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Amphiphilic block copolymers enhance the cellular uptake of DNA molecules through a facilitated plasma membrane transport

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ABSTRACT

Amphiphilic block copolymers have been developed recently for their efficient, *in vivo* transfection activities in various tissues. Surprisingly, we observed that amphiphilic block copolymers such as Lutrol® do not allow the transfection of cultured cells *in vitro*, suggesting that the cell environment is strongly involved in their mechanism of action. In an *in vitro* model mimicking the *in vivo* situation we showed that pre-treatment of cells with Lutrol®, prior to their incubation with DNA molecules in the presence of cationic lipid, resulted in higher levels of reporter gene expression. We also showed that this improvement in transfection efficiency associated with the presence of Lutrol® was observed irrespective of the plasmid promoter. Considering the various steps that could be improved by Lutrol®, we concluded that the nucleic acids molecule internalization step is the most important barrier affected by Lutrol®. Microscopic examination of transfected cells pre-treated with Lutrol® confirmed that more plasmid DNA copies were internalized. Absence of cationic lipid did not impair Lutrol®-mediated DNA internalization, but critically impaired endosomal escape. Our results strongly suggest that *in vivo*, Lutrol® improves transfection by a physicochemical mechanism, leading to cellular uptake enhancement through a direct delivery into the cytoplasm, and not via endosomal pathways.

INTRODUCTION

Gene transfer in cultured cells is, at present, in the vast majority of cases achieved using cationic lipids or polymers. However, despite having been widely used for more than 10 years for *in vitro* transfection applications, the use of these molecules has not yet been translated to application in humans because of low *in vivo* transfection efficiencies and toxicity issues. In this context, a new class of non-viral vectors has emerged for *in vivo* gene delivery, based on amphiphilic block copolymers consisting of hydrophilic blocks of poly(ethylene oxide) (PEO) and hydrophobic blocks of poly(propylene oxide) (PPO), covalently linked together in various structures. Linear non-ionic block copolymers form an A–B–A or B–A–B tri-block structure of PEO–PPO–PEO or PPO–PEO–PPO, whilst tetra-functionalized, slightly positively charged block copolymers form an X-shaped structure composed of four PEO–PPO moieties linked by the hydrophobic extremity to a central ethylenediamine core (1). Linear and X-shaped block copolymers have been used successfully to increase the deliver reporter and therapeutic genes in various rodent organs including physiological skeletal and cardiac muscle, lung and eyes (2–6) compared to that achieved with the naked DNA approach pioneered by Wolff and colleagues (7–9). Block copolymers have also been used to deliver genes to express proteins of local or systemic therapeutic interest in mouse models of human pathologies including erythropoietin (EPO) to treat anaemia in kidney failure or dystrophin in Duchenne muscular dystrophy (DMD) (10,11). More recently, an X-shaped block copolymer led to a dramatic improvement in DNA vaccination for prophylactic and therapeutic applications by reducing the amount of injected DNA by a factor of at least 50. This rendered the effective DNA dosage more compatible with human use than that achieved with naked DNA, where high amounts of DNA in the milligram range were injected, with disappointing humoral and cellular responses.

Amphiphilic block copolymers used in these various reports belong to a wide chemical family generated by

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the degree of polymerization of the ethylene oxide (EO) and propylene oxide (PO) units, leading to polymers of molecular weight ranging from 200 to 20 000 g/mol and of amphiphilic character measured by the hydrophilic/lipophilic balance (HLB) ranging from 1 to 24, corresponding to a percentage of PEO of 10 to 80%, respectively. These polymers are also characterized by their critical micellar concentration (CMC) and critical micellar temperature (CMT). These factors govern the self-assembly of these amphiphilic molecules in solution, with unimers formed below the CMC and CMT and, above the CMC and CMT, supramolecular structures including micelles with a hydrophobic core of PPO blocks surrounded by a hydrophilic corona of PEO and also lyotropic liquid crystalline mesophases of varying morphology. The physicochemical properties of block copolymers govern not only the morphology of self-assembly, but also the toxicity, which is inversely related to the percentage of PEO present in the copolymer (12). By contrast, linear PEO–PPO–PEO tri-block copolymers containing a high percentage of PEO are approved by the FDA for intravenous, oral and topical administration. Linear and X-shaped block copolymers, which have been reported to deliver genes *in vivo*, usually have a molecular weight below 15 000 g/mol, a percentage of OE ranging from 10 to 80% and are used at a dose ranging from 0.01 to 5% (w/w).

Some studies have been reported which have aimed to understand the mechanism of action of this novel class of non-viral vectors, at the physicochemical level, by investigating the interaction between block copolymers and DNA molecules, and at the physiological level by studying their biological impact on cell physiology. These studies showed that the physicochemical characterization of block copolymer/DNA complexes was not performed easily with the same techniques that have been used to describe the physicochemical properties of complexes resulting from the interaction of DNA molecules with cationic lipids and polymers. This highlights a difference in the way that DNA interacts with either block copolymers, or highly-positively-charged cationic lipids or polymers. Interactions of DNA with block copolymers probably occur mainly through hydrogen bonding, hydrophobic and some ionic interactions, but not via strong ionic interactions. On the contrary, strong ionic interactions are observed to occur between DNA and cationic lipids or polymers (13). It has also been reported that block copolymers interact with lipidic film, leading to ionic permeabilization of the reconstituted artificial membrane (14). More recently, it was reported that a linear tri-block copolymer with 50% PEO acted as a biological modifier, activating the NFκB inflammation cellular pathway leading to the enhancement of transfection efficiency by the recruitment of transcription factors on the cytomegalovirus promoter, which was used to control the transgene expression (15–17). In fact, we showed previously that PE6400, composed of 40% PEO, promoted DNA trafficking into the nucleus and increases gene expression when microinjected into the cell cytoplasm (4). Nevertheless, this mechanism for linear block copolymer of a low PEO percentage, which is associated with toxicity, does not necessarily apply to other linear tri-block copolymers consisting of a higher PEO percentage which have been reported as non-toxic and efficient for gene transfer.

In this study, we report that Lutrol®, a linear tri-block copolymer with 80% PEO and a molecular weight of 8600 g/mol, which has been reported to deliver therapeutic genes in mouse models of human diseases with a very good safety profile (10), mediates the increase of cellular internalization of DNA and siRNA molecules by a different pathway to that used by cationic lipids or polymers. The incapacity of lutrol to perform efficient transfection in cationic lipids-free *in vitro* cells strongly supports its inability to perform endosomal escape, and strongly suggest that Lutrol® acts *in vivo* via an endocytosis-independent internalization pathway. Our results obtained *in vitro* revealed Lutrol® general abilities concerning cell membrane interactions that are certainly applicable to its *in vivo* mechanism. We propose that 80% PEO linear tri-block copolymers do not promote gene transfer by the activation of inflammation cellular pathways, but rather enhance cellular uptake of DNA molecules through a facilitated plasma membrane transport.

MATERIALS AND METHODS

Plasmids, siRNA, amphiphilic block copolymers and cationic vectors

pCMV-Luc (18) and Gwizz-Luc (Genlantis, San Diego, CA) are plasmids encoding the luciferase reporter gene under the control of the human cytomegalovirus immediate-early gene promoter. pGL3 (Promega, Madison, WI) is a plasmid encoding the luciferase reporter gene under the control of the SV40 immediate-early gene promoter. pCMV-GFP (Clontech, Palo Alto, CA) is a plasmid encoding green fluorescent protein reporter gene, under the control of the human cytomegalovirus immediate-early gene promoter. Plasmids were purified from recombinant *Escherichia coli* by means of Endofree plasmid purification columns (Qiagen, Chatsworth, CA). Human anti-Lamin A/C siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Negative control siRNA (AllStars Negative Control, sense sequence: UUCUCCGAACGU GUCACGU) was provided by Qiagen (Chatsworth, CA).

F38 [80% poly(ethyleneoxide), molecular weight (MW) 4700], F68 [80% poly(ethyleneoxide), MW 8400] and F108 [80% poly(ethyleneoxide), MW 14 600] were generously provided by BASF (Mount Olive, NJ). For *in vitro* experiments, solutions of block copolymers were prepared at the given weight-to-weight (w/w) concentration in high glucose DMEM (Invitrogen) (4.5 g/l) supplemented with 10% FBS, 2 mM L-glutamine, 10 μg/ml streptomycin, 100 μg/ml penicillin. For *in vivo* experiments, stock solutions of block copolymers were prepared at the given weight-to-weight (w/w) in sterilized water. Solutions were stored at 4°C.

DOSP and BGTC were synthesized as previously described (19–22) and provided by IN-CELL-ART (Nantes, France). DOPE was obtained from Avanti

Polar Lipids (Alabaster, AL). JetPEI® was obtained from Polyplus (Illkirch, France). ICAFectin® 442 was obtained from IN-CELL-ART (Nantes, France). DOSP/DOPE (1/1, mol/mol) and BGTC/DOPE (2/3, mol/mol) cationic liposomes were prepared as previously described (23).

