Temperature-Sensitive Amphiphilic Non-Ionic Triblock Copolymers for Enhanced In Vivo Skeletal Muscle Transfection

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ABSTRACT. We report that low concentration of amphiphilictriblock copolymers of pMeOx-*b*-pTHF-*b*-pMeOx structure (TBCPs) improve gene expression in skeletal muscle upon intramuscular co-injection with plasmid DNA. Physicochemical studies carried out to understand the involved mechanism show that a phase transition of TBCPsunder their unimer state is induced when the temperature is elevated from 25°C to 37°C, the body temperature. Several lines of evidences suggest that TBCP insertion in a lipid bilayer causes enough lipid bilayer destabilization and even pore formation, a phenomenon heightened during the phase transition of TBCPs. Interestingly, this property allows DNA translocation across the lipid bilayer model. Overall, the results indicate that TBCPs exhibiting a phase transition at the body temperature is promisingto favor *in vivo*pDNA translocationin skeletal muscle cells for gene therapy applications.

1. Introduction

Gene therapy represents a great potential for curing a broad range of diseases, such as hereditary single-gene defects, cancers, viral infections, cardiovascular diseases, neurodegenerative disorders.[1-6] Replacing a mutated gene responsible of cystic fibrosis, sickle cell anemia, hemophilia, Duchenne Muscular Dystrophy (DMD) has been reported and remains the only curing opportunity for these diseases.[7-10] In the case of DMD, one of the goals is to deliver a gene encoding a functional version of the dystrophin in skeletal muscle (and, ultimately, cardiac muscle and diaphragm).[11,12] The skeletal muscle transfection with naked

plasmid DNA encoding the dystrophin gene is highly inefficient and does not result in significant dystrophin protein expression.[13] One other strategy is to edit the mutated dystrophin gene by transfection of plasmid DNA encoding CRISPR-cas9.[14] In both cases, the most critical step is the transfection efficiency of skeletal muscle cells, which requires the use of the appropriate vector or device to introduce the genetic material into the cells.

For this purpose, viral[15]and chemical[16]vectors have been developed. Current approaches make use of adeno-associated viruses (AAV)[17,18]to package the transgene, allowing efficienthost cells transfection. However, the main drawbacks are the immunogenicity, cytotoxicity, no specificity of transgene delivery, insertion mutagenesis and transgene size limitation of using viruses, even if progress in the improvement of viral vectors are performed.[15]

Chemical vectors attracted much attention and a rich library of polymers or lipids with various design strategies is proposed.[19-29]Most of the investigated carriers present positive charges,[30-34]favoring the DNA condensation and its cellular uptake. Unfortunately, transfection efficiency in skeletal muscle is much lower with cationic vectors than naked DNA when intramuscularly injected.[35]

Amphiphilic non-ionic block copolymers provided then an interesting alternative to promote significant gene transfer to skeletal and cardiac muscles.[36]The poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-*b*-PPO-*b*-PEO) family (Pluronics®, Poloxamers®, Lutrol®) increased the transfection efficiency of plasmid DNA (pDNA), administered intramuscularly.[36-42]Modifying molecular weight, EO/PO ratio and hydrophilic/lipophilic balance of the Pluronics® (L64, P85, P105) changed the levels of *in vivo* transgene expression. PEO was recently challenged in biological applications and its replacement

by poly(2-methyl-2-oxazoline) derivatives was suggested.[43]Poly(2-methyl-2-oxazoline)-*b*-poly(propylene oxide)-*b*-poly(2-methyl-2-oxazoline) (pMeOx-*b*-PPO-*b*-pMeOx) enhanced the luciferase activity by 20-times when co-administrated with pDNA compared to naked pDNA.[44]Replacing the PPO block of Pluronics® by a polytetrahydrofuran (pTHF) block reduced the toxicity.[45]Cheradame*et al.* synthesized triblock copolymers pMeOx-*b*-pTHF-*b*-pMeOx with a short hydrophobic block and suggested their potential for pDNA transfection.[46,47]*In vitro* experiments were conducted, and the poor transfection efficiencywere explained by the absence of interaction between DNA and the block copolymer. Other non-ionic amphiphilic block copolymers were developed: reverse Pluronics® PPO-*b*-PEO-*b*-PPO 25R2 was able to increase muscle transfection in a similar manner than P105[48]and a combination between poly(ethylene glycol)-*b*-poly(lactic glycolic acid)-*b*-poly(ethylene glycol) (PEO_{13}-*b*-$PLGA_{10}$-*b*-PEO_{13}) and plasmid DNA provides the potential to improve gene delivery efficiency in rat skeletal muscles.[49]

The mechanism(s) involved in the transfection efficiency of non-ionic amphiphilic copolymers is still unclear and deserves more investigations. Most of the Pluronics® do not interact with pDNA and do not protect them against DNase degradation.[50]The polymer concentration has to be tuned to transfect the targeted striated muscles (skeletal *vs.* cardiac).[50]The chemical composition and structure of the hydrophilic blocks can be modified without any alteration of the transfection efficiency *in vivo*.[44,49]The effect of the amphiphilic copolymers organization (self-assembly, micelles *vs.*unimers), and their physicochemical features (critical micellar concentration and temperature) on the transfection efficiency have to be considered.[40,41]The ability of the Pluronics® to activate *in vivo* signaling pathways involving NF-κB was proposed as a possible explanation.[51,52]The improvement of DNA diffusion in the muscle tissue by using

Pluronics® was also proposed.[38,41]Recent works stated that *in vivo*,Lutrol® directly delivered pDNA in the cell cytoplasm via a non-endocytosis process.[37]Sahay *et al.* ascertained that the internalization pathway of Pluronic® P85 in mammalian cells happens through endocytosis: unimers are internalized through caveolae-mediated endocytosis, while micelles (and their payload) are internalized through clathrin-mediated endocytosis.[53]Another assumption to explain the transfection efficiency is based on the ability of amphiphilic copolymers to span membrane lipid bilayers and to form transient pores.[54-62]

In this paper, different triblock copolymers (TBCP) of pMeOx-*b*-pTHF-*b*-pMeOx were synthesized comprising a hydrophobic block length fitting the thickness of a lipid membrane model ($\bar{X}n$(pTHF)=10). Their properties were investigated in terms of *in vivo* transfection and temperature-dependent self-assembly. The interactions between copolymers and lipid membrane model were characterized by fluorescence and electrophysiological experiments. Moreover, the translocation of pDNA through a lipid bilayer model in presence of copolymers was studied. We discussed on a relationship between the copolymers behaviors in the absence and presence of a lipid membrane and their *in vivo* biological activities.

