Relationships between the physicochemical properties of an amphiphilic triblock copolymers/DNA complexes and their intramuscular transfection efficiency

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ABSTRACT

Poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide) triblock copolymer (PEO-PPO-PEO) based plasmid delivery systems are increasingly drawing attention in the field of nonviral gene transfer because of their proven in vivo transfection capability. They result from the simple association of DNA molecules with uncharged polymers. We examined the physicochemical properties of PEO-PPO-PEO/DNA mixtures, in which the PEO-PPO-PEO is Lutrol® (PEO75-PPO30-PEO75), formulated under various conditions. We found that interactions between PEO-PPO-PEO and DNA are mediated by the central hydrophobic block within the block copolymer. Dynamic light scattering and cryo-electron microscopy showed that the mean diameter of transfecting particles as well as their stability depended on the PEO-PPO-PEO/DNA ratio and on the ionic composition of the formulating medium. The most active formulation promoting a good tissue-distribution and an optimal transfection was characterized by a reduced electrophoretic mobility, a mean hydrodynamic diameter of ~250–300 nm and by a conserved B-DNA form as shown by circular dichroism studies. Our study also revealed that the stability of these formulations strongly depended on a concentration balance between the DNA and the PEO-PPO-PEO, over which the DNA conformation was modified, micron-sized particles were generated, and the transgene expression was declined. We showed that the physicochemical properties of PEO-PPO-PEO/DNA formulations directly impact the level of gene expression in transfected muscles.

INTRODUCTION

Nucleic acids based therapy is an attractive approach for the treatment of several diseases (1–5). Although synthetic vector based delivery systems are less efficient than viral vectors in vivo, they circumvent concerns of immunogenicity, virus-mediated random integration and recombination with wild type viruses (6,7). Among developed nonviral vectors, nonionic block copolymers consisting of poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide) triblocks (PEO-PPO-PEO) hold promise as a novel class of carrier for in vivo applications. Indeed, recent investigations have reported that formulating DNA with given PEO-PPO-PEO allowed for an increase in plasmid-mediated gene expression in various tissues including muscles, heart, lungs and eyes (8–13).

Nonionic PEO-PPO-PEO triblock copolymers belong to poly(alkoxyde) family within which the largely studied poly(ethyleneglycol) (PEG) has been reported to induce significant changes in DNA solubility and structure under given conditions. Thus, DNA concentration, pH, ionic strength of the medium and presence of divalent ions have been shown to impact PEG-induced DNA precipitation (14,15). The main explanation for this phenomenon has been reported to be the change in the water activity induced by the presence of PEG causing a reduction in DNA solubility.

In contrast to the water-soluble PEG homopolymer, PEO-PPO-PEO bear amphiphilic properties due to the ability of the lipophilic poly(propyleneoxide) (PPO) block to macroscopically phase-separate from water at relatively low temperature, and to the high water solubility of the poly(ethyleneoxide) (PEO) blocks. In an aqueous solution and at a given concentration, PEO-PPO-PEO molecules remain as individual (non-associated monomers) at temperatures below their critical micellar temperature (CMT). Above the CMT the molecules become more lipophilic leading to complex self-assembling structures including micelles of various sizes, lyotropic liquid crystalline gels and rod-like micelles (16,17). The PPO block...
Hydrophobicity has been shown to be the most important factor in the formation of micelles that consist of a core of PPO blocks and a corona of PEO blocks. Temperature, molecular weight, concentration and presence of electrolytes are known to affect the phase behavior of PEO-PPO-PEO in solution. For example, the micelle formation is greatly influenced by the addition of electrolytes shifting the CMT to lower temperatures (18–20). These differences in structure and property taken together with the very high transfection efficiency of PEO-PPO-PEO prompted us to investigate the physicochemical properties of PEO-PPO-PEO/DNA formulations. In previous studies, hydrodynamic diameter and zeta potential have been used to characterize formulation made of DNA and F68 (9). Despite this, little is known about the physicochemical aspects that govern the association of the negatively charged polyelectrolyte that is DNA, with these self-organizing nonionic amphiphilic block copolymers. An understanding of those properties is needed to establish a correlation between physicochemical properties of PEO-PPO-PEO/DNA formulations and their transfection efficiency. Thus, this study was designed (i) to define the experimental conditions that lead to better association of DNA with PEO-PPO-PEO; (ii) to track the features of PEO-PPO-PEO/DNA formulations that promote high gene transfer efficiency in vivo; (iii) to investigate whether PEO-PPO-PEO/DNA formulations follow molar ratio law currently used in cationic vector based formulations. To conduct our investigations, Lutrol® was taken as a model compound of PEO-PPO-PEO because of its low levels of toxicity in muscle (21).