In vivo plasmid DNA formulations and animal experiments

Animal experiments were performed in accordance with the guidelines of the French Institut National de la Santé et de la Recherche Médicale. Eight-week-old female CD1 mice were obtained from Charles River (Chatillon-sur-Chalaronne, L'Arbresle, France). At least five mice were injected in each experimental group and each experiment was repeated two times. For intramuscular injections, mice were anaesthetized with Hypnomidate (40 mg/kg, intraperitoneal injection, Janssen-Cilag, Issy-les-Moulineaux, France). Fifty microliters of block copolymer/DNA formulations, cationic lipid/DNA lipoplexes or naked DNA were injected into shaved tibial anterior muscles at a single site, using a microfine syringe (U100; Becton Dickinson, Rungis, France).

In vitro formulation

DNA lipoplexes were formulated at a positive charge ratio of four with 1 µg plasmid. Complexes of DNA with cationic lipids were prepared by mixing equal volumes of cationic lipids in water with plasmid at the desired concentration in 300 mM NaCl. Complexes of siRNA (37.5 ng) with ICAFectin® 442 were prepared as described by the manufacturer. Hybrid DNA/siRNA lipoplexes were formulated at a charge ratio of four with 500 ng DNA mixed with 500 ng siRNA. Complexes of DNA-siRNA with DOSP–DOPE were prepared by mixing equal volumes of cationic lipids in water with nucleic acids at the desired concentration in 300 mM NaCl. In cell conditions, lipoplexes were incubated at room temperature for 15–20 min before transfection.

Cell culture, block copolymer incubation and transfection

HeLa, mouse muscle C2C12, COS-7 green monkey kidney fibroblast and H1299 human lung cancer cells were grown at 37°C in 5% CO_2/humidified atmosphere in high glucose DMEM medium (4.5 g/l) supplemented with 2 mM L-glutamine, 10 µg/ml streptomycin, 100 µg/ml/ml penicillin (GIBCO and Invitrogen Life Technologies, Carlsbad, CA) and with 10% FBS (Eurobio, Courtaboeuf, France). CHO and CHO-2241 heparan sulfate depleted (CHO HS^-) cells (a generous gift from P. Fender, Grenoble, France) were grown in the same conditions, replacing DMEM medium by Kaighn's F12 medium (GIBCO, Carlsbad, CA). One day before transfection, cells were transferred into 24-well culture plates, at 60 000 cells per well, resulting in 70–80% confluence 24 h later. Two hours before transfection, cells were incubated with block copolymers at the given w/w concentration in DMEM serum-supplemented medium or Kaighn's F12 serum-supplemented medium for CHO and CHO HS^- cells, respectively. Transfection was performed by adding 50 µl DNA complexes or siRNA/DNA complexes in 450 µl serum-free medium or 107 µl siRNA complexes in 500 µl serum-free medium to each well. For CHO and CHO HS^- transfection, 100 µl lipoplex, complexed or not with Lutrol®, was added in 450 µl serum-free medium. After 2 h, the transfection medium was replaced by 500 µl fresh serum-supplemented medium. Cells were cultured for an additional 20 h before gene expression was determined. Transfection experiments were performed in duplicate.

Luciferase and GFP assays

One day after transfection, cells were rinsed with 300 µl phosphate-buffered saline (PBS) and lysed with 300 µl reporter lysis buffer (Roche Diagnostics, Mannheim, Germany) supplemented with a protease inhibitor cocktail (Roche Diagnostics). Complete lysis was assured by one freeze–thaw (−80°C/20°C) cycle. Samples were then centrifuged at 10 000 rpm for 5 min at 4°C. Luciferase activity was measured using the Promega Luciferase Assay system (Promega, Madison, WI). Luciferase activity was measured from an aliquot of supernatant with a VICTOR^2 multilabel counter (Perkin Elmer, Les Ulis, France). Luciferase activity was assayed by measuring light emission after addition of 100 µl luciferase substrate to 20 µl supernatant. Luciferase activity was normalized to the total protein concentration of the sample. Protein content was measured with a BCA protein assay kit (Pierce, Rockford, IL).

GFP fluorescence measurements were performed on a 180 µl aliquot of supernatant using a Victor2 apparatus (PerkinElmer, Les Ulis, France). Fluorescence was normalized to the total protein concentration of the sample.

Flow cytometry experiments

Twenty hours after transfection, samples of cells (10^5–10^6) were collected and re-suspended in PBS. The resulting cell suspension was assayed for the expression of GFP by flow cytometer analysis using a FACScalibur flow cytometer (Becton Dickinson). The percentage of GFP-positive cells was determined by the flow cytometer programme.

Quantification of YOYO-1 labelled plasmid internalization was performed with luciferase encoding plasmid. Plasmid was added to a solution of YOYO-1 (0.1 mM) in dimethyl sulfoxide (DMSO) at the dye/base pair ratio 1/150. This mixture was incubated at room temperature for 10 min before self assembly of DOSP/DOPE–DNA lipoplexes at a DOSP–DNA charge ration of 4 (±). Then, HeLa cells were pre-incubated in the presence or in the absence of 3% Lutrol® for 1 h before transfection with labelled lipoplexes. Twenty four hours post-transfection, cells were analyzed for their YOYO fluorescence content by flow cytometer analysis as described above.

Real-time, quantitative RT–PCR

Total RNA was extracted from transfected cells by TRIzol® treatment. Reverse transcription was performed with total RNA using oligo(dT)20 primers and SuperScript III reverse transcriptase (Invitrogen). The

expression of lamin A/C was quantified by real-time PCR (ABI prism7000, Applied Biosystems, Foster City, CA). Experiments were performed using PCR Master Mix (Applied Biosystems) with 300 nM each primer and 250 nM TaqMan MGB probes. Primers were obtained from Applied Biosystems. The cycling conditions included a hot start at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Results were normalized to the endogenous hypoxanthine-guanine phosphoribosyltransferase (HPRT1) control gene and expressed according to the $2^{-\Delta\Delta CT}$ method (24).

Labelling of lipoplexes

The labelling procedure of lipoplexes with 15 nm aminated $\gamma Fe_2O_3@SiO_2$ nanoparticles (Nps) is described in detail elsewhere (25). Briefly, 3.68×10^{11} Nps and 0.5 µg pCMV-Luc were mixed at a ratio Np/pDNA of 5 (mole/mole) in 300 mM NaCl. Then an equal volume of cationic liposome in pure water was added, to a final lipoplex charge ratio (±) of four. The formulation was prepared in a 150 µl final volume for each well.

Conventional TEM

Cells were processed for ultramicrotomy according to standard procedures. Briefly, after trypsin treatment, cells from eight wells were pooled, pelleted and fixed for 2 h in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4). Sample pellets were then post-fixed for 1 h at 4°C with 1% osmium tetroxide in the same buffer and were dehydrated with ethanol before embedding in Epon-Araldite. Thin sections (65 nm thick) were stained successively with 5% uranyl acetate and 1% lead citrate, unless stated in the text. TEM observation was performed with a FEI tecnai F20 operated at 200 kV under low-dose conditions.

Quantification of labelled lipoplexes within cell sections

The measurement of Np areas was carried out on images of unstained sections using image J software. Np densities were selected after a manual threshold. The area corresponding to these densities was determined with the 'analyse particles' function providing a surface of Nps. To determine the area of cell sections, we drew the cell contour manually and measured the area with the 'analyse particles' function. The ratio of Np surface to cell section surface allowed the estimation of the amount of Nps per cell, considering that the 65 nm cell section is in the order of magnitude of Np size. This analysis was performed on 158 cell sections from 13 grid squares in total.

RESULTS

Amphiphilic block copolymers promote high gene transfer in muscular cells *in vivo* but not *in vitro*

In order to assess the impact of the cell environment on transfection efficiency, plasmid DNA either naked or complexed either with Lutrol®, an amphiphilic block copolymer of 80% PEO, or with cationic liposomes of DOSP/DOPE, was injected into mouse tibial anterior muscle (Figure 1) or incubated, *in vitro*, with C2C12 mouse muscle cells (Figure 1). Luciferase reporter gene expression in C2C12 and in tibial anterior muscle was evaluated 24 h and 7 days after transfection, respectively.

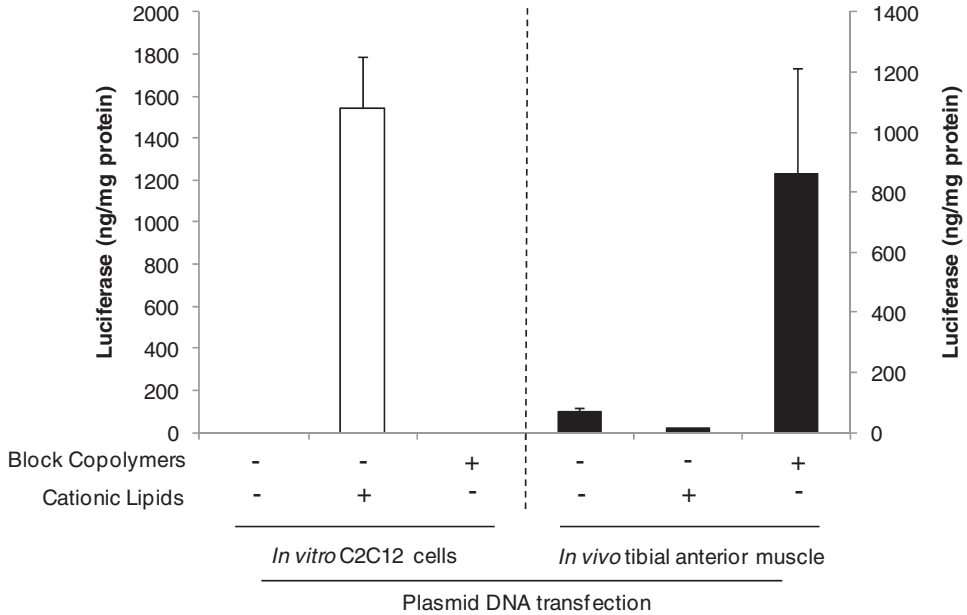

Figure 1. *In vivo* and *in vitro* transfection efficiency of amphiphilic block copolymers, cationic lipids and naked DNA. A luciferase gene expression assay was performed to compare the transfection efficiencies of an amphiphilic block copolymer, Lutrol® and cationic liposomes of DOSP–DOPE, either *in vivo* in mouse tibial anterior muscle 7 days after transfection (black bars) or *in vitro* in the C2C12 mouse muscle cell line 24 h after transfection (white bars). The luciferase gene-encoding plasmid was either naked or complexed with 3% Lutrol® or with DOSP–DOPE at a charge ratio of ±4. The amount of plasmid transfected *in vitro* and *in vivo* was, respectively, 1 and 10 µg.

