6.84 (d, 2H), 4.49–4.39 (m, 4H), 4.21 (d, 2H), 3.98–3.64 (m, 10H), 3.64–3.42 (m, 4H), 3.08–3.02 (t, 3H), 2.26–2.17 (t, 2H), 1.76–1.5 (m, 8H), 1.48–1.35 (m, 18H), 1.35–1.16 (m, 24H). LC–MS: (*m*/*z*) Found $[M+1]^+$ = 1060.62 (calcd. for $C_{51}H_{89}N_5O_{18}^+$ = 1060.62).

16-[4-(2,5-Diamino-pentanoylamino)-phenoxy]-hexadecanoica-cid{2-[2,3,5,6-tetra hydroxy-4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-hexanoyl amino]-ethyl}-amide (**Orn-C16-L**). Boc was de-protected from 50 mg of 1 using 1 ml of TFA and 2 ml CH_2Cl_2 to get the final product **Orn-C16-L** (37 mg, 91%). LC–MS: (*m*/*z*) Found $[M+1]^+$ = 860.2 (calcd. for $C_{41}H_{73}N_5O_{14}^+$ = 860.2).

{4-tert-Butoxycabonylamino-4-[2-(19-{2-[2,3,5,6-tetrahydroxy-4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-hexanoylamino]-ethylcarbamoyl}-nonadecanoylamino)-ethylcaba-moyl]-butyl}-carbamic acid tert-butyl ester (**2**). Lactonolactone (0.23 mmol, 0.078 g) was added to solution of *(1-{2-[19-(2-Amino-ethylcarbamoyl)-nonadecanoylamino]-ethylcarba-moyl}-4-tert-butoxycarbonylamino-butyl)-carbamic acid tert-butyl ester* (Jain et al., 2010) (0.14 mmol, 0.1 g) in 20 ml of methanol. Then, DIEA (1.4 mmol, 0.24 ml) was added and reaction mixture was stirred at 70 °C for about 24 h. Solvents were evaporated in vacuo and water was poured into the reaction flask. The product **2** (0.11 g, 75%) was extracted with butanol and the solvent was evaporated in vacuo. ^{1}H NMR (CD_3OD, 300 MHz): 4.49 (s, 1H), 4.36 (s, 1H), 4.22 (s, 1H), 3.95–3.67 (m, 8H), 3.61–3.47 (m, 3H), 3.06–3.02 (t, 2H), 2.21–2.15 (t, 4H), 1.61–1.51 (m, 8H), 1.44–1.43 (m, 18H),

1.42–1.28 (m, 32H). LC–MS: (*m*/*z*) Found $[M+1]^+$ = 1081.68 (calcd. for $C_{51}H_{96}N_6O_{18}^+$ = 1081.68).

Eicosanedioic acid [2-(2,5-diamino-pentnoylamino)-ethyl]-amide{2-(2,3,5,6-tetrahydroxy-4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-hexanoylamino]-ethyl}-amide (**Orn-C20-L**). Boc was de-protected from 50 mg of 2 using 1 ml TFA and 2 ml CH_2Cl_2 to get the final product **Orn-C20-L** (38 mg, 93%). LC–MS: (*m*/*z*) Found $[M+1]^+$ = 881.58 (calcd. for $C_{41}H_{80}N_6O_{14}^+$ = 881.58).

2.2. Fluorescence measurements

Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra either on FluoroMax 3.0 or Fluorolog (Jobin Yvon, Horiba) spectrofluorimeters. All the emission spectra were corrected from the background signal of the corresponding blank (corresponding solution without the fluorescent dye). In the ethidium bromide (EtBr) exclusion assay, an aliquot of calf-thymus DNA (CT-DNA, final concentration 20 μM) was added to the solution of EtBr (0.4 μM). After 2 min, increasing quantities of the corresponding bola were added from stock solutions (in DMF with 50% of water). Fluorescence intensity at 600 nm (excitation at 550 nm) was recorded 2 min after each addition of bola aliquot.

2.3. Dynamic light scattering and zeta potential measurements

The bolaplexes were prepared in 20 mM MES at pH 7 by mixing equal volumes of the solutions of pCMV-Luc plasmid (pDNA) and bolas. The bola solutions were prepared by adding aliquots of their stock solutions (in DMF with 50% of water) to the buffer. The final DNA concentration (expressed in phosphate) was 20 μM, while the bola concentration was adjusted to a desired N/P ratio. The N/P ratio between bolas and pDNA was expressed as the molar ratio between all the protonable amino groups of the bolas and the phosphate groups of the DNA. The DLS measurements were performed after 30 min of incubation at room temperature. The average size of the complexes was determined with a Zetasizer Nano ZS (Malvern Instruments) with the following specifications: sampling time, 30 s; medium viscosity, 0.8872 cP; refractive index (RI) medium, 1.33; RI particle, 1.590; scattering angle, 90°; temperature, 25 °C. For particle analysis, the statistics based on particle volume and particle number were presented. For zeta potential measurements, the same sample preparation was done using CT–DNA at 30 μM concentration. The instrumental specifications were the same, accounting for a solvent dielectric constant of 78.5.