MATERIALS AND METHODS

Plasmids

pCMV-Luc (22) and pCIK CAT (generous gift of D. Gill, Oxford) are plasmids encoding luciferase and chloramphenicol acetyl transferase (CAT) reporter genes, respectively, both under the control of the human cytomegalovirus immediately early gene promoter. They were purified from recombinant E.coli by means of EndoFree plasmid purification columns (Qiagen, Chatsworth, CA).

Formulation preparation

Lutrol® (EO<sub>75</sub>-PO<sub>30</sub>-EO<sub>75</sub>, MW 8600), F38 (EO<sub>75</sub>-PO<sub>15</sub>-EO<sub>75</sub>, MW 4700), F108 (EO<sub>75</sub>-PO<sub>63</sub>-EO<sub>75</sub>, MW 14600) and F127 (EO<sub>15</sub>-PO<sub>50</sub>-EO<sub>75</sub>, MW 12600) were gifts from BASF. Poly(ethyleneoxide), PEG (EO<sub>77</sub>, MW 8000) were from Sigma-Aldrich. Stock solutions of polymers were prepared at given weight-to-weight (w/w) concentration in sterilized water and were kept at 4°C overnight to ensure complete dissolution. Formulations of DNA with polymers were prepared by equimolar mixing of polymer at various concentrations in water with the desired concentration of DNA dissolved in saline 2× or in Tyrode 2×.

Mean diameter and zeta potential measurements

Hydrodynamic mean diameter was determined by dynamic light scattering using the intensity mean size (Z Average) with a sample refractive index of 1.59, viscosity of 0.89 cP. The system was routinely calibrated using a 220 nm standard dts 5050 from Malvern. Mean diameters values correspond to the average of 10 independent measurements performed on the same sample and calculated using the cumulant analysis mode. The zeta potential measurement based on Laser Doppler interferometry was used to assess the electrophoretic mobility of DNA, either naked or associated with polymers. Measurements were performed for 20 s using a standard capillary electrophoresis cell with zero field correction. The dielectric constant was 79 and the Smoluchowsky constant F(ka) was 1.50. The system was routinely calibrated using a −50 ± 5 mV standard (Malvern). For polymer and DNA concentration-dependent zeta potential, DNA was formulated with polymers at desired concentrations in saline or in Tyrode. Samples were then submitted to zeta potential measurement at 25°C. For the temperature-dependent zeta potential study, 100 µg DNA/ml was formulated with 0.35, 1.15 and 1.75 mM Lutrol®. Samples were injected in the capillary cell and the temperature was adjusted via the peltier controller unit adapted to the apparatus. After reaching the desired temperature, the system was stabilized for 10 min before performing measurements.

Circular dichroism

Circular dichroic (CD) spectra of the DNA alone or the DNA within formulations were obtained on a JASCO spectropolarimeter (model J-810, JASCO, Tokyo, Japan) using a 1 cm path length quartz cuvette in saline or Tyrode. Final DNA concentration within the formulations was 25 µg/ml in both media. The ellipticity values are given in millidegrees (mdeg) with the values for saline or Tyrode subtracted from the experimental values of the corresponding formulation or DNA alone.

Cryo-TEM micrography

Formation complexes were prepared at 100 µg DNA/ml and various PEG or Lutrol® concentrations either in saline or in Tyrode. Five microliters sample were deposited onto a holey carbon coated copper grid; the excess was blotted with a filter paper, and the grid was plunged into a liquid ethane bath cooled with liquid nitrogen (Leica EM CPC). Specimens were maintained at a temperature of approximately −170°C, using a cryo holder (Gatan), and were observed with a FEI Tecnai F20 electron microscope operating at 200 kV and at a nominal magnification of 50 000× under low-dose conditions. Images were recorded with a 2K × 2K Gatan slow scan CCD camera.