Injection of 10 µg plasmid DNA complexed with 3% Lutrol® led to high luciferase expression in mouse tibial anterior muscle, compared with the very low luciferase expression achieved with naked DNA or DOSP/DOPE–DNA lipoplexes. By contrast, transfection of cultured C2C12 with 1 µg of pCMV-Luciferase complexed with Lutrol® did not allow luciferase expression, whereas cationic liposomes of DOSP/DOPE led to a dramatic increase in luciferase expression. These results strongly suggest that the cell environment plays an important role in amphiphilic block copolymer and cationic liposome transfection efficiency.

Lutrol® and other 80% PEO amphiphilic block copolymers promote efficient muscular gene transfer irrespective of the plasmid promoter

Next, we investigated a possible role of amphiphilic block copolymers with the 80% PEO used in this study, in the activation of some transcription factors, as has been previously described for block copolymers containing 50% PEO (15,16). For this purpose, we compared transfection efficiencies in tibial anterior muscle using two different plasmids encoding luciferase, controlled either by the CMV or SV40 promoter. These two promoters contain different transcription factor binding sites (Table 1). The three polymers tested were all composed of 80% PEO but with various molecular weights, ranging from 4700 to 14 700 Da. Figure 2 shows that similar luciferase expression was obtained after intramuscular injection of both plasmids complexed with the various block copolymers.

These data suggest that the mechanism of action of amphiphilic block copolymers of 80% PEO does not depend on the activation of specific transcription factors, unlike that of 50% PEO copolymers.

In vitro model to study the *in vivo* mechanism of amphiphilic block copolymers

As studying the mechanism of action of block copolymers *in vivo* is very difficult, we decided to set up an *in vitro* model allowing variation of several parameters, to understand how block copolymers dramatically increased gene expression *in vivo*. To this end, since block copolymers as naked DNA alone did not transfect cells *in vitro*, we studied the influence of block copolymers on reporter gene expression by first treating cultured cells with the optimized *in vivo* concentration of block copolymers before the addition of DNA molecules complexed with cationic vectors.

C2C12 cells were pre-treated with Lutrol® and then transfected with 1 µg plasmid DNA encoding luciferase complexed with DOSP/DOPE at a charge ratio of four (±). Pre-treatment with 3% Lutrol® led to the enhancement of luciferase expression in C2C12 transfected cells compared with untreated cells (Figure 3A). Cell treatment with Lutrol® during 0.5 to 2 h before transfection, at various concentrations ranging from 0.5 to 3% led to a similar enhancement of transfection efficiency (data not shown). Of note the same optimal Lutrol® concentration was observed both *in vitro* and *in vivo*. This improvement in transfection efficiency was also observed with a plasmid encoding another reporter gene; i.e. GFP (Figure 3A). Pre-treatment with Lutrol® of various cell lines, including COS-7, C2C12, HeLa and H1299 cells, also led to a similar enhancement of transfection efficiency (Figure 3B). Various types of cationic lipids were also used and led to the same increase in reporter gene activity after pre-treatment with Lutrol® (Figure 3C). Transfection of H1299 cells with JetPEI® was also enhanced by pre-treatment with Lutrol® (Figure 3D). No toxicity was detected in the *in vitro* model as assessed by MTT experiments (Supplementary Figure S1) and by analysis of NFκB and P53 transcription activation (Supplementary Figure S2).

Altogether, these data suggest that the enhancement of transgene expression mediated by pre-treatment with Lutrol® did not depend on neither the reporter gene, the cell line, nor the cationic vector used.

Table 1. Transcription factor binding sites in the CMV and SV40 promoters [based on Promega data (15)]

Transcription factor binding sites	CMV Promotor	SV40 Promotor
Octamer-binding factor1	+	+
Activator protein 1 (AP1)	+	+
Zinc finger-containing protein SP1	+	+
NF-E2 p45	–	+
GC box elements	–	+
CRE-binding protein 1/c-Jun heterodimer	+	–
cAMP-responsive element binding protein	+	–
NFκB	+	–
c-Rel	+	–
AvianC-type LTR TATA box	+	–
Cellular and viral TATA box elements	+	–
E4BP4, bZIP domain, transcription repressor	+	–
Activating transcription factor	+	–

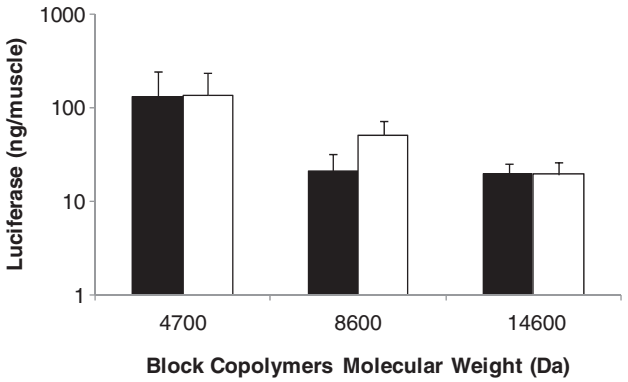

Figure 2. Plasmid promoter influence on transfection efficiency of block copolymers in mouse tibial anterior muscle as a function of the block copolymer molecular weight. Shaved tibial anterior muscles of CD1 mice were injected with 10 µg plasmid encoding luciferase under control of the CMV promoter (black bars) or SV40 promoter (white bars). Before injection, plasmid was formulated with 1% F38, 3% Lutrol® or 1% F108 in tyrode. Luciferase gene expression assay was performed 3 days after injection.