2.4. Gel electrophoresis

The bolaplexes were prepared at different N/P ratios using 60 μM pCMV-Luc plasmid (final phosphate concentration), followed by 30 min incubation at room temperature. 4 μl of loading dye (Lonza, 6x) was added to 20 μl of the prepared complexes and then, 12 μl of this mixture were loaded on 0.9% agarose gel prepared with 0.5X TAE (Tris-acetate-ethylenediaminetetraacetic acid) buffer. In parallel, a 10 kbp DNA ladder (Lonza) was loaded on the gel. Electrophoresis was carried out with 0.5X TAE buffer at a constant voltage of 100 mV. DNA bands were visualized by an UV trans-illuminator (GeneGenius, Syngene) after coloration with EtBr (0.5 μg/ml) for 15 min.

2.5. AFM measurements

AFM measurements were performed using the Solver Pro-M (NT-MDT) instrument. The measurements were performed in liquid phase (MES buffer pH 7.4) by using the tapping mode. The cantilevers used were of NSG03 type (NT-MDT) with a typical spring constant of 1.7 N/m, a resonance frequency of 32 kHz in liquid and a tip curvature radius of 10 nm. Images were acquired with a resolution of 512 × 512 and a scan rate of 2 Hz. The samples were prepared as for DLS measurements. Then, 100 μL of the solution was deposited on the freshly cleaved mica followed by the addition of 10 μl of a $MgCl_2$ stock solution to obtain a final concentration of 10 mM. The measurements were performed 10 min after sample preparation.

2.6. Transfection and cytotoxicity

COS-7 cells, HeLa cells or HepG2 cells were grown in Dulbecco's modified Eagle's medium (Gibco-Invitrogen), supplemented with 10% fetal bovine serum (FBS, Lonza) and 100 U/ml of penicillin and streptomycin (Gibco-Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded at a density of 1×10^5 cells in a 24-well plate, 24 h before transfection.

Transfection was done by using bola/pDNA (pCMV-Luc plasmid, 1 μg/well) complexes at different N/P ratios in serum-free Opti-MEM (Gibco-Invitrogen) or complete culture medium (DMEM containing 10% of FBS). For this purpose, bolaplexes were prepared in MES buffer (pH 7) as for DLS measurements and then added to the cells in Opti-MEM. Chloroquine (100 μM) or DOPE (1:1 molar ratio with respect to bola) was used to enhance transfection efficiency in some formulations. In formulations with DOPE, a mixture of DOPE and a corresponding bola in ethanol was evaporated in a round bottom flask to obtain a film. Then, MES buffer (pH 7) was added and the samples were hydrated overnight at RT. Then, the samples were vortexed vigorously for 1 min and further sonicated in an ultrasonic bath for 15 min. The obtained suspensions were mixed with an equal volume of pDNA in the buffer to obtain a desired N/P ratio. All formulations were incubated at RT for 30 min before addition to the cells. After 3 h of incubation of cells with bolaplexes at 37 °C, 10% of FBS was added to serum-free samples, while for all the samples with chloroquine the transfection medium was replaced with fresh complete culture medium and then cells were cultured for another 45 h. Total incubation time for all samples was 48 h. Luciferase gene expression of lysed cells was quantified using a commercial kit (Invitrogen) and a luminometer (CentroXS^3 LB 960, BERTHOLD Technologies). Results were expressed as relative light units integrated over 10 s per mg of total cell protein lysate (RLU/mg of total protein). The experiments were repeated six times for serum-free samples and three times for samples with serum. The total protein of lysed cells was determined by BC assay (Protein Quantitation Kit, Interchim) using the Cary 4000 spectrophotometer. As a positive control in the transfection measurements, the commercially available agent jetPEI (Polyplus) was used following protocols provided in the kit.

Repetitive transfection was done to increase the transfection efficiency of the bolaplexes. For this purpose, bolaplexes were prepared as described above. After 3 h of incubation of cells with bolaplexes at 37 °C, 10% of FBS was added to serum-free samples. The same addition of bolaplexes was repeated once after 24 h (48 h transfection) and again at 48 h (72 h transfection) and the luciferase activity was quantified at each step. Transfection efficiency determined from the luciferase assay was expressed as RLU/mg of protein. The experiments were repeated six times.

Cytotoxicity of bolas was evaluated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay (*29*). COS-7, HeLa or HepG2 cells were seeded in a 24-well plate at 1×10^5, 4×10^4 or 8×10^4 cells per well respectively. After 24 h of incubation at 37 °C, the cells were supplemented with bolas at different concentrations or jetPEI (150 μM, expressed as concentration of nitrogen residues) in serum-free Opti-MEM. Then, after incubation for 3 h at 37 °C, 10% of FBS was added and cells

were cultured for another 45 h. Cells were washed with phosphate buffer saline (PBS) and incubated with serum free medium containing 0.5 mg/mL MTT for 3 h at 37 °C. Media was discarded and formazan crystals were re-suspended in 0.5 mL MTT solvent (Sigma-Aldrich). Absorbance of formazan solution was measured at 570 nm with respect to the background at 690 nm using Cary 4000 spectrophotometer. Cell viability was expressed as relative absorbance (%) of the sample vs. control cells.