2. Experimental Section

2.1.Reagents

All reagents were purchased from Sigma (St. Quentin Fallavier, France) unless otherwise stated. Tetrahydrofuran (THF) was purified by distillation from sodium, under reflux, in the presence of benzophenone, until a persistent blue color appears. 2-Methyl-2-oxazoline (MeOx) was purified by refluxing over calcium hydride, under nitrogen, and distilled prior use. Acetonitrile (ACN) and dichloromethane (CH_2Cl_2) were dried by refluxing over CaH_2 under

$N_2$and subsequently distilled prior use. Deionized water was obtained with a Millipore Milli-Q system. Lutrol®, provided by BASF, consists in a central PPO block of 30 propylene oxide units located between two poly(ethylene oxide) blocks of 75 monomer units. L-α-phosphatidylcholine from egg yolk (EYPC) was purchased from Avanti Polar Lipids, Inc. and used as received. 5(6)-Carboxyfluorescein and Triton© X-100 (molecular biology grade) were purchased from Sigma-Aldrich.

2.2.Synthesis of the triblock copolymers TPCBs

The syntheses of the amphiphilictriblock ABA copolymers (pMeOx-*b*-pTHF-*b*-pMeOx) were performed by sequential cationic ring-opening polymerizations of tetrahydrofuran (THF) and 2-methyl-2-oxazoline (MeOx).[61,63-65]Typically, 5.8 mmol of trifluoromethanesulfonic anhydride (Tf_2O), solubilized in 10 mL of dry CH_2Cl_2, was added at -9°C to 84 mmol of dry THF. After 15 min, the polymerization was quenched by adding 19 mmol of MeOx at -9°C. Residual THF was evaporated under reduced pressure. The resulting α,ω-dioxazolinium-pTHF polymer was dissolved in 40 mL of dry acetonitrile. Then, 88 mmol of MeOx was added at 80°C. After 3 h, the reaction was quenchedby adding 4 mL of 1M Na_2CO_3. The reaction medium was stirred for another 1 h at room temperature. The triblock copolymer was obtained by chloroform extraction, evaporation of the organic phase and drying for 2 days under vacuum. The molar mass of the two blocks was adjusted with the polymerization times.

2.3.Characterization of the triblock copolymers TBCPs in solution

The molar mass of the triblock copolymers was determined by ^{1}H NMR analysis at 300 MHz in $CDCl_3$ at room temperature on a BrukerAvance AM300 spectrometer. The molar mass and dispersity (Đ) were determined by size exclusion chromatography (SEC) at 25°C with $CHCl_3$ as

eluent (1 $mL.min^{-1}$) and a copolymer concentration of 5 $mg.mL^{-1}$. The columns were a PSS GRAM 10^3 Å and a PSS GRAM 30 Å. The SEC was calibrated using PS standards.

Dynamic light scattering (DLS) measurements were performed by using a Zetasizer Nano Series ZS (Malvern) equipped with a laser He−Ne 4 mW operating at 633 nm. The data were acquired by using a BI-9000AT digital correlator fitted with the instrument. The autocorrelation functions were measured at a 173° backscattering angle and analyzed by the constrained regularized CONTIN method to obtain distributions of decay rates (Γ). The decay rates afford the determination of the distributions of apparent diffusion coefficients and the apparent hydrodynamic radii.[61]TBCPs were directly dissolved in deionized water. The solutions were filtered through Millipore Millex filters (0.45 μm pore size) and kept overnight. Critical Micellar Concentrations (CMC) were determined by plotting the hydrodynamic radius of the copolymers measured by DLS as a function of their concentration and have already been discussed.[61]To determine the possible Critical Micellar Temperature (CMT), the hydrodynamic radii of the triblock copolymers were plotted as a function of temperature, from 10°C to 70°C, at different concentrations of 0.1, 1 and 10 $mg.mL^{-1}$ in deionized water.

The CMC were also determined by fluorescence spectroscopy using Nile Red as fluorescent probe.[61]TBCP were dissolved directly in deionized waterat increasing concentrations. Then, Nile Red was added at a final concentration of 2.5 $\mu mol.L^{-1}$, and the fluorescence intensity was measured at 630 nm upon excitation at 530 nm by usinga Shimadzu RF-5000 spectrofluorimeter. The maximum emission intensity at 630 nm was plotted as a function of TBCP concentrations.

2.4.Carboxyfluorescein leakage assay

A 10 mg.mL^{-1}carboxyfluorescein solution was prepared with Tris© buffer solution (50 mM, pH 8.5); the pH of the solution was adjusted to pH 7.4 with a 0.5 mol.L^{-1}HCl solution. To make a vesicle sample, 1.0 mL of the carboxyfluorescein solution was added to 30 mg EYPC in a vial. The lipid-carboxyfluorescein mixture was subjected to vortex mixing during 5 minutes. Then, unilamellar liposomes were prepared according to MacDonald *et al.*[66]The solution was extruded through a WhatmanNucleopore polycarbonate membrane filter (19 mm diameter, 100 nm pore diameter) mounted in the LiposoFast Basic extruder apparatus (Avestin, Inc., Canada). The sample was subjected to 51-passages through the filter at room temperature.[67]An odd number of passages was performed to avoid contamination of the sample by large and multilamellar vesicles, which might have not passed through the filter. Dynamic light scattering (DLS) confirmed the narrow size distribution and determined a 120 nm diameter size of the resulting vesicles. Freecarboxyfluorescein was removed by gel filtration on a Sephadex G-25 column. The final phospholipid concentration was adjusted to 0.75 mg.mL^{-1}with Tris buffer, pH 7.4, which has been determined as the best concentration for the subsequent kinetic release experiments. The vesicle solution was kept in a sealed glass vial in the dark at 4 °C. No further treatment was applied prior to use.

Dye release from phospholipid vesicles was determined as follows: 100 µL of 50 mMTris buffer, pH 7.4 containing TBCP was injected in 2.5 mL of the vesicle solution in a quartz cuvette to reach the final concentration of 0.77 mg.mL^{-1}. This value is not too far from the copolymer concentration giving the highest *in vivo* transfection and the release time is compatible with the experiment treatment. After homogenization, the fluorescence was measured by using aVarian Cary Eclipse fluorescence spectrophotometer with excitation at 490 nm and emission at 520

nm.[68]The excitation and emission band widths were 3 and 5 nm, respectively. The maximum fluorescence intensity was determined by adding 0.1 mL of a 1 % Triton X-100 solution.

2.5. Black Lipid Membrane Experiments

Interactions of TBCPs with a lipid membrane modelmade of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Inc.) were studied according to the 'Black Lipid Membrane' (BLM) technique.[57]The electrolyte used was a 1M KCl (the measured intensity is related to the salt concentration, and this salt concentration is required to get enough information in the data analysis step), 5mM HEPES, buffer pH 7.4. The BLM technique consists in measuring the current between two Ag-AgCl electrodes, each being dipped into one of the two chambers of the measurement device connected by a 150 μm diameter hole, on which is painted the lipid bilayer from a decane solution (10 mg/mL)(Warner Instruments, Hamden, CT).The capacitances of the membranes were above 50 pF, to ensure the formation of a membrane bilayer. Ionic current measurements were conducted to assess the formation of the bilayer and then to characterize the interactions of TBCPs with the membrane. Increasing concentrations of the copolymer was added to the*cis* compartment only. All the presented BLM results were obtained at least for two different experiments.

For the data acquisition, the ionic current through the membrane was measured by using a BLM 120 amplifier (Biologic, Axon Instruments, Sunnyvale, CA, USA), the sampling frequency being 1500 Hz and the filter frequency 300 Hz.