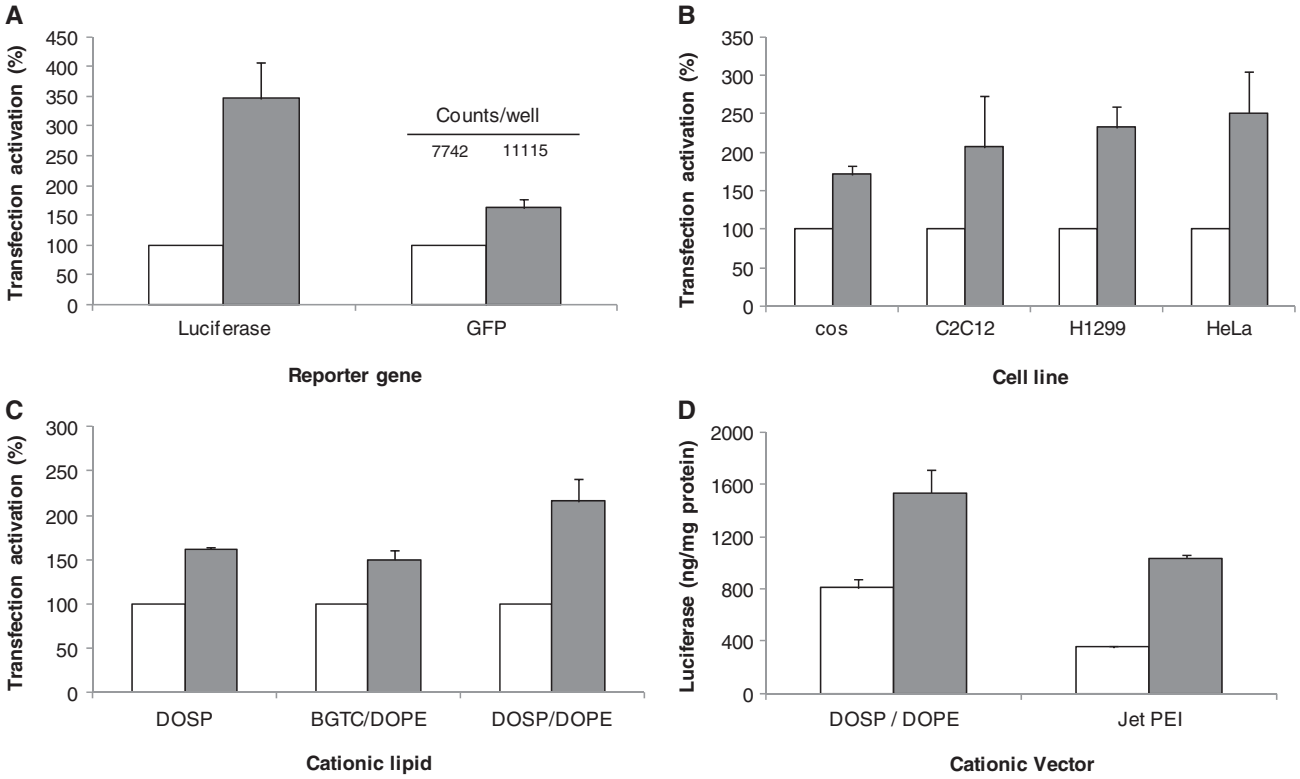

Figure 3. Effect of amphiphilic block copolymers on *in vitro* transfection efficiency, as a function of (**A**) the reporter gene, (**B**) the cell line, (**C**) the cationic lipid and (**D**) the chemical structure of the cationic vector. Reporter gene expression assay was performed in transfected cells after Lutrol® treatment (gray bars) or without Lutrol® treatment (white bars). Treated cells were incubated with Lutrol® diluted at optimized concentration in culture medium for 2 h before transfection. (A) C2C12 cells were transfected with 1 μg luciferase or GFP encoding plasmid complexed with DOSP/DOPE at a charge ratio of ±4. (B) COS-7, C2C12, HeLa or H1299 cells were transfected with 1 μg luciferase encoding plasmid complexed with DOSP–DOPE at a charge ratio of four (±). (C) HeLa cells were transfected with 1 μg luciferase encoding plasmid complexed with DOSP, DOSP/DOPE and BGTC/DOPE at a charge ratio of four (±). (D) H1299 cells were transfected with 1 μg luciferase encoding plasmid complexed with DOSP/DOPE at a charge ratio of four (±) or with JetPEI® according to manufacturer's instructions.

Amphiphilic block copolymers enhanced *in vitro* transgene expression, irrespective of their molecular weight and the plasmid promoter

Next, in order to validate our *in vitro* model and to provide an insight into the *in vivo* mechanism of action of block copolymers, we investigated the influence of the plasmid DNA promoter and the molecular weight of the block copolymer on transfection efficiency in cultured cells. Figure 4 shows that, as observed *in vivo* (Figure 2), pre-treatment of cultured cells with block copolymers of various molecular weights led to a similar increase in luciferase expression (Figure 4A). We also showed that either CMV or SV40 promoters led to similar reporter gene expression enhancement in cells pre-treated or not with Lutrol® (Figure 4B). These data strongly suggest that, as observed *in vivo*, Lutrol® and other 80% PEO block copolymers did not activate promoter-specific transcription factor signalling pathways, but increased gene expression by another mechanism.

Lutrol® enhanced DNA cellular transport and not reporter gene expression

To investigate a possible role of amphiphilic block copolymers in the stimulation of transcription and translation, cells were treated with Lutrol® after the intracellular internalization of DNA molecules had occurred. Figure 5A shows that enhancement of luciferase expression was observed only when cells were pre-treated with Lutrol®, suggesting that Lutrol® did not promote reporter gene expression stimulation at the transcription or translation level, but rather stimulated steps involved in DNA internalization, endosomal escape or nuclear targeting.

To strengthen this hypothesis, we studied the influence of temperature on luciferase expression obtained after pre-treatment of cells with Lutrol®. As expected, in the absence of pre-treatment with Lutrol®, we observed that cationic lipid-mediated transfection was partially inhibited at 4°C. By contrast, transfection enhancement by Lutrol® was not affected by the transfection temperature, suggesting that the main mechanism of block copolymers is probably due to the improvement of the different steps involved in DNA transfection by a physicochemical process (Figure 5B).

Did Lutrol® increase cytoplasmic or nuclear delivery?

To analyse the contributions of endosomal escape and nuclear import in the enhancement of transgene expression after cell pre-treatment with Lutrol®, we studied the

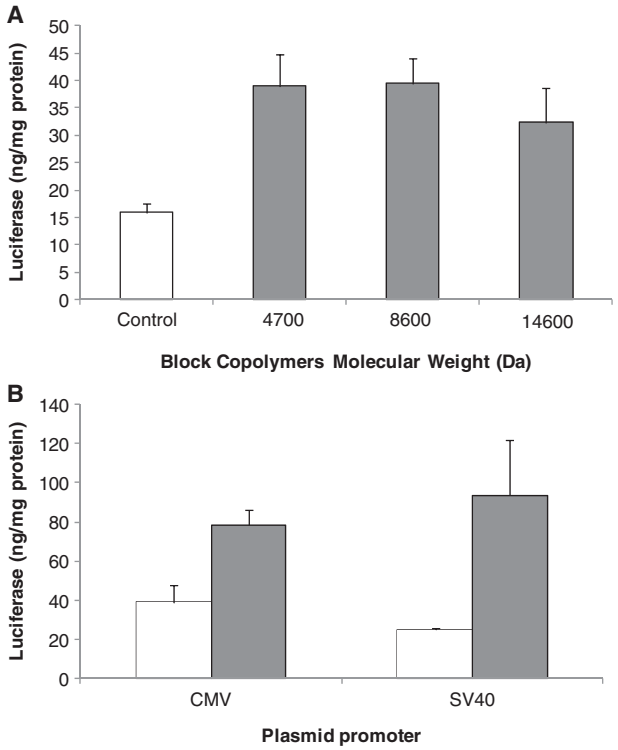

Figure 4. Effect of amphiphilic block copolymers on *in vitro* transfection as a function of (**A**) the block copolymer molecular weight or (**B**) the plasmid promoter used. Cells were transfected with 1 µg luciferase encoding plasmid complexed with DOSP/DOPE at a charge ratio of ±4. Transfection was performed after block copolymer treatment (gray bars) or without block copolymer treatment (white bars). Luciferase reporter gene assay was performed 24 h after transfection. (A) Treated cells were incubated with F38, Lutrol® or F108 diluted at optimized concentration in culture medium for 2 h before transfection. (B) Cells were incubated with 3% Lutrol® diluted in culture medium for 2 h before transfection with two different plasmids with a CMV or SV40 promoter.

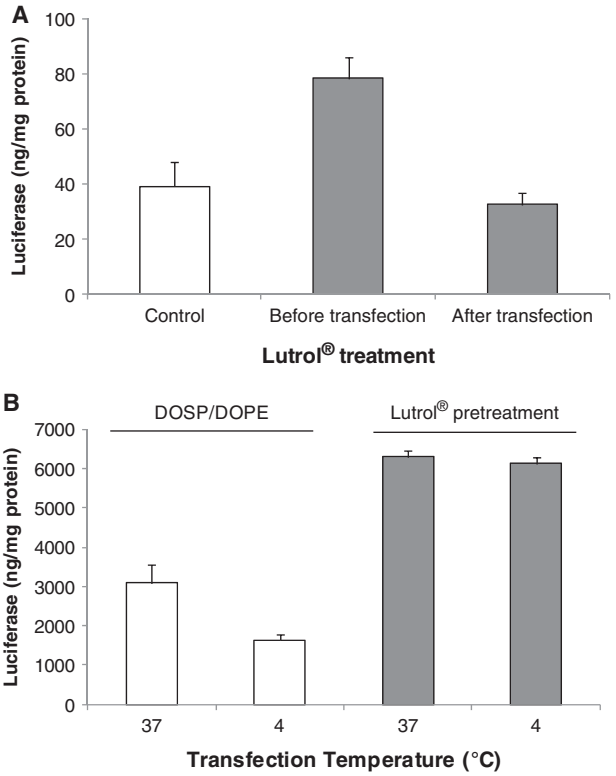

Figure 5. Effect of amphiphilic block copolymers on *in vitro* transfection as a function of (**A**) the block copolymer treatment period or (**B**) the transfection temperature. Cells were transfected with 1 µg luciferase encoding plasmid complexed with DOSP/DOPE at a charge ratio of ±4. Transfection was performed with Lutrol® treatment (gray bars) or without Lutrol® treatment (white bars). Luciferase reporter gene assay was performed 24 h after transfection. (A) Cells were treated with Lutrol® diluted at optimized concentration in culture medium for 2 h before or after addition of DOSP/DOPE–DNA lipoplexes. (B) Cells were incubated for 2 h at 37 or 4°C, just after the addition of DOSP/DOPE–DNA lipoplexes.

influence of cell pre-treatment with Lutrol® on gene silencing efficiency (Figure 6). Indeed, gene silencing occurs after the delivery of siRNA in the cell cytoplasm and not in the nucleus. Thus, if Lutrol® pre-treatment increases nuclear import of the transfected nucleic acids, efficiency of gene silencing, i.e. the residual lamin A/C expression would not be modified by Lutrol®. On the contrary, if nuclear import is not the limiting step overcome by Lutrol®, but rather the common steps of plasmid and siRNA transfection, i.e. cellular internalization or endosomal escape, Lamin A/C inhibition would also be enhanced by Lutrol® pre-treatment. H1299 cells were pre-treated with Lutrol® and then transfected with anti-lamin A/C siRNA complexed with ICAFectin® 442 Reagent, in conditions that partially inhibited lamin A/C expression. In the absence of Lutrol® pre-treatment, RT–PCR results showed that the percentage of lamin A/C inhibition was 31%, whereas Lutrol® pre-treatment led to a percentage of inhibition of 58%, supporting the notion that Lutrol® also enhanced the siRNA transfection process. Next, we decided to study in a single experiment the effect of Lutrol® pre-treatment on both siRNA and DNA transfection using particles similar to the one used for plasmid DNA experiments. In this condition using