2.7. Bolaplexes internalization

For the internalization studies, the pCMV-Luc plasmid DNA was labeled with YOYO-1, which was added to the solution of DNA in milli-Q water at a molar ratio of 1 YOYO-1 molecule for 50 DNA phosphate groups and vortexed overnight in the dark at 4 °C. All experiments were performed in liquid conditions at room temperature on an ibidi dish (μ-Dish35 mm, ibiTreat, Biovalley). The bolaplexes prepared at different N/P ratios using 1 μg YOYO-1 stained pCMV-Luc plasmid (final phosphate concentration) were incubated for 30 min at room temperature. Cell binding and internalization of the complexes was followed by incubating the labeled bolaplexes with *HeLa* cells for different times at 37 °C. Images were taken on a Leica DMIRE2 microscope equipped with an 63x HCX PLAPO (1.32 NA) objective and a Leica DC350FX CCD camera piloted by the Leica FW4000 software. Leica GFP filter cube (excitation 480/40 nm, emission 527/30 nm) was used. The microscope was also equipped with a Life Imaging Services (Basel, Switzerland) control system maintaining the cells at 37 °C.

3. Results and discussion

3.1. Interactions with DNA

The interaction of bolas with firefly luciferase plasmid DNA (pDNA) was characterized using gel electrophoresis. The two bolas (Orn-C16-L and Orn-C20-L) showed similar results (Fig. 2). Indeed, at low N/P (ratio between cationic groups of bola and phosphate groups of DNA), almost no changes are observed as compared to the free pDNA. At the intermediate N/P ratios (2–5), the band of free pDNA disappears, while some smear moving slower than free pDNA could be noticed. The latter could be related to bola/pDNA complexes with partial negative charge. At N/P = 10, this smear disappears completely for both bolas and only immobile complexes at the top of the gel can be detected. Thus, an excess of bola molecules is needed to achieve complete pDNA binding. The binding efficiency of Orn-C16-L and Orn-C20-L appears thus lower than that of their

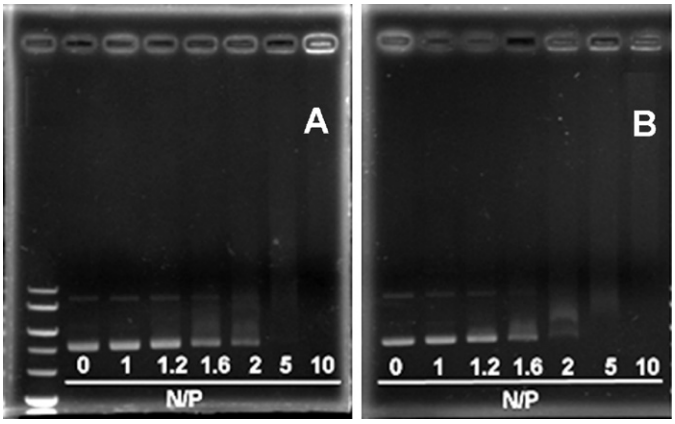

Fig. 2. Agarose gel electrophoresis (0.9%) of Orn-C16-L (A) and Orn-C20-L (B) complexed with pDNA at different N/P ratios. Band at the left of gel (A) correspond to 10 kbp DNA ladder.

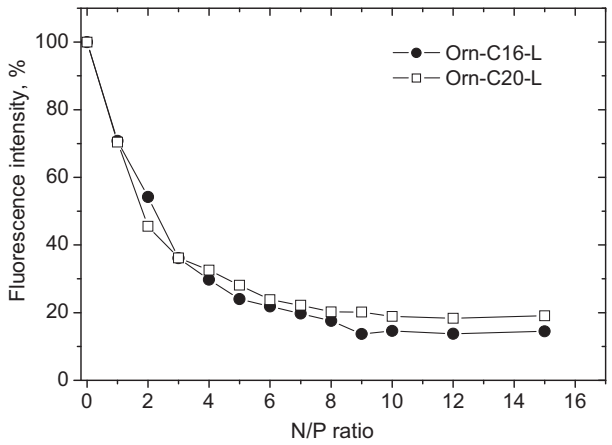

Fig. 3. Exclusion of EtBr from CT–DNA complexes with bolas at different N/P ratios. The fluorescence intensity was normalized to 100% of the initial intensity.

gluconic analogues, Orn-C16-G and Orn-C20-G (Jain et al., 2010). This difference can be explained by the larger size of the lactonic polar head-group compared to the gluconic one, which increases the bola solubility and thus decreases the self-assembling abilities of the bolas. The latter probably weakens the formation of its complexes with DNA, because formation of these complexes relies both on bola–DNA and bola–bola interactions.

To evaluate the level of DNA condensation within the complexes, the ethidium bromide (EtBr) exclusion assay is commonly used (Lleres et al., 2001). This dye, when intercalated into DNA, is highly fluorescent while after DNA condensation, it is expelled from its intercalation site thus resulting in a strong decrease of its fluorescence intensity (up to 90%). For these studies, a linear DNA from calf-thymus (CT–DNA) was used as a model. According to our data, an increase in N/P values decreases the fluorescence intensity of EtBr (Fig. 3). Moreover, a fast decrease in the fluorescence intensity is observed around N/P 2, which corresponds to the initial steps of the complex formation. This observation is in line with the gel electrophoresis data also showing partial disappearance of free pDNA around this N/P ratio. The decrease in the fluorescence intensity is completed at N/P > 8, where a relatively efficient DNA condensation with a 80% drop of EtBr intensity is observed.