In vivo transfection

Female Swiss mice were obtained at 8 weeks of age from Janvier (Le Genest Saint Isle, France). Following anaesthesia with hypnomidate (40 mg/kg, intraperitoneal), 50 µl of formulation in saline or in Tyrode, were injected into shaved tibialis anterior muscles at one site, using a microfine syringe with a small 30 gauge needle (U100; Becton Dickinson, Sandy, UT). Injections were performed parallel to the muscle fibers. At least six tibialis anterior muscles were included in
each individual set of experiment. Reported gene expressions values are the average of five and two independent experiments for luciferase and CAT expression, respectively. Seven and three days after injection of luciferase and CAT reporter gene, respectively, muscles were dissected and snap-frozen in liquid nitrogen. The muscles were homogenized in 1 mL of luciferase reporter lysis buffer (Promega, Madison, WI) or CAT reporter lysis buffer (Roche Diagnostics, Mannheim, Germany) both supplemented with a protease inhibitor cocktail (Roche Diagnostics). Samples were then centrifuged at 10,000 r.p.m. for 5 min. Luciferase activity was measured from a 10 μl aliquot of supernatant with VICTOR2 (PerkinElmer, Les Ulis, France), using a luciferase assay system (Promega) based on light emission after addition of 100 μl of luciferase assay substrate. Each muscle extract was analyzed in duplicate. A standard curve, prepared with non-injected mouse tibial anterior muscle, was included with each microplate reading, using a purified luciferase (Sigma, St. Louis, MO). CAT activity was measured from diluted aliquots of supernatant with VICTOR2 using the CAT Elisa kit (Roche Diagnostics) following instructions of the supplier. Each muscle extract was analyzed in duplicate. A standard curve, prepared in standard sample buffer (Roche Diagnostics), was included on each microplate using the CAT enzyme standard included in the kit. To study the influence of cardiotoxin and hyaluronidase on gene transfection efficiency, 4 μg of cardiotoxine (Latoxan, Valence, France) and 1 unit of hyaluronidase (Sanofi-synthelabo, Le Plessis Robinson, France) were injected intramuscularly 5 days and 3 h prior to formulation injections, respectively.

Fluorescence microscopy

pCMV-Luc was Rhodamine-labelled by nick translation according to the supplier protocol (Molecular Probes). Mice were injected with formulations made of 100 μg/ml of the labelled DNA with or without 3.5 mM Lutrol® and sacrificed 12 h after injection. Immediately after euthanasia of mice, tibial anterior muscles were harvested and fixed in 4% paraformaldehyde for 10 min. They were then embedded in TissueTek and snap-frozen in liquid nitrogen cooled in isopentane. Thick sections (8 μm) were cut from the entire muscle and reviewed under a Zeiss AxioVision fluorescence microscope.

RESULTS

Characterization of Lutrol®/DNA formulations in saline

Compared to PEG, which is a polymer of ethyleneoxide units, Lutrol® consists of a succession of 30 propyleneoxide moieties flanked on both sides by 75 ethyleneoxide moieties. We first investigated whether the presence of the central PPO block within Lutrol® could impact DNA light scattering property by using a PEG of about the same number of alkoxide as control. For this purpose, since PEG-POO-PEO alone, but not PEG, can be subject to self-light scattering at high concentrations, DNA was formulated in saline at 10 μg/ml with low subscttering Lutrol® concentrations (0.11–0.35 mM). The results were compared with those obtained from the corresponding PEG made formulations. As showed in Figure 1A, the scattered light intensity in Lutrol®/DNA formulations increased as the Lutrol® concentration was raised, while in PEG made formulations, the scattered light intensity did not change. In the absence of DNA, both polymers exhibited very low and similar light scattering patterns. This observation showed that the presence of Lutrol® changed the light scattering property of the DNA or that new molecular species may be generated in the presence of Lutrol®, but not in the presence of PEG. To check the meaning of these characteristic changes from the viewpoint of DNA hydrodynamic diameter, quasi-elastic light scattering (QELS) studies were performed under the same conditions. Results were expressed as global mean diameter of particles scattering in solution. As showed in Figure 1B, cumulant mode analysis of QELS data revealed that increasing the Lutrol® amount led to a steep decrease in the mean diameter of DNA from 400 to ~225 nm. Such a reduction in DNA hydrodynamic diameter did not occur in PEG/DNA formulations at low concentration of polymer (Figure 1C). However, raising both polymer amounts to Lutrol® scattering range concentrations (1.1–10.5 mM) allowed for further mean diameter reduction in both formulations (Figure 1B and C). We also noted that particles within Lutrol®/DNA formulations were smaller than those in PEG/DNA formulations.

For further characterization, we then compared both type of formulations by studying the electrophoretic mobility of DNA, expressed as the zeta potential. The zeta potential increased as the Lutrol® concentration was raised, reaching above the neutrality at 7.0 mM Lutrol®. In contrast increasing PEG concentration did not lead to a zeta potential reduction (Figure 1D). To further understand the importance and the role of the PPO block in the observed DNA mobility reduction, we measured the zeta potential of formulations prepared with a PEO-PPO-PEO with the same percentage of ethyleneoxide (80%) as Lutrol®, but two-times less (F38) and two-times more (F108) propyleneoxide moieties. The zeta potential reduction did not occur for F38/DNA formulations even at 7.0 mM polymer (~19.0 mV), while it was faster with F108/DNA formulations; at 1.1 mM (Figure 1E). Furthermore we investigated the zeta potential of formulations made of F127 which bears about the same amount of polypropylene moiety as F108 but 70% of ethyleneoxide. The results showed that the zeta potential reduction occurred at lowest polymer concentrations (Figure 1E). We thus found a hydrophobicity dependence on the DNA mobility reduction that strongly suggested a PPO block-dependent interaction between the DNA and PEO-PPO-PEO copolymers.