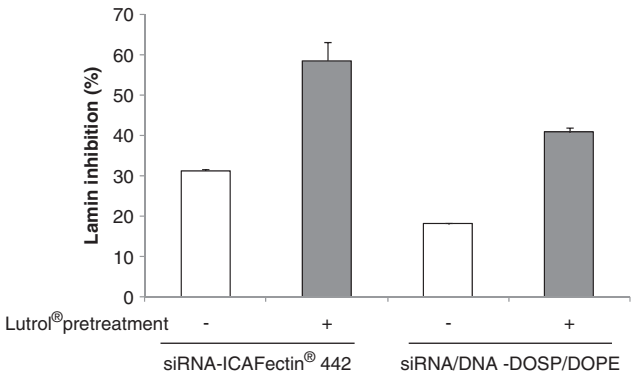

Figure 6. Effect of block copolymers on gene silencing. Anti lamin A/C siRNA transfection was performed on cells after Lutrol® treatment (gray bars) or without Lutrol® treatment (white bars). Treated H1299 cells were incubated with Lutrol® diluted at optimized concentration in culture medium for 2 h before transfection. H1299 cells were transfected either with 37.5 ng siRNA complexed with ICAFectin® 442, or with 500 ng siRNA mixed with 500 ng plasmid DNA complexed with DOSP–DOPE at a charge ratio of ±4. Real-time quantitative RT-PCR analysis of human lamin A/C mRNA was performed 24 h after transfection. Values were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT1). Values are relative to cells transfected under the same experimental conditions with a control siRNA.

mixed particles would allow us to compare Lutrol® impact on gene silencing and reporter gene expression using a single particle containing DNA and siRNA molecules. To this purpose, 500 ng siRNA were mixed with 500 ng plasmid DNA to obtain a total dose of 1 μg nucleic acids, complexed with DOSP/DOPE at a charge ratio of four (±). H1299 cells were pre-treated with Lutrol® and then transfected with hybrid siRNA/DNA particles. The results showed that anti-lamin siRNA transfection allowed an inhibition of lamin RNA expression of 18% and, most importantly, Lutrol® pre-treatment increased this inhibition to 41%. We also observed that luciferase expression was enhanced by Lutrol® pre-treatment (data not shown).

These data strongly suggest that Lutrol® enhanced the common steps of plasmid DNA and siRNA transfection, i.e. cellular internalization and endosomal escape, and did not enhance the terminal steps of plasmid transfection, including nuclear import, transcription and translation.

Did Lutrol® increase the transfected cell number?

To confirm that block copolymers enhance transfection by DNA molecule internalization and/or endosomal escape, we measured the percentage of transfected cells by FACS analysis using plasmid DNA encoding GFP. FACS analysis showed a similar percentage of GFP-expressing cells in the presence or absence of Lutrol® pre-treatment (Figure 7). As a control, luciferase expression was strongly enhanced after pre-treatment with Lutrol® during the same experiment (Figure 7). These results were also in good agreement with the experiment using β-galactosidase as a reporter gene, as shown by the presence of the same number of blue cells (data not shown) irrespective of pre-treatment with Lutrol®. However, in these two experiments, we noticed that cells pre-treated with Lutrol® expressed the reporter gene at a higher level, as shown by the blue intensity of the cells (data not shown).

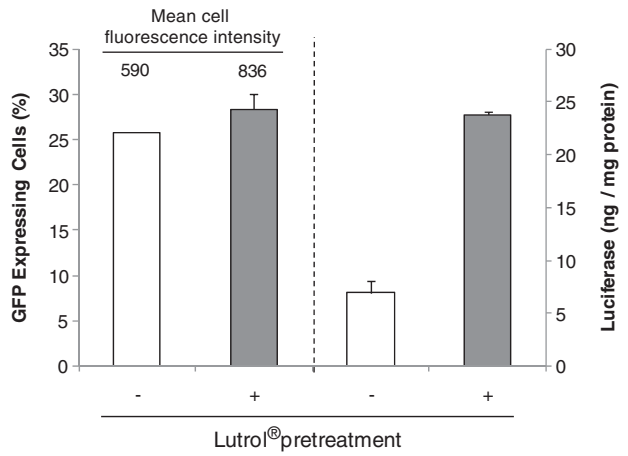

Figure 7. Effect of block copolymers on the percentage of transfected cells. C2C12 cells were transfected after Lutrol® treatment (gray bars) or without Lutrol® treatment (white bars). Treated cells were incubated with Lutrol® diluted at optimized concentration in culture medium for 2 h before transfection. One μg of GFP or luciferase encoding plasmid was complexed with DOSP/DOPE at a charge ratio of ±4.GFP expressing cells were counted by flow cytometry and a luciferase gene expression assay was performed 24 h after transfection.

The same observation was made in GFP-transfected cells pre-treated with Lutrol® (data not shown). These results strongly suggest that, even if the same number of cells were transfected in the presence of Lutrol®, the number of plasmids entering each cell would certainly be increased.

Lutrol® enhanced DNA cellular uptake: lipoplex labelling and TEM imaging

Next, we investigated if the transfection enhancement observed after lutrol® pre-treatment was associated with an increase in lipoplex internalization. In order to detect DNA cellular uptake by TEM, lipoplexes were labelled with NPs. Cells were incubated for 2 h in the presence of labelled lipoplexes and then submitted to a fixation procedure. Labelled lipoplexes were observed in both untreated and treated cells and possessed similar morphological aspects indicating that Lutrol® treatment did not modify lipoplex structure (Figures 8C and F). To quantify the cellular uptake of lipoplexes in both conditions, we estimated the Np amount per cell by measuring the Np surface per cell surface (see 'Materials and methods' section). The analysis was performed on 158 cell sections from 13 grid squares**.** Our quantitative analysis revealed an increase of DOSP-lipoplex uptake by treated cells, as shown in Figure 8E and I. This result suggests that Lutrol® promoted an enhanced DNA internalization through the cell membrane. As a control, cells transfected with labelled and unlabelled lipoplexes showed similar luciferase activities (data not shown). Moreover, a strong transfection enhancement after cell treatment with Lutrol® was also observed with labelled lipoplexes (data not shown), indicating that Nps did not influence transfection efficiency, confirming findings of Le Bihan (25). In addition, for both untreated and treated cells, we also determined that the surface of cells containing Nps represented 30% of the total area of cells, which corresponds to the percentage of transfected cells as indicated by FACS analysis and microscopy on GFP transfected cells. We also analysed the lipoplexes internalization by means of YOYO-1-labelled DNA. Flow cytometry analysis showed that cells pre-treated with Lutrol® exhibit a 2-fold increase in fluorescence intensity compare to cells that were not pre-incubated with Lutrol® (Figure 9). Altogether, these results indicated clearly that Lutrol® enhanced the cellular uptake of nucleic acids.

Lutrol® promoted DNA interaction with cell membranes

As efficient, cationic vector-mediated transfection requires the condensation of DNA in positively charged particles, it has been inferred that anionic proteoglycans are potential receptors (26). Direct evidence for the involvement of heparan sulfate proteoglycans (HSPGs) in transfection has been obtained by several groups (27,28). Figure 10 shows clearly that transfection of heparan sulfate-deficient CHO (CHO HS^-) cells by DOSP/DOPE–DNA lipoplexes was strongly decreased compared with that obtained in normal CHO cells. By contrast, CHO HS^- transfected by lipoplexes in the presence of Lutrol® exhibited an enhanced luciferase expression. These data strongly