3.2. Characterization of bolaplexes

Using dynamic light scattering (DLS), we studied the size of the bola/pDNA complexes at different N/P ratios in the absence and in the presence of the helper lipid DOPE. It can be seen that bolas without DNA self-assemble in buffer into relatively small structures of 50–70 nm (Fig. 4). In the presence of DOPE, both bolas assembled in slightly larger structures of about 100 nm (Fig. 4), which are probably of vesicular nature. In the presence of DNA, the particle size showed a strong dependence on N/P ratio. At N/P = 3 without DOPE, Orn-C16-L formed relatively small complexes of about 200 nm. The effect of free DNA in this measurement could be neglected, as its scattering signal was much weaker (data not shown). In contrast, at higher N/P ratios 5 and 10, the size of the complexes increased up to 1.8 μm. A similar increase in the bolaplex sizes was previously observed for gluconic analogues (Jain et al., 2010). For Orn-C20-L without DOPE, the size of the complexes was above 1 μm for all tested N/Ps (Fig. 4). Addition of DOPE affected the size of bolaplexes only at N/P = 10, where a strong (5-fold) drop in the size of the complexes was observed (200–250 nm).

To better understand the observed size changes, we measured the zeta-potential of the bolas and their complexes (Fig. 5). Bolas

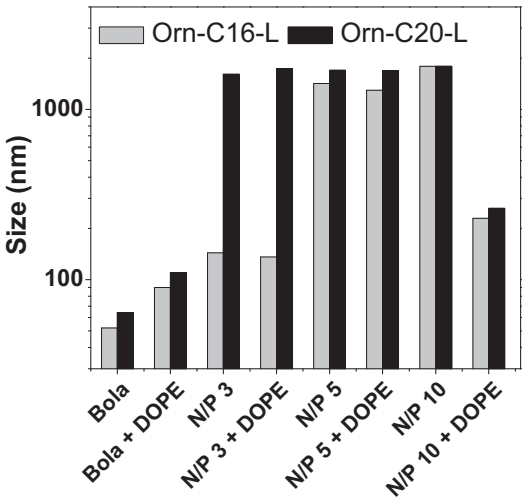

Fig. 4. Size measurements by DLS of bolaplexes with pDNA in MES buffer (pH 7.0) for different formulations. The experimental error is ±10%.

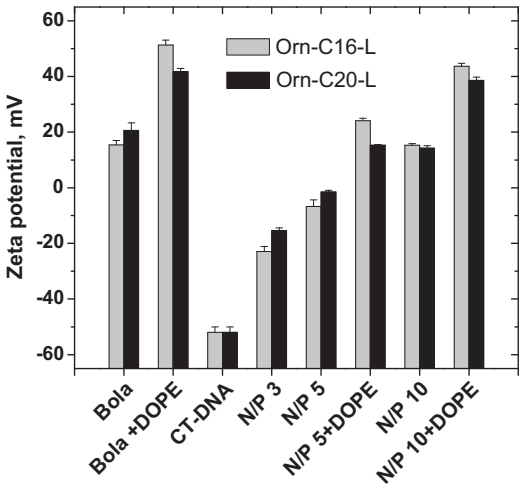

Fig. 5. Zeta-potential of nanostructures formed by bolas alone and by their complexes with CT–DNA in MES buffer (pH 7.0) at different N/P ratios.

show a positive zeta potential without DNA, which is nearly half of that observed for the gluconic-analogues Orn-C16-G and Orn-C20-G (Jain et al., 2010). Without DNA, bolas are probably organized into membrane structures exposing both cationic and neutral sugar residues. As the lactonic group is much more bulky than the gluconic one, the positive surface charge of the ornithine residues is probably screened more efficiently by the former. The presence of DOPE increases nearly twice the observed zeta potential of bola assemblies. Probably, the surface density of the bulky lactonic group decreases in the presence of DOPE, thus exposing the cationic groups of bolas and contributing to larger zeta potential values.

For the bola–DNA complexes (bolaplexes) without DOPE, we found that the zeta potential at N/P = 3 is negative, which explains the relatively small size of the complexes at this N/P. At N/P = 5,