Effect of physiological mimicking medium on Lutrol®/DNA physicochemical properties

Tyrode’s solution (Tyrode) has been described as a physiological medium that mimics the extracellular ionic composition and consequently the ionic environment of Lutrol®/DNA formulation after in vivo delivery. Since both DNA and PEO-PPO-PEO intrinsic properties depend upon their concentration and upon the nature and the concentrations of ions in solution, we compared the physicochemical properties of Lutrol®/DNA formulations made in Tyrode
with those obtained in saline. We also studied the effect of an increase in the DNA amount to a concentration compatible with luciferase assay following an intramuscular injection (100 µg/ml of DNA). Results showed that, at a low concentration of DNA (10 µg/ml), particles within formulations shared the same mean diameter as the polymer concentration increased irrespective of the medium used (Figure 2A). In contrast, when using 100 µg/ml of DNA, particle mean diameter was minimum at 0.35 mM Lutrol®, then increased progressively when the Lutrol® concentration increased (Figure 2A). Interestingly, in Tyrode, while at 10 µg/ml of DNA particle sizes were similar to overall Lutrol® concentrations, at 100 µg/ml of DNA and 10.5 mM Lutrol® particles appeared as 1.1 µm aggregates (Figure 2A). Moreover, while from 0.35 to 7.0 mM Lutrol®, particle mean diameters were similar in both media, the increase in zeta potential occurred more quickly in Tyrode, reaching a maximum of -7.0 mV at 3.5 mM Lutrol®. This increase in zeta potential was significantly slower in saline (-25 mV at 3.5 mM Lutrol®). The zeta potential did not change with increasing the PEG concentration in control PEG/DNA formulation with 100 µg/ml DNA (Figure 2B).
Cryo-TEM Micrography of Lutrol\textsuperscript{®}/C210/DNA formulations

To check if micrography of formulations could be in agreement with the observed changes in particle hydrodynamic diameter, Cryo-TEM micrography was performed. Structures were analyzed at the reliable concentrations of 0.35, 3.5 and 10.5 mM Lutrol\textsuperscript{®} or PEG using 100 μg/ml of DNA. At 0.35 and 3.5 mM Lutrol\textsuperscript{®}, no condensed DNA structures were observed both in saline and in Tyrode (data not shown). At 10.5 mM Lutrol\textsuperscript{®}, globular features of 100–200 nm in size with a very low contrast were visible in saline (Figure 3A–F), while micron-sized large compact DNA structures with a round-shaped feature were observed in Tyrode (Figure 3G–J). At a higher magnification, DNA molecules appeared condensed within these structures (Figure 3B–F and H–J). Although DNA molecules are tightly packed, they did not exhibit regular pattern like lamellar or hexagonal packing described with cationic lipids (23–28). However, linear features were visible on the edge of the condensates, likely revealing a rough linear organization of DNA strands. In the middle of the condensates, no peculiar pattern was observed probably because of the thickness increase.

**Figure 2.** Characterization of Lutrol\textsuperscript{®}/DNA formulations in Tyrode’s solution. (A) DNA was formulated at 10 μg/ml (open bars) or 100 μg/ml (closed bars) in both saline and Tyrode with increasing amounts of Lutrol\textsuperscript{®}. Formulations were then submitted to mean diameter measurement using the zetasizer 3000HSA. Polydispersity indexes (PI): *PI = 0.4–0.5, **PI = 0.3–0.5, #PI = 0.6–0.7. (B) DNA was formulated at 100 μg/ml with Lutrol\textsuperscript{®} in saline (filled squares) or Tyrode (closed triangles) or with PEG (open circles) in Tyrode, zeta potential of the formulations were measured by using the Zetasizer 3000HSA.
Complexes made of PEG allowed observation of condensed DNA even at lowest polymer concentrations. Figure 3K and L–M shows representative structures obtained at 3.5 and 10.5 mM PEG, respectively. These DNA condensates resemble a small dense core surrounded by fine elongated structures (Figure 3M). Their size did not change significantly while increasing PEG concentration. These results showed that Lutrol/C210 and PEG triggered the condensation of DNA molecule in a notably different manner. With PEG, DNA condensation occurred at a lower concentration and the global shape and size of these condensed structures remained unchanged even at high PEG concentration. With Lutrol/C210, condensed DNA structures were formed only at high polymer concentration and their shape were very different from those formed with PEG. Such compact structures were never observed with DNA alone either in saline or in Tyrode (data not shown). With Lutrol, compact and dense structures were formed while with PEG DNA was condensed into expanded and branched structures. If the morphologies of the condensates were different, the DNA organization induced in the presence of Lutrol/C210 and PEG looked similar. This non-regular DNA condensation induced by PEG has already been reported with linear DNA (29). The presence of propylene oxide moiety plays a crucial role in the morphology of the DNA condensed structure.