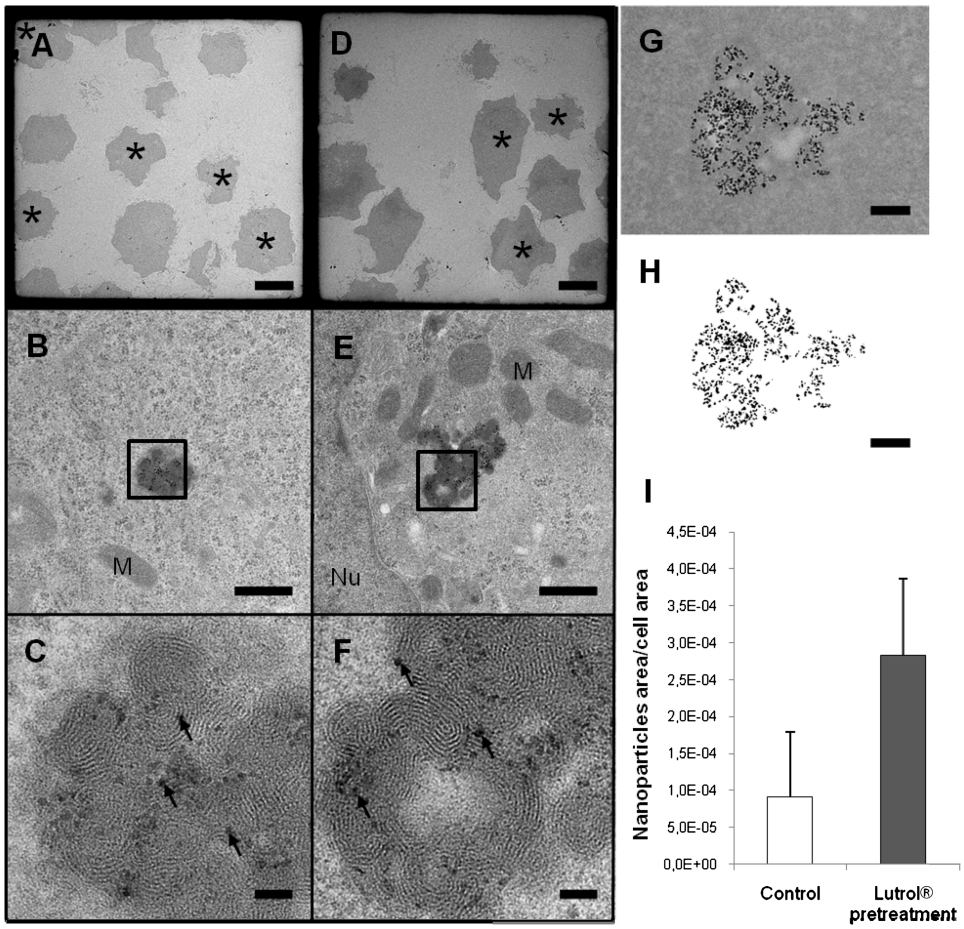

Figure 8. TEM visualization of H1299 cells cellular uptake of DOSP–DOPE/plasmid/Nps (**A–C**) and after a Lutrol® treatment (**D–F**) observed 2 h after transfection. Cells were transfected with luciferase encoding plasmid complexed with Nps (1/5) and with DOSP–DOPE at a charge ratio of ±4. Ultrathin sections were observed at low (A, D), medium (B, E) and high (C, F) magnification and revealed the presence of labelled lipoplexes containing electron-dense Nps (black arrows). Cell sections were screened for the presence of Nps (asterisks in A and D). Labelled lipoplexes were observed within the cytoplasm and formed multilamellar assemblies (C, F enlargement of marked areas with black squares in B and E). The measurement of Np amount per cell section was performed on unstained sections (**G**) and analysed after threshold (**H**). The result is expressed as the ratio of Np surface per cell surface. This ratio increased after Lutrol® treatment (**I**). Scale bars are 10 µm (A, D) 500 nm (B, E) and 50 nm (C, F). Nu Nucleus, M Mitochondria. The analysis was performed on 158 cell sections from 13 grid squares.

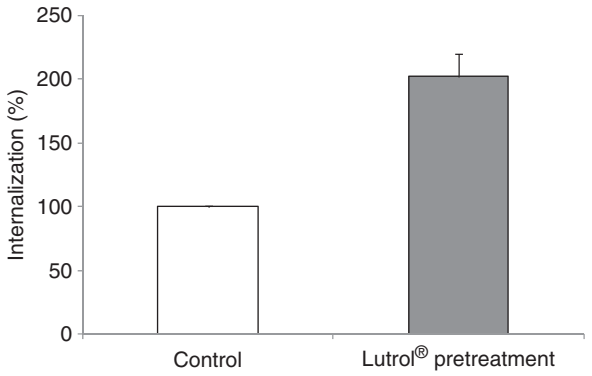

Figure 9. Cellular uptake of DNA in the presence or in the absence of Lutrol® pre-treatment. DNA molecules were labelled with YOYO-1 (one molecule every 150 base pair). Hela cells were transfected after 1 h 3% Lutrol® treatment or without Lutrol® treatment. One µg of labelled luciferase encoding plasmid was complexed with DOSP/DOPE at a charge ratio of ±4. Then, cells were analysed by FACS 24 h after transfection.

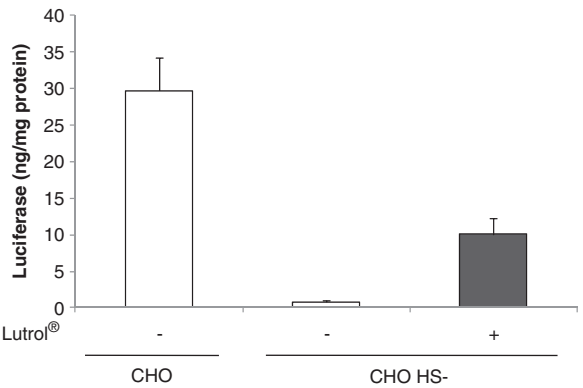

Figure 10. Effect of block copolymer complexation on lipoplex transfection efficiency. CHO cells and heparan sulphate-deficient CHO-2241 cells (CHO HS^-) were transfected with 1 µg luciferase-encoding plasmid complexed with DOSP/DOPE at a charge ratio of ±4. Lipoplexes were formulated with Lutrol® (gray bars) or without Lutrol® (white bars). After 24 h, the luciferase gene expression assay was performed.

suggest that Lutrol® enhanced the interaction of DNA particles with the cell membrane, improving their uptake.

Lutrol® promoted DNA internalization but not endosomal escape *in vitro*

To study whether Lutrol® allows DNA internalization in cultured cells without cationic lipids, we labelled DNA molecules with NPs and observed cell section by TEM. Thus, H1299 cells were incubated with labelled DNA complexed with Lutrol®. After 3 h cells were fixed and submitted to the resin embedding method. TEM visualization of cell sections shows that labelled DNA was localized inside endosomal/lysosomal vesicles (Figure 11C and D) but not in the cell cytoplasm. TEM visualization was repeated 24 h after transfection and never revealed free DNA molecules in the cell cytoplasm (data not shown). However, as presented in the companion paper (25) labelled DNA molecules could be observed in the cell cytoplasm only in the presence of cationic lipids. Of note, identical observations were made on two different cells lines (data not shown). These results strongly suggest that Lutrol® acts as an enhancer of nucleic acids cell-entry *in vitro* as well as *in vivo*. The incapacity of Lutrol® to perform efficient transfection in cationic lipids-free *in vitro* cells strongly support its inability to perform endosomal escape, and strongly suggest that Lutrol® acts *in vivo* via an endocytosis-independent internalization pathway.

DISCUSSION

Amphiphilic block copolymers consisting of EO and PO represent a novel class of non-viral vectors for *in vivo* nucleic acid transfer into various organs. Indeed, numerous studies have shown clearly that *in vivo* injection of DNA complexed with various linear non-ionic block copolymers including Lutrol® and PE6400, or tetra-functionalized X-shaped block copolymers, leads to a dramatic increase in transgene expression, using either a reporter or therapeutic gene, in skeletal and cardiac muscle and in lung, as well as in the corresponding pathological tissues such as DMD muscles and cystic fibrosis lungs (4,6,11,29). Proof of the effectiveness of PEO/PPO-based formulations was also reported previously by Lemieux *et al.*, who described the mixture of L61 and F127—named SP1017—for the transfection of skeletal muscle (2). More-recently, P85 was also reported to increase transfection in muscle successfully (15), and F68/DNA formulations were shown to efficiently transfect eyes by drop delivery (3). Despite the great interest of these block copolymers when applying gene transfer *in vivo* setting, their mechanism of action is still ill defined. Even if it is tempting to assume that needle play a role in DNA transfection in the muscle since muscle cells form a specific anasthamosis cell tissue network, this hypothesis cannot be applied to the mechanism of action of the used block copolymers. Indeed, we also described the large improvement of block copolymer over naked DNA in other organs including heart and most importantly lungs (6) where the injection is performed without needle, just by microspraying the solution containing polymer/DNA complexes. Therefore, even if we cannot definitively rule out that needle could play a role in muscle transfection, it is likely that block copolymers molecules could also act by themselves as vectors improving the *in vivo* internalization process of DNA molecules.

In the present study, we aimed to elucidate the precise mechanism underlying amphiphilic block copolymer transfection efficiency, which would facilitate an understanding of the differences in transfection abilities *in vitro* and *in vivo*. *In vivo*, cells have at their disposal a vascular system that brings them essential nutrients, whilst highly dividing cell lines *in vitro* need to develop a strong endocytotic activity to internalize culture medium nutrients to sustain their rapid growth. This particular behaviour of *in vitro* cell lines leads to the facile, non-specific internalization of DNA/cationic lipid or polymer complexes. The cationic vectors currently used—cationic lipids or cationic polymers—complex DNA to form stable and positively charged particles, which interact