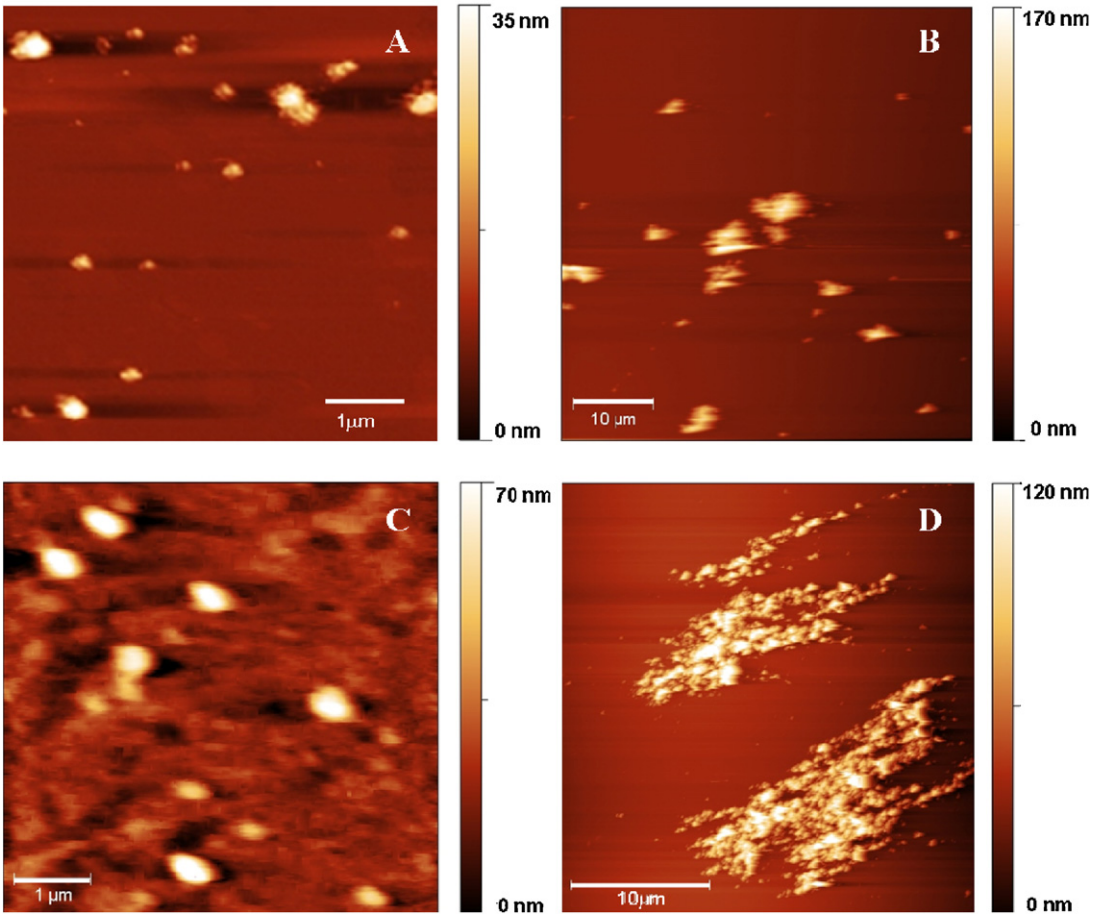

Fig. 6. AFM images of Orn-C16-L (A and B, N/P 10) and Orn-C20-L (C and D, N/P 10) complexed with pDNA in the presence (A and C) and the absence of DOPE (B and D). Images were obtained by the tapping mode.

complexes show a zeta potential close to zero, in line with the observed large size of the bolaplexes (Figs. 4 and 5). Usually, at this N/P ratio cationic lipids and polymers give positively charged complexes (Pitard et al., 1999; Xu et al., 1999). In our case, the observed neutral complexes at higher N/Ps originate probably from the structure of bolaamphiphiles, having both positively charged and neutral ends. Thus, DNA–bola interaction should produce particles exposing predominantly neutral sugar groups (Fig. 1). At N/P = 10, the zeta potential is positive, being similar to that of bola alone. Since the particles are very large (Fig. 4), it is likely that the bolaplexes remain neutral at this high N/P, while the positive zeta potential results from the excess of bolas in solution. In the presence of DOPE, the zeta potential increases strongly with N/P. At N/P = 10, the zeta potential is highly positive (around 40 mV) for both bolas. The presence of lipid could change the bola organization within the bolaplex, exposing cationic ornithine groups, which explains the formation of stable small particles suggested by DLS (Fig. 4). In addition, the presence of the excess of bolas–DOPE structures without DNA could also contribute to the high positive zeta potential.

The nanoscopic structure of the complexes was further characterized by atomic force microscopy (AFM) in liquid (buffer) phase on a mica surface. As the complexes are completed at N/P > 8, we studied only formulations at N/P = 10, with and without DOPE (Fig. 6). It is clear that, without DOPE, very large aggregates are observed for both Orn-C16-L and Orn-C20-L. A careful examination reveals that these large complexes are composed of small particles of ca. 200 nm. Thus, the large complexes are likely aggregates of smaller DNA/bolas complexes, which due their neutral surface charge may have a strong tendency to aggregate. In contrast, in the presence of DOPE, we observe particles of about 350 nm in width and 70 nm in height, which probably correspond to spherical particles distorted by their adsorption on the mica surface. Indeed, from the volume of the observed particles (6.7×10^{-21} m^3), the calculated diameter of hypothetical spherical particles is found 230 nm, which is close to that measured by DLS. Thus, AFM data confirmed the formation of small complexes of ca. 200 nm at N/P = 10, in the presence of DOPE.