Circular dichroism of Lutrol/C210/DNA formulations

To investigate whether this first set of data, including cryo-TEM observations, had any significance on the molecular organization of the DNA within the formulations, circular dichroism studies were performed at 0.35, 3.5 and 10.5 mM Lutrol/C210 or PEG. No change in the DNA spectrum was observed for PEG made formulations either in saline or in Tyrode regardless of the presence and the concentration of the polymer (data not shown). In saline, at all Lutrol/C210 concentrations, the typical spectrum of DNA B-form with a negative band at 245 nm and a positive band at 272 nm was obtained with both polymers (Figure 4A) (30,31). Similarly, the DNA spectrum did not change for Lutrol/C210 made formulations at 0.35 and 3.5 mM in Tyrode (Figure 4B). However, at 10.5 mM Lutrol/C210, changes in both the negative and the positive peaks of DNA occurred with shifts to 250 and 288 nm, respectively (Figure 4B). Such shifts indicate a transition of the secondary DNA structure from a B- to a C-type. Moreover, simultaneous decrease in the positive peak and the increase in the negative peak were observed (Figure 4B). The decrease of the positive value has been explained by dehydration and/or neutralization of the phosphate group and might thereby indicate difference in hydration state of DNA either free or associated with Lutrol/C210. The increase in the negative peak of the DNA spectra suggested the presence of DNA helices oriented parallel to each other in the condensed DNA, called the Ψ-form of DNA (32–34).

Study of hydrophobicity mediated Lutrol/C210/DNA interaction

Based on the zeta potential reduction as a function of PEO-PPO-PEO concentrations and PPO block length and also on the absence of zeta potential reduction in the presence of PEG, we hypothesized that DNA and PEO-PPO-PEO interact through the central hydrophobic PPO arm leading to the zeta potential reduction. By changing artificially the hydrophobicity of Lutrol/C210 molecules, we should thus be able to show
the interaction between the negatively charged DNA phosphate groups and Lutrol® at lowest concentrations. Temperature-induced hydrophobicity was used to argue our hypothesis. Experiments were performed at 1.75 mM Lutrol and $T<55^\circ C$, conditions under which the scattered light intensity of the Lutrol® did not change while the temperature increased. Formulations were then prepared at various concentrations of DNA and Lutrol® and zeta potential was measured as a function of the temperature. Figure 5A showed that at a fixed DNA concentration of 100 $\mu$g/ml, 0.35 and 1.16 mM Lutrol® did not induce zeta potential increase from 15 to 45°C, while a moderate increase occurred between 45 and 55°C. At 1.75 mM Lutrol®, such temperature-induced zeta potential increase occurred earlier, between 25 and 35°C (from $-24$ to $-7.0$ mV). Further temperature increase did not affect the zeta potential value. Zeta potential of PEG/DNA control formulation did not change with increasing the temperature (Figure 5A). Figure 5B showed that at the given Lutrol® concentration of 1.75 mM, a 3-fold increase in DNA amount (300 $\mu$g/ml of DNA) delayed the temperature-induced zeta potential neutralization observed previously. Furthermore, a 10-fold reduction of DNA amount (10 $\mu$g/ml) did not allow highly negative zeta potential values. These results demonstrated a strong impact of the polymer hydrophobicity on the DNA mobility reduction and also the importance of the concentration balance between the DNA and the polymer on this phenomenon.

**Figure 5.** Study of hydrophobicity mediated Lutrol®/DNA interaction. (A) Formulations were prepared in Tyrode, at a final DNA concentration of 100 $\mu$g/ml with 0.35 mM (closed circles), 1.16 mM (closed squares) and 1.75 mM (closed triangles) of Lutrol® or 1.75 mM PEG (open diamonds). (B) Formulations were prepared with 1.75 mM Lutrol® at the final DNA concentration of 0 (open diamonds), 10 $\mu$g/ml (closed circles), 100 $\mu$g/ml (closed triangles), 300 $\mu$g/ml (closed diamonds). Temperature was raised and the zeta potential was measured by allowing 10 min equilibration between each temperature.