Ionic current measurements at 37°C were performed by using a dedicated set-up. The volume of the cell measurement was about 100 μL. The Teflon cell, embedded in a copper holder, was

thermalized at a temperature varying from 5 to 70 ± 0.1°C using a Peltier module connected to a temperature controller (Newport, Irvine, CA).

2.6.Translocation of plasmid DNA through a model lipid bilayer

After formation of the lipid bilayer, TBCP was added in the *cis* chamber at a concentration of 10 µg.mL^{-1} and ionic current measurements were then recorded for a voltage of -100 mV. Once the first interactions between TBCP and the lipid membrane were observed, the pGL3-ctrl (Promega Madison, WI, USA; 5256 base pairs) plasmid DNA (pDNA) was added in the *trans* chamber at a concentration of about 160 µg.mL^{-1}. The ionic current was then applied again with a voltage of -100 mV. After 35 min, the solutions in *cis* and *trans* chambers were carefully collected to avoid any disruption of the membrane. The quantity of pDNA in each chamber was finally determined by using the QuantiTect SYBR Green qPCR kit (Qiagen, Chatsworth, CA, USA) in accordance with the manufacturers' recommendations, as previously reported.[57]

2.7.Intramuscular gene transfer

Transfection efficiency was measured by analyzing the luciferase activity in the injected tibialis anterior muscles. Plasmid pGWIZ-Luc (Gelantis) encoding luciferase under the CMV promoter was amplified in E. coli and purified by using Qiagen Endo free kits (Qiagen).

Eight weeks old female Swiss mice were purchased from Janvier (Le Genest Saint Isle, France) and were housed in conventional conditions according to INSERM (Institut National de la Santé et de la RechercheMédicale) guidelines. All animal experiments were performed in accordance with the recommendations of French Ministry of Higher Education and Research, and approved by an ethics committee on animal experimentation under reference APAFIS#7897

and 4118. Mice were anesthetized and injected in each shaved tibialis anterior muscle with 50µl of DNA/amphiphilictriblock copolymer formulations prepared in Tyrode. Seven days after injection muscles were collected, homogenized in 1 mL of reporter lysis buffer (Promega), supplemented with protease cocktail inhibitor (Roche). Luciferase expression was measured in the supernatant, in duplicate and expressed as counts per second (CPS) in the whole muscle.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism Software (L Jolla, CA, USA). Values are expressed as the mean +/SEM. Significant differences between groups are indicated *<p0.05, **p<0.01, *** p<0.001 by multiple t tests.

3. Results

3.1. Synthesis and characterization of pMeOx-*b*-pTHF-*b*-pMeOx (TBCPs).

Three amphiphilictriblock ABA copolymers, composed of poly(2-methyl-2-oxazoline) (pMeOx) as hydrophilic block A and polytetrahydrofuran (pTHF) as hydrophobic block B, were synthesized by sequential cationic ring-opening polymerization of tetrahydrofuran then 2-methyl-2-oxazoline (Figure 1).[61]

The experimental conditions used for the synthesis are reported in Table S1. TBCPs were analyzed by SEC and ^{1}H NMR to confirm their polymeric structure and access to their molar mass (Figures S2-S3). The dispersity of the copolymers is higher than expected due to the reversible polymerization of THF. As previously reported, ^{1}H NMR provided molar mass more

reliable for this block copolymer family, and will be used for the discussion.[61]The low $\bar{X}n$ of the pTHF block led to a contour length of the hydrophobic block close to the thickness of the hydrophobic part of lipid bilayers and cell membranes. The molar mass of the pMeOx blocks was varied to modify the Hydrophilic-Lipophilic Balance (HLB) of the block copolymers. TBCP8 has a high HLB with a relatively high molar mass. TBCP1 and TBCP3 have approximately the same HLB, with slightly different block molar masses.

The critical micellar concentrations (CMC) were determined by dynamic light scattering (DLS) and fluorescence spectroscopy of copolymers solubilized in deionized water.[61]Table 1 gathers the molecular parameters of TBCPs and their main important physicochemical properties.

3.2.*In vivo* transfection.

The ability of TBCPs to improve pDNA transfection in the skeletal muscles of mice was evaluated and compared to naked DNA and Lutrol® at 30 $mg.mL^{-1}$, the optimized concentration for this copolymer.[36]Ten µg of pDNA encoding the luciferase gene were mixed with various concentrations of TBCPs. The mixtures were injected into shaved tibialis anterior muscles of Swiss mice. Seven days after injection, the muscles were harvested and the luciferase activity was measured (Figure 2). The Lutrol® concentration was set at a value giving the best gene transfer results.[36]During this short period of time, there was no modification of the body weight of the injected mice.

TBCP3 of intermediate HLB and number of THF units in the hydrophobic block was selected for concentration dependence study. At 10 $mg.mL^{-1}$, the luciferase activity was comparable to the one of naked DNA. Luciferase activity in harvested muscles transfected with 1 $mg.mL^{-1}$ of

TBCP3 was of the same order than the one measured with Lutrol® at its optimal concentration of 30 mg.mL^{-1}. At 0.1 mg.mL^{-1}, the luciferase activity was 10-fold and 2.5-fold higher than the one of naked DNA and Lutrol®, respectively. Transfection efficiency increased when TBCP3 concentration decreased. However, no effect on transfection was observed for 0.01 mg.mL^{-1}.

The three TBCPs were then used at 1 mg.mL^{-1} to study the effect of the N_{THF}/N_{MeOx} ratio on pDNA transfection, for a given molar mass of pTHF. TBCP8 lead to luciferase activity 4-fold higher than naked pDNA, and was comparable to the one of Lutrol® at 30 mg.mL^{-1}. The luciferase activity of pDNA with 1 mg.mL^{-1} of TBCP3 was of the same order than the one measured with TBCP8. However, the luciferase activity with TBCP1 was 8-fold higher than the one of naked DNA and 2-fold higher than the one of Lutrol® 30 mg.mL^{-1}. Lower TBCP1 concentration did not improve the transfection efficiency.

Overall, co-administration of pDNA with TBCPs, especially TBCP3 provided enhanced gene expression in skeletal muscle as obtained with pDNAand Lutrol® but at lower concentration for TBCP(1 mg.mL^{-1}*vs.* 30 mg.mL^{-1}).

3.3. Self-aggregation of TBCP as a function of temperature.

During the intramuscular injection, the TBCP undergoes a temperature elevation, from 20°C to 37°C that could modify itsphysicochemical properties. The aggregation of TBCP3 was first studied by DLS measurements. Figure 3 reports the hydrodynamic radius of TBCP3 at three different concentrations in deionized water as a function of temperature.