3.3. Transfection efficiency and cytotoxicity

The transfection efficiency of bolaplexes was studied using the firefly luciferase expression assay (Fig. 7). Formulations at different N/P with and without DOPE were tested on three different cell lines: HepG2, HeLa and COS-7. HepG2 cells were selected because they express the receptor of endocytosis for galactose (Remy et al., 1995) and thus could specifically recognize our bolaplexes. The other two cells lines are among the most commonly used in cell transfection experiments, though they do not express the galactose receptor. We found that for all cells lines, bolaplexes without DOPE at N/P ratios 3 and 5 showed almost no transfection. Only at N/P = 10, a moderate transfection was observed for Orn-C16-L in COS-7 cells. Addition of chloroquine increased the transfection efficiency of bolaplexes with Orn-C20-L (at N/P = 10) for all three cell lines, but only for HeLa cells in the case of bolaplexes with Orn-C16-L.

Importantly, in the presence of the helper lipid DOPE, bolaplexes showed a dramatic increase in the transfection efficiency. Thus, for N/P = 10, DOPE increased the transfection efficiency >100-fold for both bolas in all cell lines. At N/P = 10, the transfection efficiency was comparatively higher for Orn-C16-L than for Orn-C20-L. Importantly, the observed transfection efficiencies were only 4–6 fold lower than that of jetPEI, one of the most efficient in vitro transfection agent, and were among the highest reported to date for bolaamphiphiles, being for instance about 100-fold more efficient than their gluconic-analogues Orn-C16-G and Orn-C20-G (Jain et al., 2010). Surprisingly, the transfection efficiency of bolaplexes with both Orn-C16-L and Orn-C20-L, was significantly lower in HepG2 cells than in HeLa and COS-7 cells, suggesting that the galactose-specific receptors of the Hep G2 cells do not play a major role in the endocytosis of the bolaplexes. However, as lactonic-bearing bolas are about 100 times more efficient than bolas bearing gluconic group, the lactonic group probably improves the structure of the bolaplexes and plays some positive role in the transfection process, as it has already been reported for other nonviral vectors (Fajac et al., 1999).

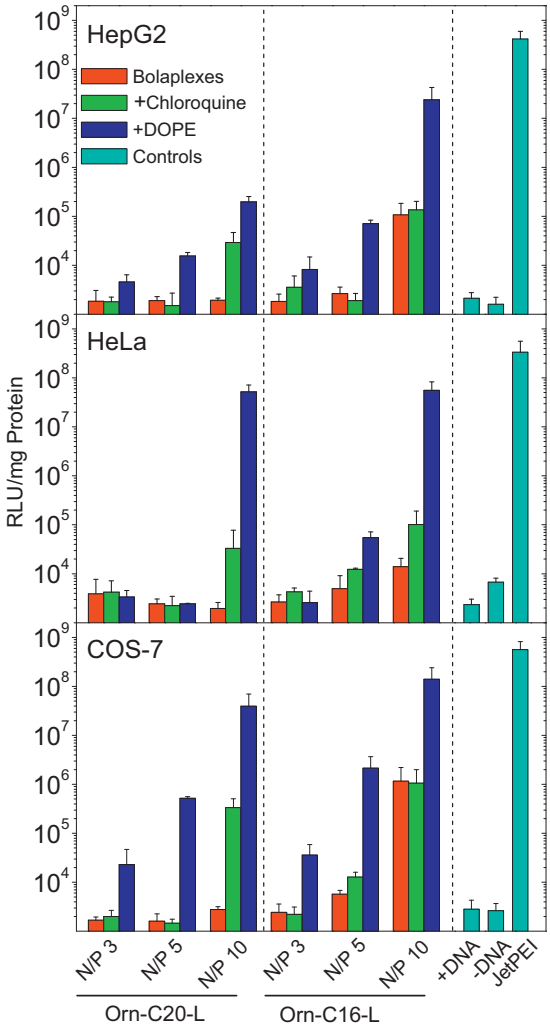

Fig. 7. Transfection efficiency of the Orn-C20-L and Orn-C16-L bolaplexes at different N/P ratios in HepG2, HeLa and COS-7 cells without serum. Cells were incubated in serum-free Opti-MEM with bolaplexes composed of plasmid DNA (1 μg per well), bola and DOPE (when indicated). For some samples, the medium contained 100 μM chloroquine. After 3 h, 10% of FBS was added while, for all the samples in the presence of chloroquine, the transfection medium was replaced with fresh complete culture medium. The luciferase activity quantification was performed after 48 h of incubation. Transfection efficiency determined from the luciferase assay was expressed as RLU/mg of protein. The negative controls were non-treated cells (−DNA), those transfected with naked pDNA (+DNA), while the positive control was commercial transfecting agent jetPEI. The experiments were repeated six times.