**Reporter gene expression and tissue-distribution**

To gain insight into the relationship between the transfection potential of formulations and their aforementioned physicochemical properties, they were injected into mice tibial anterior muscles. In saline, the presence of 3.5 mM Lutrol® increased luciferase activity by a factor 10 over that of naked DNA while no significant increase was observed with 0.35 mM. Increasing the Lutrol® concentration to 10.5 mM led to a decrease in luciferase expression to levels lower than that obtained with naked DNA (Figure 6). In Tyrode, the same concentration/luciferase activity pattern was observed, excepted that luciferase activity was higher.
tested concentrations (Figure 6). To better understand the main reason underlying such an improved transgene expression, muscles were injected with 100 µg/ml of rhodamine-labelled DNA alone or formulated with 3.5 mM Lutrol®. As shown in Figure 7, naked DNA molecules were poorly distributed in the muscle and were mainly located around the injection site (Figure 7A). The presence of Lutrol® ensured an even spread of the DNA throughout the muscle with fluorescent spots observed in numerous muscle fibers (Figure 7B). As control, muscle injection with non-labelled molecules, did not show fluorescence (data not shown).

We then investigated whether the observed improved transgene expression was only due to a better tissue-distribution allowing an increase in the number of transfected fibers. Cardiotoxin and hyaluronidase, which have been reported to improve naked DNA-based transgene expression in muscle, were injected prior to formulation injections. In contrast to naked DNA, both cardiotoxin and hyaluronidase injection failed to increase the transfection efficiency of Lutrol®/DNA formulations, suggesting that the important reduction of the DNA charge achieved by formulating the DNA with Lutrol® leads to a different type of mechanism that contribute to the better DNA uptake by the fibers.

Study of Lutrol®/DNA formulation stoichiometry

The results described above showed that for 100 µg/ml of DNA, raising the Lutrol® concentration to 10.5 mM triggered micron-sized aggregates formation and reduced the transfection efficiency. As a reflex of nonviral system formulators, we thought that varying amount of polymer and DNA by keeping constant Lutrol®/DNA ratio could overcome aggregate formation and then prevent the loss in transgene expression. We then varied DNA concentrations from 10 to 1000 µg/ml in Tyrode within 0.35, 3.5, 7.0 and 10.5 mM Lutrol® in order to study the stoichiometry impact on the formulation. For this purpose we used a CAT encoding DNA since CAT activity detection Kit is more sensitive than luciferase assay. Figure 8A shows that, at 0.35 mM Lutrol®, there was no significant variation in size with increased DNA amount and that at 3.5 and 7.0 mM Lutrol®, ~500 nm sized particles were generated for DNA concentrations >100 µg/ml. Surprisingly, increasing the amount of DNA in 10.5 mM made formulations did not impair nor reduce aggregates formation as would be expected, but generated larger aggregates for highest DNA concentrations. Formulations made with 0.35 mM Lutrol had zeta potentials that sharply decreased as the DNA concentration ranging from 5 to 100 µg/ml, and for higher DNA concentrations, the zeta potential decreased and stabilized around ~17 mV (Figure 8B). The results obtained at 7.0 mM Lutrol® were similar to those obtained at 3.5 mM (data not shown).

Finally, we evaluated the influence of DNA concentration on transfection efficiency. Injection of Lutrol®/DNA formulations led to a significant increase in CAT expression when the amount of formulated DNA increased up to 50 µg/ml. A further increase in the amount of DNA led to a plateau of gene expression. Most importantly, naked DNA led to a maximum gene expression with 300 µg DNA/ml, whereas
with Lutrol® the same expression level was achieved with 10–20 μg DNA/ml (Figure 8C). Expressing the results as the ratio of CAT activity obtained from Lutrol®/DNA complexes versus that obtained from naked DNA, showed that the maximum increase in CAT expression over naked DNA was observed with 50 and 100 μg DNA/ml (Figure 8C, inset).

**DISCUSSION**

In this article, we have investigated the physicochemical properties of the new promising nonviral system resulting from the association of DNA molecules with nonionic amphiphilic block copolymers. We attempted to understand the key parameters involved in the association of both molecules and the behavior of the DNA within the formulation. Thereby we aimed to draw correlations between their physicochemical properties and their transfection efficiency, which is of crucial importance for the development of nonviral vectors. In our previous works, we have reported that mixing DNA with PE6400 block copolymer or Lutrol® increased either reporter and therapeutic genes expression in skeletal or cardiac muscles and in lungs, including the corresponding pathological tissues such as Duchenne Muscular Distrophy muscles and cystic fibrosis lungs (10,12,13). Proofs of PEO-PPO-PEO-based formulations effectiveness have also been reported by Lemieux et al. (8) who have used mixture of L61 and F127 to transfect muscle. More recently P85 was reported to successfully increase transfection in muscle (35). Similarly, F68/DNA formulations have been shown to efficiently transfect eyes by drop delivery (9). So far no thorough investigations have been done to characterize this type of formulations and to study the relationships between the formulation features, the transfection efficiency and the tissue-distribution of the DNA following intramuscular injection.