At 0.1mg.mL^{-1}, the hydrodynamic radius of TBCP3 was close to 2.5 nm at room temperature, witnessing the presence of unimers only. The temperature elevation triggered a significant

enhancement of the hydrodynamic radius due to the aggregation of TBCP3 into micelles. Since pMeOx is soluble in water whatever the temperature, we suggest a dehydration of the pTHF block to form the corona of the micelle.[50]The transition temperature occurred in the 25-50°C range, which was too large to suggest a clear critical micellar temperature (CMT). At 1 $mg.mL^{-1}$, the effect was similar, i.e. the enhancement of the hydrodynamic radius of TBCP3 as a function of temperature. The observed sharp transition suggested a CMT of 25°C at 1 $mg.mL^{-1}$. At 10 $mg.mL^{-1}$, no variation of the hydrodynamic radius was observed, and the 6 nm value suggested the presence of aggregates as the main population, even at room temperature. In the case of TBCP8, no CMT was detected in the range of studied temperatures, at the concentration used for the gene transfer experiments, i.e. 1 $mg.mL^{-1}$. For TBCP1, a sharp increase of the hydrodynamic radius was observed as a function of the temperature, leading to a CMT of 42°C at 10 $mg.mL^{-1}$ (Figure S4). A slight CMT decrease could occur under physiological salt conditions (150 mMNaCl) as reported for PEO-*b*-PPO-*b*-PEOL64,[69]that has a CMT close to 60°C at a 1% concentration, a temperature range much higher than the body temperature (as reported for most pluronic polymers).[70]

3.4.Effect of TBCP on membrane bilayers.

Phospholipid liposomes are useful biomembrane models.[72]Unilamellar EYPC liposomes of ~100 nm were prepared by extrusion through pores in a polycarbonate filter (50 nm) in the presence of carboxyfluorescein in Tris buffer. According to Balgavy*et al.*,[71]EYPC liposomes have a bilayer thickness of 4.2 nm. Carboxyfluorescein (CF) is an anionic dye at pH 7.4 that was widely used to monitor pore formation and membrane stability.[72-74]Gokel*et coll.*[75]have determined that CF is no more than ≈ 10 Å in any dimension according to CPK and computer

models. External carboxyfluorescein was removed by size exclusion chromatography. The concentration of the dye inside the vesicles was high enough to promote self-quenching of the fluorescence. An aqueous solution of the amphiphilic copolymers was added to the liposome suspension to reach a final concentration of 0.77 $mg.mL^{-1}$, a value close to the lowest copolymer concentration used in the gene transfer assay, and providing signals compatible with the timescale of the experiment. The carboxyfluorescein release was monitored as a function of time by fluorescence measurements. When the dye is released from the vesicles and diluted into a much larger extra-vesicular volume, the fluorescence intensity increases and is proportional to the dye concentration (Figure 4).

After completion of the monitoring, the liposomes were lysed by addition of Triton X100 to measure the total fluorescence intensity in order to calculate the percentage of CF release. The CF release curves increased rapidly according to an exponential equation to reach a pseudo-plateau (Figure 4). A time constant was determined for the percent of CF release with a previously described equation using an ordinary least squares method:[75-77]

$$F_{\lambda=520} = F_{\lambda=520}^{t=0} + A \times \left(1 - e^{-t/\tau}\right) + B \times t$$

With $F_{\lambda=520}$ measured fluorescence at 520 nm; $F_{\lambda=520}^{t=0}$ fluorescence at the time of polymer injection; A the size of the exponential component; B the slope of the linear portion of the curve and τ the time constant for the pore activation. We have also chosen an arbitrary time point at 50 min to compare the copolymer CF releases (Table 2).

CF release allowed detecting pore formation or membrane leakage. According to Gokel*et coll.*,[75]the exponential portion of the curve corresponded to the vesicles that empty from a single pore activation. This pore formation was therefore the rate-limiting step, which could be

described by the time constant τ. According to Table 2, the order of transport efficiency is TBCP1 > TBCP3 > TBCP8 at 0.77 $mg.mL^{-1}$. After 50 min, TBCP1 promoted the highest CF release, as well as the highest transfection efficiency. At this concentration, the CF release efficiency induced by the copolymer was in line with the gene transfer results.

The temperature effect on membrane permeation was therefore investigated. To mimic the *in vivo* gene transfection experiments, the TBCP solutions were prepared at 20°C and added to the vesicles at the investigated temperatures. The copolymer undergoes anincreased temperature directly in the presence of the lipid membranes, as it happens after intramuscular injectionof copolymer-pDNA formulations. At 0.77 $mg.mL^{-1}$, TBCP3 exhibiting a sharp CMT was then selected for the study (Figure 5).

The temperature varied from 20°C to 40°C. At the highest temperature, the liposomes remained intact in the time scale of the experiments. The polymer addition in the vesicle solution resulted in a significant increase of the release rates (Table 3). The release was low at 20°C. S-shaped release curves were observed when TBCP3 was added at temperatures higher than the CMT. The increasing temperature decreases the solvent-polymer interactions, favoring the liposome-TBCP3 interactionsinstead of the formation of TBCP3-aggregates in the presence of vesicles (suggested by the variation of CF release as a functionof temperature).

3.5.Ionic current measurements in the presence of TBCPs.

The formation of pores was investigated by 'Black Lipid Membrane' (BLM) measurements.[57] This technique allows the observation of single events occurring in lipid bilayers. A current is measured through a lipid membrane model when a voltage is applied. The lipid membrane is separating two compartments filled with 1 M KCl solutions and is acting as an insulator.

Perturbation of the membrane leads to the appearance of a current witnessing interactions or pore formation. The current measured during the BLM experiments gives information on the size of the pore, by comparison with the current value measured in the presence of α-hemolysin, a biological nanopore used as reference.[78]The internal diameter of α-hemolysin is about 2 nm at 1M KCl, resulting in a current of -100 pA for an applied voltage of -100 mV.

TBCPs alone were first studied with the diphytanoylphosphatidylcholinemodelmembrane, a standard phospholipidfound in the skeletal muscle membrane[57](Figure 6). Auvray*et coll.*[55], reported that Lutrolforms nanopores in such membrane through a carpet mechanism, involving few PEO-*b*-PPO-*b*-PEOmolecules. One recalls that the $\bar{X}n$ of the hydrophobic pTHF block (Table S1) was designed to fit with the thickness of the lipid membrane model (4-6 nm). In order to avoid membrane disruption and to be in a single-molecule scale necessary for this technique, the final concentration of copolymers was set in the range 10-100 µg.mL^{-1}. A much lower concentration was set compared to the one used for *in vivo* transfection in order to isolate the measured events and to avoid any fast lipid membrane disruption.

Figure 6-A represents a typical ionic current trace, current plotted as a function of time, obtained in presence of TBCP3 (10 µg.mL^{-1}) at -100 mV. TBCP3 allowed an ion flux through the lipid membrane giving a maximal current of ~ -20 pA corresponding to an average pore diameter of 0.4 nm based on the values obtained for α-hemolysin.[78]

Figure 6-B gives the usual data analysis of the ionic current trace, allowing plotting a histogram of current distribution, i.e. occurrence (number of events obtained at this current) as a function of current. On the histogram profile, the population present at 0 pA corresponds to the lipid membrane (insulator). Then, a population of different events, at almost all the currents, was

observed indicating that the structures of permeation are not well-defined, as already mentioned by using amphiphilic copolymers in presence of lipid membrane.[55,57,58]

The BLM data measured in presence of TBCP8 and TBCP1 were similar at the same concentration. The three TBCPs thus induce lipid membrane perturbation. By using different copolymer concentrations, it was possible to determine the number of macromolecules involved in the pore formation, thanks to Hill equation.[79]

$$\text{Log } Y = n \log C_M - n \log K_D$$

with Y: fractional activity (or P_0)

C_M: macromolecule concentration,

n: Hill coefficient,

K_D: dissociation constant of the supramacromolecules.