MTT-based assay suggested that the new bolas show limited cytotoxicity with COS-7 cells. In the concentration range 3–20 μM of bolas and with 48 h of incubation, the observed cell viability was >75%, while for jetPEI (15 μM, expressed in nitrogen residues) it was only about 50% (see Supporting information). The only exceptions were Orn-C16-L at high concentration (20 μM) in HeLa cells and Orn-C20-L in HepG2 cells, where cell viability dropped to ~60%. The measured total cellular protein content, which can also be

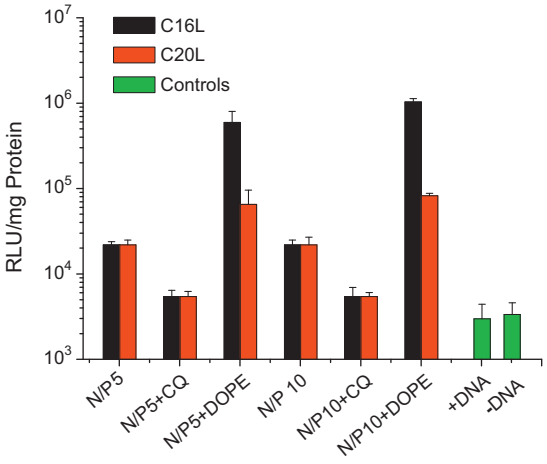

Fig. 8. Transfection efficiency of the Orn-C16-L and Orn-C20-L bolaplexes at different N/P ratios in COS-7 cells in the presence of serum. Cells were incubated in complete culture medium, DMEM containing 10% of FBS, with bolaplexes composed of plasmid DNA (1 μg per well), bola and DOPE (when indicated). For some samples, the medium contained 100 μM chloroquine (CQ). After 24 h, the transfection medium was replaced with fresh complete culture medium. The luciferase activity quantification was performed after 48 h of incubation. Transfection efficiency determined from the luciferase assay was expressed as RLU/mg of protein. The negative controls were non-treated cells (−DNA) and those transfected with naked pDNA (+DNA). The experiments were repeated three times.

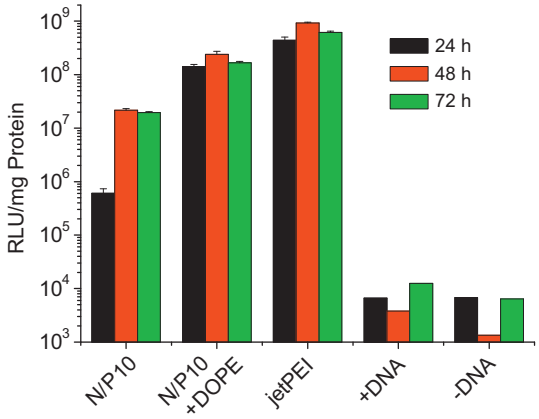

Fig. 9. Repetitive transfection of COS-7 cells with Orn-C16-L bolaplexes with and without DOPE. Cells were incubated in three 24-well plates, in a serum-free Opti-MEM with bolaplexes composed of plasmid DNA (1 μg per well), Orn-C16-L and DOPE (when indicated). After 3 h, 10% of FBS was added. The same addition of bolaplex dose was repeated once after 24 h (48 h transfection) and again at 48 h (72 h transfection) and their luciferase activity was quantified at each step. Transfection efficiency determined from the luciferase assay was expressed as RLU/mg of protein. The negative controls were non-treated cells (Cells) and those transfected with naked pDNA (DNA), while the positive control was jetPEI.

used as an estimate of cell viability, was relatively high (>75% with respect to control, Fig. S1) after 48 h of transfection with bolaplexes, in line with the MTT data. Remarkably, low total protein was observed for Orn-C16-L with HeLa cells and Orn-C20-L with HepG2 cells, while for corresponding formulations with DOPE, the toxicity was no more observed. Thus, the obtained results show the potential of the new bolas molecules for the elaboration of highly efficient nonviral vectors.

3.4. Effect of serum

As it is highly desirable that vectors work in the presence of serum (Audouy et al., 2000; Li et al., 2011), which is an essential component of both growth media and blood, we checked the effect of serum on the transfection efficiency of the bolaplexes (Fig. 8). For this purpose, the bolaplexes were added to the cells in presence of 10% of serum. Serum was found to decrease dramatically the transfection efficiency of the bolaplexes of both Orn-C16-L and Orn-C20-L. The strong negative effect of serum on the bolaplexes (N/P = 10 with DOPE) could be connected with their relatively small size and presence of DOPE in the formulation (Li et al., 1999; Ma et al., 2007). Serum could extract DOPE lipid from the bolaplexes, thus decreasing drastically their transfection efficiently. Nevertheless, formulations of Orn-C16-L with DOPE preserved a transfection efficiency in the presence of serum (around 10^6 RLU), which is 10-fold higher than that observed for Orn-C20-L and 350-fold higher than that observed for naked pDNA.

