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Research Paper

Synthesis and Evaluation of Amphiphilic Poly(tetrahydrofuran-*b*-ethylene oxide) Copolymers for DNA Delivery into Skeletal Muscle

Catherine Pomel,[1] Christian Leborgne,[2] Hervé Cheradame,[1] Daniel Scherman,[2] Antoine Kichler,[2,3] and Philippe Guegan[1,3]

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Purpose. Amphiphilic triblock copolymers such as the pluronic poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide) L64 (pEO_{13}-pPO_{30}-pEO_{13}) have been shown to mediate more efficient gene transfer in muscle as compared to naked DNA. We were interested in studying the effect of a chemical change of the central block of pluronic polymers on the transfection activity.
Methods. We synthesized new amphiphilic copolymers in which the hydrophobic pPO block was replaced by poly(tetrahydrofuran) (pTHF) chains. The resulting triblock pEO–pTHF–pEO polymers have been characterized by NMR and SEC and assayed for *in vitro* and *in vivo* gene transfer.
Results. The animal experiments showed that the new copolymers are able to significantly increase the transfection efficiency of plasmid DNA after intramuscular injection.
Conclusions. These results indicate that the capacity to enhance plasmid DNA transfection in skeletal muscle is not restricted to pEO–pPO–pEO arrangements.

KEY WORDS: amphiphilic copolymer; non-viral gene transfer; poloxamer; polytetrahydrofuran; skeletal muscle.

INTRODUCTION

Gene therapy consists in delivering nucleic acids into cells in order to obtain a therapeutic effect. Such an approach has a great potential for the treatment of genetic and acquired diseases, as well as for the development of new vaccines. One of the major barriers that hinder the development of gene therapy is the lack of efficient and safe delivery methods of the expression vector, which can be plasmid DNA or mRNA. Indeed, since plasmid DNA is a polyanionic macromolecule which crosses membranes with a low efficiency and has a short half life in biological fluids, it requires association with a transporter to enter cells. During the last two decades many nucleic acid delivery systems were developed and tested, including viruses and physical- or chemical-based methods. Among the latter, various compounds such as mono- (1) and poly-cationic lipids (2), linear (3) and branched polymers (4,5), and amphipathic peptides (6) have been synthesized and evaluated. Cationic polymers, as for example the linear polyethylenimine of 22 kDa, have been found to be efficient *in vitro* on many cell lines and, following tail vein injection in mice, significant transgene expression was found in the lung (3). However, none of these compounds is able to promote gene transfer after intra-muscular administration.

Skeletal muscle is an important target for gene therapy approaches since many diseases affect this tissue. In addition, muscle can be used either as bioreactor to produce a secreted therapeutic protein or as production site for antigens for vaccines. Thus, there is a real interest for developing efficient methods for muscle gene transfer.

It is only recently that chemicals were discovered that are able to significantly increase the level of transgene expression in muscle as compared to naked DNA. In particular, Lemieux and colleagues (7) improved transfection by using a mixture of two pEO_k–*block*–pPO_m–*block*–pEO_k copolymers (Pluronic L61 and F127) differing by their molecular weight and their hydrophilic–hydrophobic balance. Following this pioneering work, Pitard *et al.* (8) showed that intramuscular administration of DNA formulated with the non-ionic amphiphilic Pluronic L64 (pEO_{13}–*block*–pPO_{30}–*block*–pEO_{13}) resulted in 35% of fibres expressing the transgene at day 7.

In this context, we synthesized new copolymers characterized by the presence of a central hydrophobic polytetrahydrofuran (pTHF) block instead of the polypropylene block (pPO). As reference for the size of the hydrophilic and hydrophobic blocks of the new polymers, we chose Pluronic L64. The synthesis strategy which was used is based on the previous work made by Goethals and co-workers (9). Briefly, a bifunctional polyTHF was first synthesized, and the living species were then quenched by a telechelic α-hydroxy-ω-methyl-poly(ethylene oxide). The resulting pEO–pTHF–pEO polymers were characterized by NMR and SEC and then assayed for *in vitro* and *in vivo* gene transfer. The animal experiments showed that the new copolymers are able to significantly increase DNA transfection efficiency after intramuscular injection.