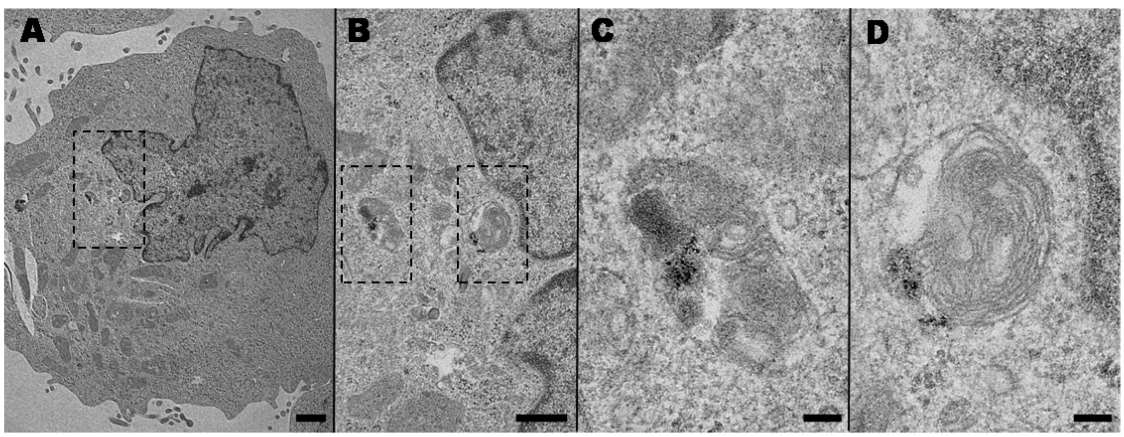

Figure 11. TEM visualization of H1299 cells cellular uptake of Lutrol®/labelled-NpDNA observed 3 h after incubation. Cells were transfected with luciferase plasmid complexed with Nps (1/5) and with Lutrol® at 3%. Ultrathin sections were observed at low (**A**), medium (**B**) and high (**C** and **D**) magnification and revealed the presence of labelled complexes containing electron-dense Nps (black points). Labelled DNA were observed within endosomal/lysosomal structures (C and D). Scale bar represents 1 μm, 500 and 100 nm in A, B and C–D respectively.

electrostatically with negatively charged molecules such as proteoglycans present at the surface of plasma membranes, and are internalized by adsorptive endocytosis. DNA molecules, then, escape from the endosomes by mechanisms which have been proposed to be dependant of the cationic vector used. Cationic lipids would promote endosomal membrane disruption by a 'flip–flop' mechanism, as has been proposed by Xu and Szoka (30). Cationic polymers such as poly(ethylenimine) (PEI) would promote endosome escape by over-acidification, ultimately leading to rupture of endosome membranes by a proton sponge mechanism (31). After endosomal escape, DNA molecules present in the cell cytoplasm reach the nucleus to be transcribed and translated.

In order to attempt to understand the *in vivo* mechanism of action of amphiphilic block copolymers, we designed an *in vitro* model that consisted of treating cultured cells with various amphiphilic block copolymers of different molecular weights, but with a constant percentage of EO. After incubation with block copolymers, DNA molecules were added with a cationic lipid to ensure efficient endosomal escape, one of the main barriers to transfection. We chose the amphiphilic block copolymer subfamily of 80% PEO because of its high *in vivo* transfection efficiency and good tolerance (10,12). We showed by electron microscopy studies and flow cytometry analysis that cell pre-treatment with Lutrol® dramatically increased DNA molecule internalization into each transfected cell, subsequently leading to an overall increase in transgene expression for a constant percentage of transfected cells. Lutrol® allowed enhancing transfection efficiency for concentration lower than 5%, corresponding also to the efficient *in vivo* concentration dose. As increasing concentration of amphiphilic block copolymers leads to the formation of micelles, transfection enhancement is probably related to the presence of unimers *in vivo* as well as *in vitro*. Interestingly, toxicity assays showed that optimized Lutrol® concentration do not affect cell survival *in vitro*, confirming its *in vivo* good tolerance. In addition the observed transfection enhancement was not restricted to DNA molecules, as lower molecular weight nucleic acids displaying the A conformation, such as siRNA, also led to a better gene expression inhibition in Lutrol® pre-treated cells. These results strongly support a role of Lutrol® in the enhancement of the common step of DNA and siRNA transfection, which is cellular internalization, and not in the processes of intracellular trafficking or transcription and translation, as siRNA has to be located in the cytoplasm and DNA in the nucleus in order to be active.

This is in contrast with the report of Yang *et al.* (15), using P85 [poly(ethyleneoxide)$_{26}$-poly(propyleneoxide)$_{40}$-poly(ethyleneoxide)$_{26}$] containing 50% PEO, which activated the NFκB signalling pathway and promoted DNA transfection in a promoter-dependant manner after intramuscular injection. Indeed, it has been proposed that P85 allows transfection of plasmids with viral promoters containing binding sites for specific transcription factors such as NFκB by the improvement of plasmid nuclear targeting due to the NLS sequences present within the transcription factors bound to the plasmid promoter (15). This signalling pathway activation mechanism was also described by Sriadibhatla *et al.*, who demonstrated that P85 activates *in vitro* luciferase transcription in engineered cells expressing luciferase (17). More recently, Yang *et al.* showed that P85 cell pre-treatment also promotes transfection enhancement in a promoter-dependant manner, suggesting a signalling pathway activation-dependent mechanism (32). A previous study with PE6400 containing 40% PEO also showed that microinjected DNA molecules complexed with PE6400 in the cell cytoplasm led to an increase in transfection efficiency (4). This was probably due to the activation of inflammation pathways resulting in the binding of transcription factors to the CMV promoter, as described with P85.

By contrast, the present study shows that block copolymers of 80% PEO enhance DNA cell internalization without displaying any promoter dependence, either *in vivo* or *in vitro*. Transcription factor binding elements present on CMV and SV40 promoters did not influence the transfection efficiency, underlying the absence of promoter-specific signalling pathway stimulation, notably inflammatory such as NFκB signaling pathways. Of note, Q-PCR experiments did not reveal any NFκB transcript enhancement in Lutrol® pre-treated cells, contrary to what has been described with more hydrophobic block copolymers. Therefore, the present study strongly suggests that these 80% PEO block copolymers promote nucleic acid internalization without the activation of previously described inflammatory signalling pathways highlighting their use for the expression of protein of therapeutic interest, as we previously described with EPO expression by intramuscular EPO gene transfer which lasted for at least 250 days (10), and with dystrophin expression in mdx mice (11).

Previous results, and those obtained in this study, have allowed us to propose the *in vivo* mechanism of action of these 80% PEO block copolymers. Block copolymers interacting with DNA molecules would not only enhance the tissue distribution of DNA molecules, but also their cellular internalization by a passive physicochemical mechanism that does not involve an endocytotic process, but rather by a direct delivery to the cytoplasm by fusion with the cell membrane. Indeed, if DNA was internalized via an endocytic pathway *in vivo*, PEO–PPO–PEO block copolymers would be unable to facilitate endosomal escape, as shown by the absence of free DNA molecules in the cell cytoplasm as seen by TEM and efficient transfection in cultured cell lines. Thus, one can imagine that Lutrol® could favour the transmembrane passage of DNA molecules, because it has been shown that, on the one hand, polymers are able to interact with cell membranes by their PPO hydrophobic moiety (33), and on the other hand, polymers are also able to directly interact with DNA by their PPO blocks (13). This explanation is also supported by results described in this paper using heparan sulfate-depleted CHO cells, where transfection of cationic lipid/DNA lipoplexes was only observed in the presence of Lutrol®, whereas as clearly demonstrated by Kopatz *et al.*, cation-mediated transfection requires heparan sulfate proteoglycan (28). In addition, fusion process has already been described *in vitro* by using negatively charged

lipoplexes (34). In the reported study, Resina and colleagues demonstrated that cationic but also anionic lipoplexes are both internalized, but that only cationic objects enter cells through temperature-dependant endocytosis. Indeed, anionic objects were able to enter the cell in a temperature-independent manner. Moreover, Lu *et al.* also recently showed that siRNA lipid based carriers mostly enter the cell through endocytosis-dependent pathway (95%), but also through a minority endocytosis-independent process, which was probably achieved by fusion processes. Interestingly, they observed that this minority endocytosis-independent uptake was responsible of most of the silencing effect, underlying the importance of endosomal sequestration (35). In the present study, the passive enhancement observed with Lutrol® may be attributed to the enhanced attachment of nucleic acids to cell membrane, which could lead to an enhanced internalization through endocytosis in *in vitro* conditions, but may also be attributed to an enhanced fusion process. In the light of these observations, fusion processes might make sense to a passive and endocytosis-independent role of Lutrol® during *in vivo* transfection.

All together, these results show that all block copolymers cannot be considered to have a common mechanism of action regarding gene delivery but, depending on their physicochemical characteristics, they can promote gene expression either by direct fusion with the plasma membrane, or by acting as a biological modifier.