**Figure 8.** Study of Lutrol®/DNA formulation stoichiometry (A) Lutrol®/DNA formulations were prepared with 0.35 mM (closed circles), 3.5 mM (closed diamonds), 7.0 mM (closed squares) and 10.5 (closed triangles) of Lutrol® and increasing amount of DNA in Tyrode. Mean diameters were measured on the zetasizer 3000HSA. Polydispersity indexes (PI): *PI = 0.4–0.5, **PI = 0.3–0.5, #PI = 0.6–0.7, ##PI = 0.7–0.8. (B) Lutrol®/DNA formulations were prepared with 0.35 mM (filled triangle) and 3.5 mM (filled diamond) of Lutrol® and increasing amount of DNA in Tyrode. Zeta potential were measured on the zetasizer 3000HSA. (C) Lutrol®/DNA formulations were prepared with 3.5 mM Lutrol® (closed bars) and various concentrations of DNA. Equivalent amounts of naked DNA (open bars) were used as control. A constant 50 μl volume was injected per tibial anterior muscle. (inset) Each bar represents the ratio of CAT activity obtained with Lutrol®/DNA complexes divided by the CAT activity of naked DNA.
Focusing our study on Lutrol® (PEO17-PPO30-PEO75), we showed that the presence of the PPO arm in a nonionic PEO-PPO-PEO copolymer may markedly modify both electrical and hydrodynamic properties of DNA in solution when compared to PEG homopolymer. We also showed that by opposition to PEG, PEO-PPO-PEO were able to interact with DNA in a concentration-dependent manner and that this interaction was significantly increased in a physiological mimicking medium such as Tyrode. We demonstrated that the interaction between DNA and PEO-PPO-PEO was mediated by the hydrophobic POP block. This is shown by the difference in zeta potential reduction while using PEO-PPO-PEO containing shorter or longer size of PPO block and by the temperature-induced zeta potential reduction. We noticed that the polymer concentrations under which the maximum zeta potential reduction was obtained did not correspond to their CMC (36). We have also found that, although the ion types contained in Tyrode allowed a better interaction of Lutrol® with DNA, such ionic composition can favor formation of aggregated DNA forms when using higher PEO-PPO-PEO amount. Generated aggregates failed to improve in vivo gene expression. On the other hand, at lower PEO-PPO-PEO concentration, the transfection efficiency was declined. Thus, we have evidenced an optimal 3.5 mM Lutrol® concentration, under or over which the transfection efficiency was lost. However, when the formulation balance is reached, Tyrode made formulation displayed higher transfection efficiency. The difference in ionic composition between saline and Tyrode plays probably a central role in improving transgene expression. The presence of divalent cations such as calcium and magnesium as well as the buffered feature of Tyrode may also contribute positively to the plasmid delivery into the cells. Previous works using others PEO-PPO-PEO block copolymers have shown that a mixture of L61 and F127 (8) or P85 alone (35) led to an increase in reporter gene expression by a factor 10 or 15 as compared to naked DNA, respectively. In the same experimental conditions, i.e. 5 µg of DNA per muscle and 7 days post-injection, Lutrol® can lead to up to 36-fold increase in transgene expression (12). Compared to our previous data in which PE6400 (PEO13-PPO30-PEO13) was used (10), the present results suggest that the intrinsic hydrophilic–hydrophobic balance is a key parameter in selecting such polymers for efficient intramuscular gene expression. Nevertheless, we previously reported only a 2-fold increase in transgene expression in a dystrophic mouse model (mdx) (12). Thus the nature of the muscle influences the efficiency of PEO-PPO-PEO mediated gene expression.