P_0 is the pore opening probability, defined as the ratio between time of interactions and time of acquisition. The Hill coefficient n obtained from the curve $\log(P_0)$ versus concentration corresponds to the number of macromolecules involved in the pore formation. n equal to 1 corresponds to a monomolecular channel and $n>1$ corresponds to a multimolecular channel.

The Hill equation is commonly used for biological pores that have a definite structure. Modifying the concentration and determining the opening probability versus the concentration of TBCPs was performed (Figure S5). Only one macromolecule of each TBCP seemed to be necessary to let ions passing through the lipid membrane, which was consistent with the unimer phase of the copolymers at this concentration.

3.6. TBCP-assisted pDNA translocation through lipid bilayers.

The 'BLM' results demonstrated a TBCP-assisted lipid membrane permeation allowing the translocation of ions through pore of diameter around 0.4 nm. The question whether macromolecules, especially DNA, could also pass through pores thanks to TBCPs is of main interest for transfection application. Another question concerned the effect of the temperature on the TBCP3-mediated translocation.

BLM experiments in the presence of a well-defined DNA i.e. plasmid DNA of 9680 kbp and TBCPs were performed. First, we demonstrated that no pDNA translocation through this lipid bilayer was observed in our experimentalconditions. Next, TBCP was first added at a final concentration of 10 $\mu g.mL^{-1}$ in the *cis* chamber to allow its interaction with the membrane under -100 mV. Then, pDNA at the final concentration of ~ 160 $\mu g.mL^{-1}$ was added in the *trans* chamber and a voltage of -100 mV was appliedagain. This allowed continuing to study the interactions of the polymer with the membrane, while promoting DNA translocation from one chamber to the other according to the direction of the applied voltage. The current, representing the interactions between the polymer and the lipid membrane, was recorded during 35 min, in a way to observe the pore formation. Then, the solutions in the *cis* and *trans* chambers were carefully collected to avoid the membrane disruption, and the amount of pDNA in both chambers was quantified by qPCR. It is noteworthy that the concentration of pDNA in *trans* was intentionally high to have a better chance to observe its translocation.

Table 4 summarizes the BLM experiments and the quantification of pDNA in each chamber after 35 min. Concerning the BLM data, Figure S6-A represents the current *versus* time during

the translocation experiments and Figure S6-B represents the analysis of the observed events, for TBCP8, at room temperature. Interactions with important current jumps (-100 pA for an applied voltage of -100 mV) occurred (Figure S6-A). The all-points histogram (occurrence *versus* current) allowed demonstrating that a large range of current values is measured at the beginning of the acquisition (Figure S6-B, logarithmic scale), meaning that interactions were not well-defined. However, a long-lasting porewith a current jump of approximately -2 pA for an applied voltage of -100 mV was obtained at the end of the acquisition (1500s) (visible on Figures S6-B and S6-C).

In the presence TBCP1 at 25°C, interactions with important current (-100 pA at -100 mV) were also observed (Figure S7-A). However, well-defined discrete current jumps were observedfor TBCP1 on the histogram of events varying from -2 pA (Figure S7-C, linear scale) to -20 pA (Figure S7-B, logarithmic scale). Black arrows on the figures highlight their multiples (Figure S7-B). Such discrete current signals were not observed for TBCP8.

For TBCP3, interactions with important current jumps (-100 pA at -100 mV) occurred at 25°C (Figure S8-A) with also large current distribution (Figure S8-B). Same type of events were observed at 37°C, except that the current jumps were much higher (-400 pA at -100 mV) (Figure S9-A). Experiments with TBCP1 at 1 $mg.mL^{-1}$ were not performed because its mild critical temperature transition occurring at 42°C caused thermal instability of the lipid bilayer leading to disruption.

qPCR analysis demonstrated that translocation of pDNA occurred in presence of TBCPs; the highest translocation efficiency being reached with TBCP3 at 37°C. It is important to note that in each case, pDNA was not detected in the *cis* chamber when no copolymer was added in the *trans*

chamber thus validating the role of TBCPs in pDNA translocation through the lipid membrane model.

4. Discussion

Three TBCPs were synthesized and their capacity to increase the transfection efficiency of pDNA encoding the luciferase gene in mice skeletal muscles was tested upon intramuscular injection in the tibialis anterior muscles of Swiss mice. These triblock copolymers were found to enhance the transfection efficiency as determined through luciferase activity compared to naked pDNA, whatever their molar mass or theirHLB.This category of *in vivo* synthetic delivery vectors consisting of amphiphilic block copolymers of triblock copolymers including different compositions of the hydrophilic and hydrophobic blocks, has been widely used for efficient and safe *in vivo* delivery of DNA. Indeed, formulations composed of pDNA and triblock copolymer have been described to transfect skeletal and cardiac muscles[80-83]. Ability of those block copolymers to deliver DNA in muscles for expression of gene of therapeutic interest has been demonstrated in mouse model of hepatocellular carcinoma[84], of allergic asthma[85] or of colorectal cancer[86]. Importantly, results of all of those studies show that amphiphilic block copolymer/DNA formulations were well tolerated and did not induce mortality nor toxicity when compared to control animals injected with saline solution**.**

The copolymers with lower molar mass (TBCP3 at 0.1 $mg.mL^{-1}$ and TBCP1 at 1 $mg.mL^{-1}$) offered better transfection efficiencies than Lutrol® at 30 $mg.mL^{-1}$ selected as the copolymer reference. TBCP8 at 1 $mg.mL^{-1}$ gave a transfection efficacy comparable to Lutrol®.

The good transfection efficiency is correlated to the presence of TBCP unimers at room temperatureat the concentration providing the best transfections. These results are in line with those reported for Pluronic® L64.[40]The authors showed that the conditions favoring interactions between unimers (medium, temperature) displayed maximal efficiency *in vivo* after intramuscular injection in normal and dystrophic muscles.[40]At 37°C, the temperature of the animal body, we demonstrate that TBCP8 and TBCP3 were in a micellar stateat 1 $mg.mL^{-1}$, whereas TBCP1 was in an unimer state. However, TBCP3 undergoes a mild transition from unimer to micelles at 0.1 $mg.mL^{-1}$in the range of 27 to 45 °C. Thus, the good transfection efficiency is likely ascribed to the TBCP3 concentration at which a CMT close to 37 °C is observed. In this assumption, TBCP3 undergoes a phase transition during intramuscular injection, and the water depletion from the hydrophobic pTHFblock may force the copolymer to interact with the cell membrane.