CONCLUSION

In vitro cells present a particular behaviour respective to *in vivo* cells. In fact, as they do not have at their disposal a vascular system to bring them the essential nutrients, they developed an enhanced endocytosis to catch the nutrients present in the medium. This extended endocytosis process is the main route used by cationic vectors such as lipoplexes and polyplexes to enter the cell. Their ability to escape from endosomal sequestration (through flip-flop or proton sponge mechanisms) ensures an efficient gene transfer *in vitro*. *A contrario*, non-ionic vectors such as amphiphilic block copolymers like Lutrol® can not promote endosomal disruption, leading to lysosomal degradation of the carried nucleic acid molecules. This lack of endosomal escape ability allowed us to propose an *in vivo* mechanism of internalization through an endocytosis independant pathway. In fact, *reductio ad absurdum*, if Lutrol® was internalized *in vivo* through endocytosis, its inability to escape from endosomes could not lead to any transfection signal. We support (i) that amphiphilic block copolymers act *in vivo* independently of an endocytosis mechanism, but also (ii) that they do not enhance nucleic acid transfection at the same level. In fact, we showed here that high HLB polymers, such as Lutrol®, facilitate the first step of transfection, i.e. nucleic acid diffusion and cell membrane interaction. Low and intermediate HLB polymers such as P85 have been shown to enhance *in vivo* transfection through gene expression stimulation and nuclear import facilitation by activating inflammatory signalling pathways (15,16,17,32). This classification is in good agreement with previous data obtained by Batrakova *et al.* concerning polymers structure influence on membrane interaction behaviour and signalling pathways activation (33). Moreover, this rational analysis of the different transfection steps stimulated by these three vectors (high HLB polymers, low HLB polymers and cationic lipids) could explain the enhanced effect promoted by their combination as demonstrated with SP1017 polymeric formulation (2) or with multimodular lipid-polymer based systems (36). The identification of the mechanism of action of this novel class of vectors for *in vivo* gene delivery should aid in guiding the future design and synthesis of new block copolymers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Pitard,B., Bello-Roufai,M., Lambert,O., Richard,P., Desigaux,L., Fernandes,S., Lanctin,C., Pollard,H., Zeghal,M., Rescan,P.Y. *et al.* (2004) Negatively charged self-assembling DNA/poloxamine nanospheres for in vivo gene transfer. *Nucleic Acids Res.*, **32**, e159.
2. Lemieux,P., Guerin,N., Paradis,G., Proulx,R., Chistyakova,L., Kabanov,A. and Alakhov,V. (2000) A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Ther.*, **7**, 986–991.
3. Liaw,J., Chang,S.F. and Hsiao,F.C. (2001) In vivo gene delivery into ocular tissues by eye drops of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles. *Gene Ther.*, **8**, 999–1004.
4. Pitard,B., Pollard,H., Agbulut,O., Lambert,O., Vilquin,J.T., Cherel,Y., Abadie,J., Samuel,J.L., Rigaud,J.L., Menoret,S. *et al.* (2002) A nonionic amphiphile agent promotes gene delivery

in vivo to skeletal and cardiac muscles. *Hum. Gene Ther.*, **13**, 1767–1775.
5. Kabanov,A.V., Lemieux,P., Vinogradov,S. and Alakhov,V. (2002) Pluronic block copolymers: novel functional molecules for gene therapy. *Adv. Drug Deliv. Rev.*, **54**, 223–233.
6. Desigaux,L., Gourden,C., Bello-Roufai,M., Richard,P., Oudrhiri,N., Lehn,P., Escande,D., Pollard,H. and Pitard,B. (2005) Nonionic amphiphilic block copolymers promote gene transfer to the lung. *Hum. Gene Ther.*, **16**, 821–829.
7. Wolff,J.A., Malone,R.W., Williams,P., Chong,W., Acsadi,G., Jani,A. and Felgner,P.L. (1990) Direct gene transfer into mouse muscle in vivo. *Science*, **247**, 1465–1468.
8. Wolff,J.A., Williams,P., Acsadi,G., Jiao,S., Jani,A. and Chong,W. (1991) Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques*, **11**, 474–485.
9. Wolff,J.A., Ludtke,J.J., Acsadi,G., Williams,P. and Jani,A. (1992) Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.*, **1**, 363–369.
10. Richard-Fiardo,P., Payen,E., Chevre,R., Zuber,J., Letrou-Bonneval,E., Beuzard,Y. and Pitard,B. (2008) Therapy of anemia in kidney failure, using plasmid encoding erythropoietin. *Hum. Gene Ther.*, **19**, 331–342.
11. Richard,P., Bossard,F., Desigaux,L., Lanctin,C., Bello-Roufai,M. and Pitard,B. (2005) Amphiphilic block copolymers promote gene delivery in vivo to pathological skeletal muscles. *Hum. Gene Ther.*, **16**, 1318–1324.
12. Johnston,T.P. and Miller,S.C. (1985) Toxicological evaluation of poloxamer vehicles for intramuscular use. *J. Parenter. Sci. Technol.*, **39**, 83–89.
13. Bello-Roufai,M., Lambert,O. and Pitard,B. (2007) Relationships between the physicochemical properties of an amphiphilic triblock copolymers/DNA complexes and their intramuscular transfection efficiency. *Nucleic Acids Res.*, **35**, 728–739.
14. Gau-Racine,J., Lal,J., Zeghal,M. and Auvray,L. (2007) PEO-PPO block copolymer vectors do not interact directly with DNA but with lipid membranes. *J. Phys. Chem. B*, **111**, 9900–9907.
15. Yang,Z., Zhu,J., Sriadibhatla,S., Gebhart,C., Alakhov,V. and Kabanov,A. (2005) Promoter- and strain-selective enhancement of gene expression in a mouse skeletal muscle by a polymer excipient Pluronic P85. *J. Control Release*, **108**, 496–512.
16. Kabanov,A.V., Batrakova,E.V., Sriadibhatla,S., Yang,Z., Kelly,D.L. and Alakov,V.Y. (2005) Polymer genomics: shifting the gene and drug delivery paradigms. *J. Control Release*, **101**, 259–271.
17. Sriadibhatla,S., Yang,Z., Gebhart,C., Alakhov,V.Y. and Kabanov,A. (2006) Transcriptional activation of gene expression by pluronic block copolymers in stably and transiently transfected cells. *Mol. Ther.*, **13**, 804–813.
18. Ferrari,S., Moro,E., Pettenazzo,A., Behr,J.P., Zacchello,F. and Scarpa,M. (1997) ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther.*, **4**, 1100–1106.
19. Vigneron,J.P., Oudrhiri,N., Fauquet,M., Vergely,L., Bradley,J.C., Basseville,M., Lehn,P. and Lehn,J.M. (1996) Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. *Proc. Natl Acad. Sci. USA*, **93**, 9682–9686.
20. Desigaux,L., Sainlos,M., Lambert,O., Chevre,R., Letrou-Bonneval,E., Vigneron,J.P., Lehn,P., Lehn,J.M. and Pitard,B. (2007) Self-assembled lamellar complexes of siRNA with lipidic aminoglycoside derivatives promote efficient siRNA delivery and interference. *Proc. Natl Acad. Sci. USA*, **104**, 16534–16539.
21. Sainlos,M., Hauchecorne,M., Oudrhiri,N., Zertal-Zidani,S., Aissaoui,A., Vigneron,J.P., Lehn,J.M. and Lehn,P. (2005) Kanamycin A-derived cationic lipids as vectors for gene transfection. *Chembiochem.*, **6**, 1023–1033.
22. Belmont,P., Aissaoui,A., Hauchecorne,M., Oudrhiri,N., Petit,L., Vigneron,J.P., Lehn,J.M. and Lehn,P. (2002) Aminoglycoside-derived cationic lipids as efficient vectors for gene transfection in vitro and in vivo. *J. Gene Med.*, **4**, 517–526.
23. Pitard,B., Oudrhiri,N., Vigneron,J.P., Hauchecorne,M., Aguerre,O., Toury,R., Airiau,M., Ramasawmy,R., Scherman,D., Crouzet,J. *et al.* (1999) Structural characteristics of supramolecular assemblies formed by guanidinium-cholesterol reagents for gene transfection. *Proc. Natl Acad. Sci. USA*, **96**, 2621–2626.
24. Livak,K.J. and Schmittgen,T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402–408.
25. Le Bihan,O., Chèvre,R., Mornet,S., Garnier,B., Pitard,B. and Lambert,O. (2010) Probing the mechanism of action of cationic lipid/DNA lipoplexes at a nanometric scale. doi:10.1093/nar/gkq921.
26. Labat-Moleur,F., Steffan,A.M., Brisson,C., Perron,H., Feugeas,O., Furstenberger,P., Oberling,F., Brambilla,E. and Behr,J.P. (1996) An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther.*, **3**, 1010–1017.
27. Mislick,K.A. and Baldeschwieler,J.D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl Acad. Sci. USA*, **93**, 12349–12354.
28. Kopatz,I., Remy,J.S. and Behr,J.P. (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J. Gene Med.*, **6**, 769–776.
29. Piron,J., Quang,K.L., Briec,F., Amirault,J.C., Leoni,A.L., Desigaux,L., Escande,D., Pitard,B. and Charpentier,F. (2008) Biological pacemaker engineered by nonviral gene transfer in a mouse model of complete atrioventricular block. *Mol. Ther.*, **16**, 1937–1943.
30. Xu,Y. and Szoka,F.C. Jr (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*, **35**, 5616–5623.
31. Kichler,A., Leborgne,C., Coeytaux,E. and Danos,O. (2001) Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.*, **3**, 135–144.
32. Yang,Z., Sahay,G., Sriadibhatla,S. and Kabanov,A.V. (2008) Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. *Bioconjug. Chem.*, **19**, 1987–1994.
33. Batrakova,E.V., Li,S., Alakhov,V.Y., Miller,D.W. and Kabanov,A.V. (2003) Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.*, **304**, 845–854.
34. Resina,S., Prevot,P. and Thierry,A.R. (2009) Physico-chemical characteristics of lipoplexes influence cell uptake mechanisms and transfection efficacy. *PLOS ONE*, **4**, e6058.
35. Lu,J.J., Langer,R. and Chen,J. (2009) A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery. *Mol. Pharm.*, **6**, 763–771.
36. Letrou-Bonneval,E., Chevre,R., Lambert,O., Costet,P., André,C., Tellier,C. and Pitard,B. (2008) Galactosylated multimodular lipoplexes for specific gene transfer into primary hepatocytes. *J. Gene Med.*, **10**, 1198–1209.