Due to the absence of charged atoms on PEO-PPO-PEO block copolymers, it was difficult to imagine an interaction between the DNA and these uncharged polymers. However, it has been demonstrated elsewhere that uncharged poloxamer pyrrolidone (PVP) polymer can interact with DNA, creating a hydrophobic surface that protects the DNA from degradation and increases interaction with cell membranes (37,38). In contrast to PVP that has hydrophobic and hydrophilic sites located together along the same molecule, block copolymer structure allows a clearly distinct and separated localization of hydrophobic and hydrophilic segments. Then, the good colloidal stability of PEO-PPO-PEO/DNA formulations, even though the particles exhibit neutral zeta potential, could be due to the presence of a hydrated outer corona made of PEO blocks. Supramolecular organization of PEO-PPO-DNA clearly contrasts with that obtained with non-viral complexes resulting from the association of DNA with cationic molecules, where neutral complexes formation led to colloidal instable formulation and then to aggregated complexes (23,39). Previous structural studies of cationic polymers and lipids based complexes have shown a pronounced change in DNA CD spectrum with a Ψ-DNA form (40,41). The results obtained here suggest that the lack of transfection efficiency of cationic systems following intramuscular injection may be caused by this important change in the DNA molecular structure. Indeed, as shown by the DNA CD spectrum of a 10.5 mM Lutrol® made formulation, a B- to Ψ-DNA form change occurred and Lutrol®/DNA formulation failed to increase the transgene expression, whereas at the optimal 3.5 mM Lutrol® concentration, the native B-DNA form was maintained. Thus, unmodified non-aggregated DNA structure, neutrally charged formulation seem to be the sine qua none conditions required for PEO-PPO-PEO block copolymers mediated transgene expression improvement. Our results indicate that the optimal feature of active PEO-PPO-PEO/DNA, and Lutrol®/DNA formulations in particular, is an interactive non-condensing associated complex. This feature is in agreement with the absence of decrease in ethidium bromide fluorescence in the presence of 3.5 or 7.0 mM Lutrol® (data not shown). Another important contrasting feature that belongs to PEO-PPO-PEO/DNA made complexes is the weak interaction force between the DNA and the PEO-PPO-PEO shown by the lack of retardation of the DNA in the presence of the copolymer while using agarose gel retardation assay (data not shown). This weak interaction force could favor fast release and/or increased availability of the DNA once the formulation is injected.

Moreover, the present results showed that the PEO-PPO-PEO/DNA concentration ratio directly impacted the level of gene expression in vivo. Indeed, increasing Lutrol® amount over the ideal Lutrol®/DNA ratio led to the formation of large aggregates, probably responsible for the decrease in the transfection efficiency due to a poor diffusion. It then appeared that, decreasing this optimized ratio by increasing the DNA concentration allowed the formation of negatively charged formulations. In these conditions, negative charges of DNA were not sufficiently masked and large aggregates were formed. The benefit due to the presence of the carrier is lost in that circumstance, as shown by the reduction of the ratio of formulated/naked DNA transgene expression.

When compared with naked DNA, formulated DNA is greatly spread in the muscle after injection. This suggests that, shielding the negative charge of DNA affects its interaction with the negatively charged extracellular matrix plasma membrane allowing an improved tissue-distribution of the DNA as shown by the even fluorescence in Lutrol®/Rhodamine-labelled DNA injected muscles. The shielding may facilitate the DNA cellular uptake by decreasing the electrostatic repulsion. Putative receptors present in the intact extracellular matrix may be involved in the Lutrol®/DNA complex uptake by a mechanism different from that implicated in naked DNA internalization. Then, the assembled Lutrol®/DNA complex interacting with the plasma membrane could be directly delivered to the cytosol via diffusion.
through the plasma membrane or by endosome from where they may efficiently escaped. In both hypothetical cases the presence of amphiphilic PEO-PPO-PEO may play a central role. It has been indeed reported that PEO-PPO-PEO block copolymers have the ability to perturb the plasma membrane by insertion into the acyl chain portion of the lipid bilayers. Another important finding of this study is that, overcoming high-concentrations induced DNA aggregation was not possible by balancing the Lutrol amount with the appropriate DNA concentrations, a procedure that can be used in cationic carriers based formulations. PEO-PPO-PEO block copolymers seem to have a completely different feature that does not follow the basic rule of stoichiometric ratio. In conclusion, we have drawn out three physicochemical formulation zones. An electronegative, small and stable particles zone generated by ~0.35 mM Lutrol that failed to increase transfection efficiency; an electroneutral, small and stable particle zone triggered by 3.5 and 7.0 mM Lutrol where particles transfact efficiently; and an electroneutral and aggregated particles zone obtained at 10.5 mM Lutrol in which the transfection efficiency is lost. A recent study on the concentration effect showing the decline in transgene expression at concentrations higher or lower than the optimal dose has been found with P85, and strongly supports our finding. This study provides a better understanding of nonionic PEO-PPO-PEO/DNA complexes formation and their impact on in vivo transfection efficiency. Future experiments will aim to extend this study to other PEO-PPO-PEO family members in order to define a structure–activity relationship between structurally different PEO-PPO-PEO. This structure–activity relationship will be used to design novel block copolymers that lead to enhanced gene expression in various organs.

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