TBCPs interact with lipid membrane (Figure 6). Indeed, they provided CF release from liposomes with efficacy at 0.77 $mg.mL^{-1}$ and at room temperature in the following order TBCP1 > TBCP3 > TBCP8; TBCP1 providing the highest release after 50 minutes. This result was in line with the observed gene transfer efficiency. BLM revealed that the active concentration of the copolymers was in the range of 10-100 $\mu g.mL^{-1}$, lower than the one used for fluorescence experiments and *in vivo* assays. TBCPs destabilize the lipid membrane but the permeation mechanism is not well-defined, as already mentioned for other amphiphilic copolymers.[55,57,58]The Hill plot indicated that only one macromolecule can induce the pore formation, which is in line with their unimer phase at this concentration. This copolymer insertion in the lipid membrane is favored by the good fit between the hydrophobic contour length of the pTHF block and the hydrophobic thickness of the lipid bilayer. Small Angle X-Ray

Scattering showed that when the hydrophobic PPO block length of Pluronics® is lower than the thickness of dimyristoyl-sn-glycero-3-phosphocholine or DMPC bilayer, few amounts of copolymer are integrated in the bilayer and the PEO blocks are oriented laterally to the membrane surface, which may seal defects on the surface of the membrane.[87]When the PPO block and the bilayer thickness have the same size, the copolymer is well anchored in the membrane and the hydrophilic PEO blocks is perpendicular to the surface of the membrane. Two processes are discussed for the pore formation of Pluronic® L64.[55]The barrel stave mechanism was comparable to biological channel permeation structures, where pores form a defined structure like α-hemolysin for instance.[78]This mechanism could occur with block copolymers when the hydrophobic length block is fully extended in the lipid bilayers, and then stabilizes the inner surface of the resulting pore. The carpet mechanism corresponds to a partial insertion of the block copolymer in the lipid membrane. The adsorbed polymer induces a local change of the lipid membrane curvature forming a transient hole in the bilayer.

qPCR analyses validate the pore-assisted pDNA penetration through the membrane via TBCP insertion in the membrane. At the concentration used in the BLM experiments and at 25°C, the three TBCPs are in the unimer phase and the translocation (although significant) is really weak. Higher translocation efficiency is observed with TBCP3 at 37°C, the polymer that actually allows the best transfection efficiency in our experiments carried out in mice. It is thus noteworthy that the highest I_{mean} measured for TBCP3 at 37°C corresponds to a higher pore diameter and correlates with the highest translocation efficiency (Table 4). BLM, under experimental settings close to physiological conditions can be an interesting technique to understand phenomena occurring *in vivo*. Before injection, in the mixture containing pDNA, TBCP3 exhibits a unimer phase at 25°C. After intramuscular injection, it is organized in micelles

at 37°C and/or better interacts with cell membranes. The temperature-dependent self-assembly of TBCPs thus is likely an essential parameter for DNA translocation through cell membrane providing high transfection efficiency.

5. Conclusions

The present work relies on the design of new competitive amphiphilictriblock copolymers of pMeOx-*b*-pTHF-*b*-pMeOxstructure that improve gene delivery in skeletal muscles. Several lines of evidences indicate that the temperature elevation during intramuscular injection is crucial for their positive impact on the transfection efficiency. The best TBCP undergoes a phase transition at temperature between 25-40°C and physicochemical studies support the insertion of TBCP in lipid bilayer allowing DNA translocation. A CMT between 25-40°C appears to be one of the driving forces that helps single molecule of copolymer to interact with the membrane of muscle cells after intramuscular injection and induces enough membrane destabilization to improve pDNAtranslocation in the cells. Altogether, our results highlight the interest of pMeOx-*b*-pTHF-*b*-pMeOxblock copolymers exhibiting a phase transition around the body temperature to improve gene delivery in skeletal muscle cells for gene therapy and vaccine applications.

FIGURES.

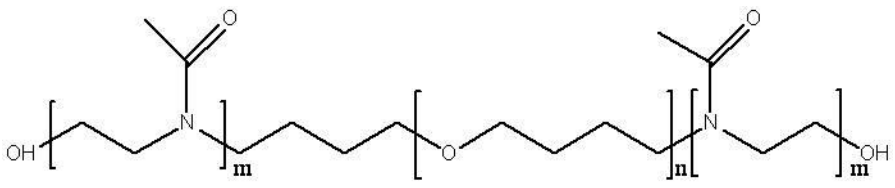

Figure 1. Chemical structures of amphiphilictriblock copolymers pMeOx-*b*-pTHF-*b*-pMeOx (TBCP).

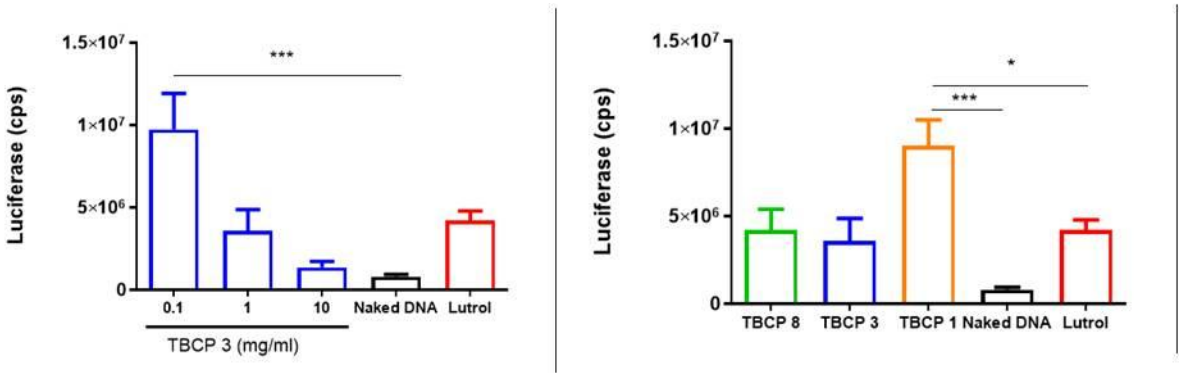

Figure 2.*In vivo* luciferase expression (cps/ muscle) in the mouse tibialis anterior muscle. (A) Luciferase activity was measured after intramuscular injection of 10 µg of DNA associated with 0.1 mg.mL^{-1}, 1mg.mL^{-1} and 10 mg.mL^{-1} of the triblock copolymer TBCP3 and (B) 1 mg.mL^{-1} of TBCP8, TBCP 3 and TBCP1. As controls, 30 mg.mL^{-1} of Lutrol® and naked DNA were used. Luciferase expression was monitored 7 days post-injection. Data were analyzed using multiple t tests *** $p<0.001$, * $p<0.05$