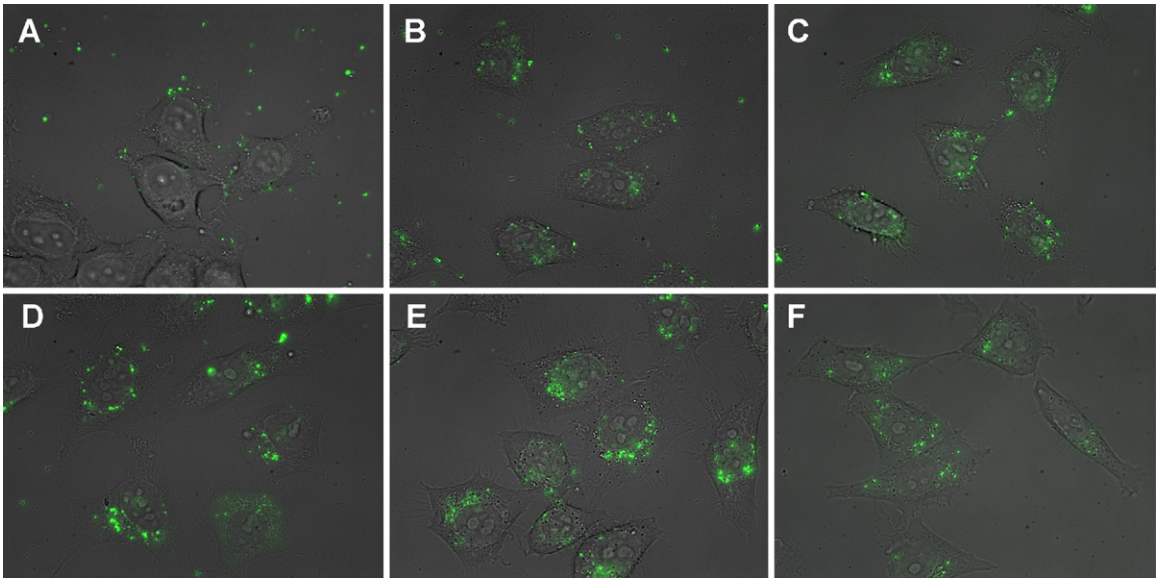

Fig. 10. Fluorescence microscopy images of Orn-C16-L + DOPE bolaplexes at N/P 10 stained with YOYO-1 at different incubation times: 10 min (A), 1 h (B), 2 h (C), 4 h (D), 6 h (E) and 24 h (F) in HeLa cells. The images size was 141×105 μm.

3.5. Effect of continuous transfection

Since bola Orn-C16-L showed a relatively low cytotoxicity for most formulations, repetitive transfection assays could improve the transfection efficiency, as it was shown for lactosylated polyethylenimine (Grosse et al., 2004). For this purpose, COS-7 cells were transfected with Orn-C16-L bolaplexes at N/P 10 with and without DOPE and their transfection efficiency and cytotoxicity were studied after 24 h (Fig. 9). Then, a second dose of the bolaplexes was added to the cells and further incubated for 24 h, and the same was done for a third assay (72 h in total). After the second transfection assay, the transfection efficiency of Orn-C16-L bolaplex without DOPE at N/P 10 increased remarkably from 6×10^5 to 2×10^7 RLU/mg protein, while with DOPE it increased from 1.4×10^8 to 2.4×10^8 RLU/mg protein. However, the third transfection did not further improve the transfection efficiency, which could be explained by a too high cell density, which inhibited their proliferation. For all three transfection steps, the cell viability estimated from the total protein was very high (>85% with respect to control, Fig. S2), while for jetPEI, it decreased sequentially. Thus, our results showed that a continuous mediation of transfection by bolaplexes appears more efficient than a single transfection and it is associated with low cytotoxicity.

3.6. Internalization of bolaplexes

Finally, the internalization of Orn-C16-L/DOPE bolaplexes at N/P 10, presenting high transfection efficiency, was studied by fluorescence microscopy (Fig. 10). Fluorescence imaging of HeLa cells with added bolaplaxes containing a YOYO-1 stained pDNA was performed at different incubation times. The binding of bolaplexes to cells was rapid, as after 10 min of incubation, fluorescent bolaplexes were observed at the membrane surface. After 1 h, the amount of fluorescent particles at the membrane further increased, and intracellular fluorescence starts to be visible. Then, between 1 h and 6 h, the particles accumulate around the nucleus. These changes are typical for endocytotic internalization of the particles. Moreover, for longer incubation times, larger fluorescent dots were observed, indicating the formation of late endosomes containing bolaplexes. After 24 h, the fluorescence remains concentrated in dots, which are less numerous and smaller in size. Thus, our studies suggest that bolaplexes interact rapidly with the cells and internalize probably through endocytosis. However, we could not detect the release of bolaplexes from the endosomes. The endosomal escape of bolaplexes is probably a very slow process, not easy to detect. The disassembly of the bolaplexes followed by DNA degradation could explain the decrease in the fluorescence signal after 24 h of incubation. It should be noted that it remains difficult to determine at which step the bolaplexes disassemble, since we followed only the fluorescently labeled pDNA, but not the bola molecules. A multi-labeling strategy will be needed to address this question.

4. Conclusions

In the present work, new bolaamphiphiles bearing neutral lactonic and cationic ornithine residues were synthesized. Bearing a larger neutral head group as compared to their gluconic-analogues, the new bolas bind and condense DNA at higher N/P ratios. In the presence of DOPE, the formulations at high N/P showed relatively small particle size (ca. 200 nm). Finally, the new bolas formulated with DOPE showed high transfection efficiency, comparable to that of jetPEI, with relatively low cytotoxicity. Without DOPE, higher transfection efficiency could be achieved by a repetitive transfection protocol. Fluorescence imaging studies suggested that the bolaplexes internalize through an endosomal pathway. Since their transfection efficiency is improved in the presence of DOPE or chloroquine, the endosomal escape remains a key barrier for transfection. This is one of the first examples of bola-based vectors exhibiting a high transfection efficiency, showing the important potential of these classes of molecules as non-viral vectors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.12.026.

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