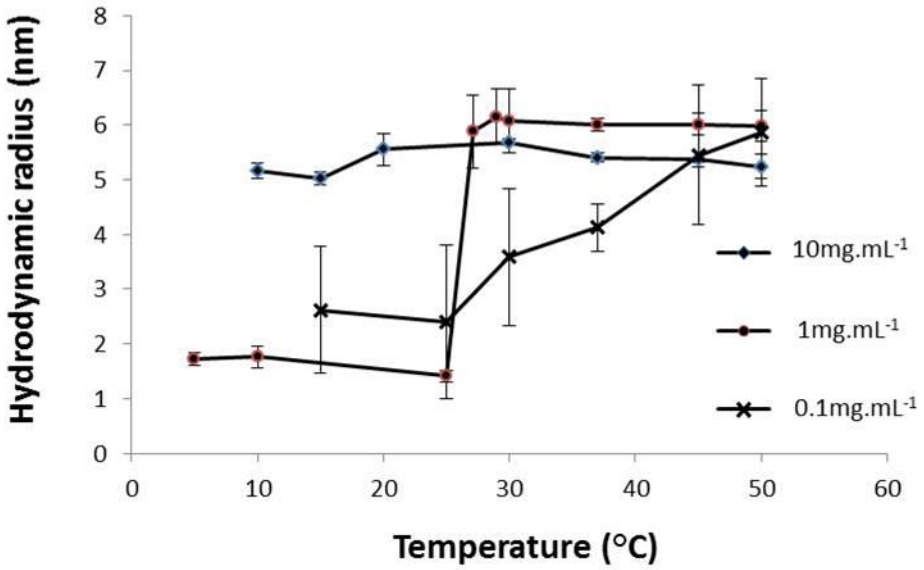

Figure 3. Hydrodynamic radius of TBCP3 in deionized water as a function of temperature and concentration.

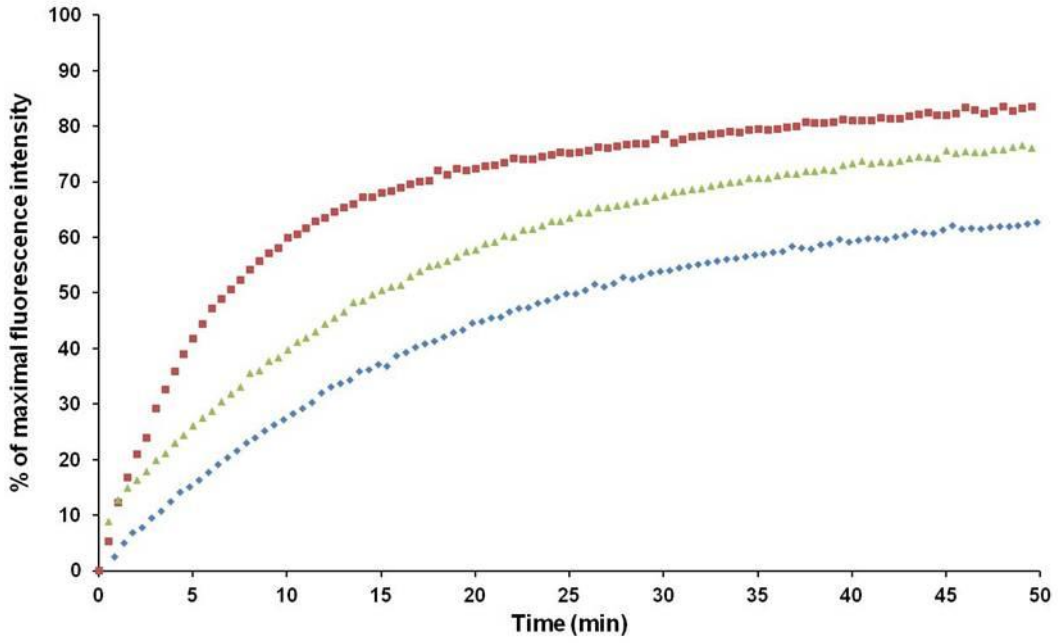

Figure 4. Carboxyfluorescein release from lipid vesicles at 20°C in the presence of TBCP8 (blue), TBCP1 (red) or TBCP3 (green). The copolymers were added to the vesicles solution, at a final concentration of 0.77 $mg.mL^{-1}$, followed by a stirring of 2 seconds before fluorescence measurements.

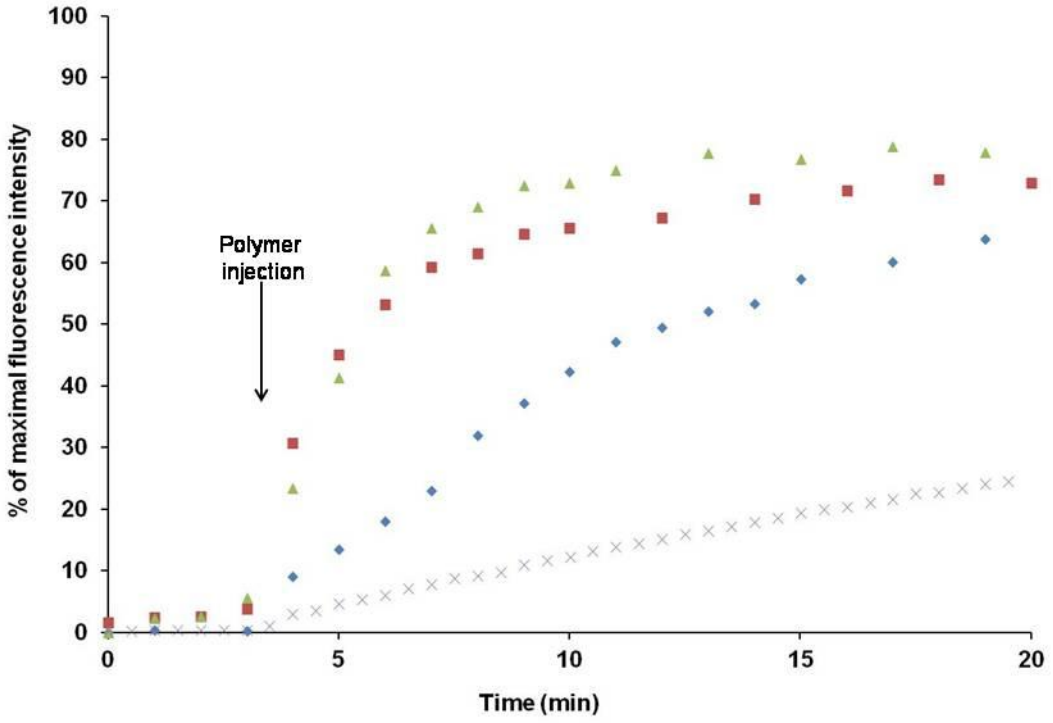

Figure 5. Carboxyfluorescein release from lipid vesicles in the presence of TBCP3 at (grey) 20°C, (blue) 30°C, (red) 35°C and (green) 40°C. The copolymers were added at 0.77 $mg.mL^{-1}$to the vesicles solution without any stirring.

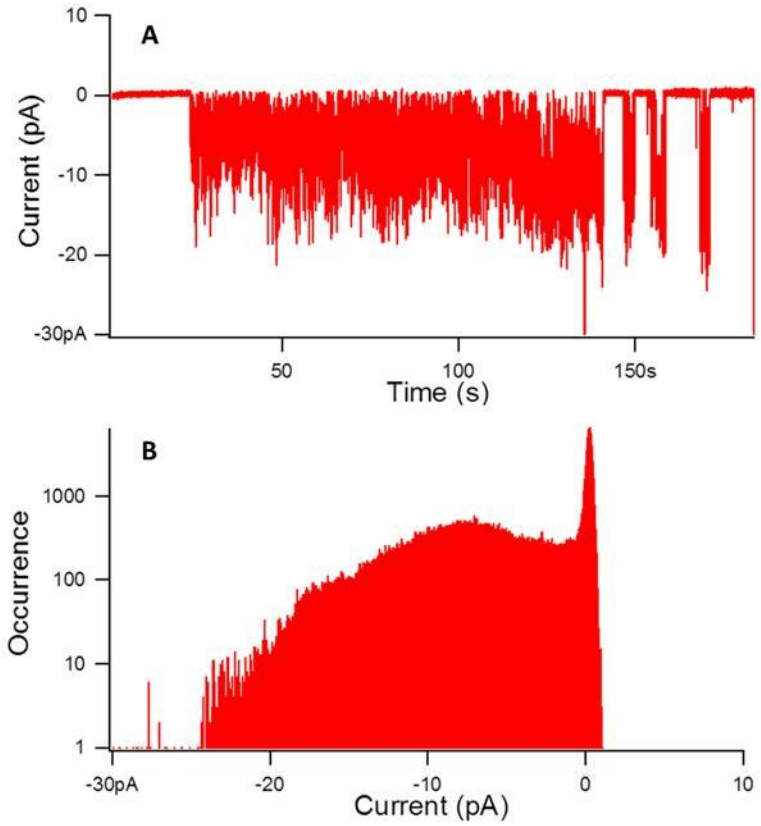

Figure 6. BLM results for TBCP3 at 10 μg.mL^{-1}, for an applied voltage of -100 mV, in 1 M KCl, 5 mM HEPES, pH=7.4, at room temperature (**A**) Current as a function of time. (**B**) Occurrence as a function of current (current distribution).

TABLES.

Table 1. Characteristics of pMeOx-*b*-pTHF-*b*-pMeOxamphiphilictriblockcopolymersTBCPs.[61]

TBCP	HLB	N_{THF}	N_{MeOx}	$\overline{Mn}$ (^{1}H NMR) [g.mol^{-1}]	CMC^{a,60} [mol.L^{-1}]	CMC^{a,60} [mg.mL^{-1}]	CMC^{b,60} [mol.L^{-1}]	20°C 1 mg.mL^{-1}
8	18	13	2*48	9200	$1.1.10^{-4}$	1	$2.7.10^{-4}$	Micelles
1	13	7	2*6	1600	$6.3.10^{-3}$	10	$6.7.10^{-3}$	Unimer
3	12	11	2*8	2200	$5.3.10^{-4}$	1.2	$5.3.10^{-4}$	Unimer

[a]In deionized water (DLS)

[b]In deionized water (Fluorescence Spectroscopy)

Table 2. Kinetic constants and percentages of CF released from liposomes, determined from the release experiments of Figure 4.

TBCP	$F_{\lambda=520}^{t=0}$	*A*	*B* [min^{-1}]	τ [min]	% CF release at 50 min
8	109.92	122.28	0.0179	18.47	62.6
1	114.82	133.37	0.5978	5.73	83.5
3	128.56	125.43	0.2602	14.46	75.8

Table 3. Kinetic constants *versus* temperature, determined from the release experiments in presence of TBCP3 (Figure 5).

T (°C)	$F_{\lambda=520}^{t=0}$	*A*	*B* (min^{-1})	τ (min)
20	154.65	150.99	0.065	47.62
30	182.39	126.20	0.313	7.67
35	204.17	94.116	0.365	2.14
40	205.92	102.43	n.d.	2.58

Table 4. BLM Data and qPCR results for the triblock copolymers.

TBCP	T [°C]	P_0 [%]	I_{mean} [pA] -100mV	Pore diameter [nm]	$[ADN]_{trans}$ [ng.µL^{-1}]	$[ADN]_{cis}$ [ng.µL^{-1}]	Translocation Efficiency [%]
8	25	56	-12	0.24	280 ± 31	$4.5.10^{-2}$ $\pm 2.7.10^{-2}$	0.02
1	25	41	-2.5	0.05	71 ± 38	$4.21.10^{-3}$ $\pm 6.96.\ 10^{-4}$	$5.9.10^{-3}$
3	25	88	-5.5	0.11	170 ± 13	10^{-4} $\pm 6.8.10^{-5}$	$5.9.10^{-5}$
3	37	73	-42.3	0.85	90 ± 9	3 ± 0.3	3.3

TBCP concentration in cis: 10 µg.mL^{-1}

T: Working temperature during the translocation experiments

P_0: ratio between time of interactions and time of acquisition

I_{mean}: mean current obtained during the acquisition

Pore diameter: determined by a calculation with the values concerning α-hemolysin

Translocation efficiency: calculated as the ratio between $[DNA]_{cis}$ and$[DNA]_{trans}$

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge : characterization of TBCPs and BLM experiments in presence of DNA and TBCPs are supplied as Supporting Information.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript, and do not have any conflict of interest.

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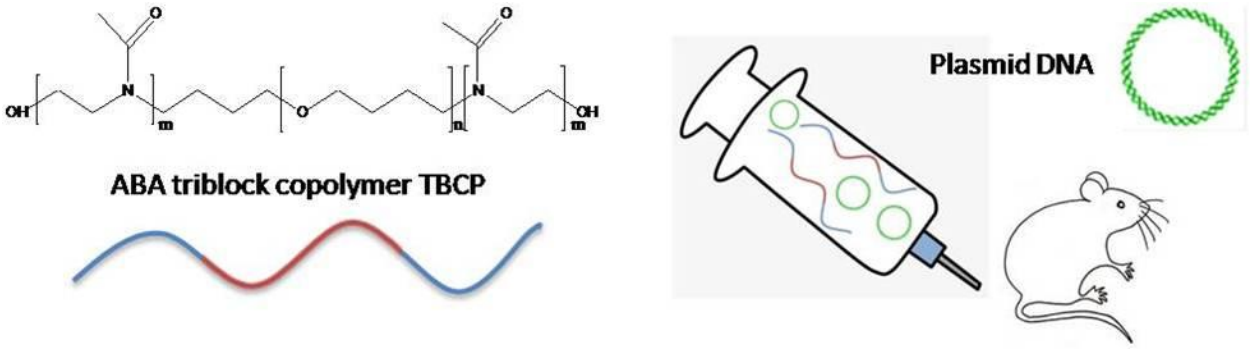
TOC Figure.



TOC Texte :

Amphiphilic neutral block copolymers having a LCST close to 37°C are shown to improve gene transfer via *in vivo* intramuscular administration at low concentration.Physico-chemical

experiments demonstrate that permeation of lipid membrane by this block copolymers is the factor that improves the gene delivery, when temperature is increased from 20°C to 37°C.

KEYWORDS. Amphiphilic copolymers; *in vivo* transfection; LCST; poly(2-methyl-2-oxazoline); skeletal muscle.