[1] Laboratoire Matériaux Polymères aux Interfaces–UMR CNRS 7581, Université d'Evry, 91025 Evry, France.
[2] Genethon–FRE 3087 CNRS, BP60 91002 Evry, France.
[3] To whom correspondence should be addressed. (e-mail: akich@genethon.fr; Philippe.Guegan@chimie.univ-evry.fr)

MATERIALS AND METHODS

Materials. THF (SDS, 99.7%) was purified from sodium-benzophenone ketyl just prior polymerization. Trifluoromethanesulfonic anhydride (triflic anh., Avocado) was cryodistilled and kept in an inert atmosphere before use. α-Hydroxy-ω-methyl-poly(ethylene oxide) (pEO-monoOH; 550 g mol^{-1}) was purchased from Aldrich and the molecular weight was checked by SEC prior use. The polymers were dried under vacuum at room temperature for 10 h (10^{-5} mbar). Toluene (SDS, 99.3%) and CH_2Cl_2 (SDS, 99.95%) were overnight reacted with CaH_2 and distilled prior use. CMV-Luc (7.6 kb) is an expression plasmid encoding the firefly *luciferase* gene under the control of the human cytomegalovirus (CMV) immediate-early promoter.

Polymer characterization. Size exclusion chromatography (SEC) was carried out with a Waters apparatus equipped with a HR5E column (Styragel) with THF as eluant (flow rate, 0.3 ml/min). Detection was ensured by UV (345 nm) and refractometric detectors. A pEO calibration was used to determine the molecular weight of the synthesized copolymers. ^{13}C and ^{1}H NMR measurements were conducted on a Bruker 300 MHz spectrometer in DMSO-d_6 (T=300°K). MALDI-TOF experiments were performed using a Perspective Biosystems Voyager-DE Pro STR time of flight mass spectrometer equipped with a nitrogen laser (=337 nm). External calibration using pEO-monoOH was performed with the same matrix as in the experiments. Data were collected with a laser power set just above the ionization threshold of the matrix to avoid fragmentation and to maximize the resolution. Typically, five acquisitions corresponding to 50 shots were realized for each sample to ensure representative mass spectra. All investigated matrices and analyses were dissolved in THF. The concentration of the polymer solutions was 10^{-3} M. Solutions of matrix had a concentration of 10^{-3} M. For all analyses 20 μl of the sample containing the polymer were added to 20 μl solution of the matrix and 10 μl of the salt solution (sodium acetate in MeOH/H_2O) thoroughly mixed and then centrifuged. About 0.5 μl of the resulting mixture was spotted onto the sample plate and allowed to air dry at room temperature. This method of sample preparation is usually called dried droplet and is easier to process than the sandwich sample preparation method. The method allowed an estimation of the polymerization degrees as a function of the EO and THF units in spite of the complexity of samples.

Synthesis of block copolymer pEO_k–block–$pTHF_m$–block–pEO_k. The triblock copolymers were synthesized using a pressurized nitrogen method. All glass wares were oven dried at 80°C and flame-dried before use. Copolymerizations were carried out as follows. The copolymerization of the pEO_k–*block*–$pTHF_m$–*block*–pEO_k polymer having a molecular weight of 660–2380–660 is given as an example. THF (15.4 g, 213.9 mmol) was first distilled into an addition burette to permit the precise determination of the monomer weight used for the copolymerization. The required amount of pEO-monoOH (7.4 g, 13.5 mmol) was introduced in a second addition burette and dried for 10 h. Purified solvent (5 ml) toluene (or CH_2Cl_2) was then cryodistilled into the burette and complete dissolution of the dried oligomer was allowed. The addition burettes were attached to the baked reactor, which was flamed-dried and stored under dynamic vacuum (10^{-5} mbar) for 1 h. The reactor was then flushed with nitrogen and the temperature was decreased to −9°C. THF was then introduced in the reactor and allowed to reach the desired temperature. The 6.6 mmol of triflic anh. were injected in the reactor through a septum. Polymerization was conducted for 12 min. and the solution of pEO-monoOH was quickly added to the reactive mixture to allow the coupling reaction between the reactive THF and the hydroxy-functionalized pEO-monoOH. Temperature was then increased up to room temperature. Coupling reaction was conducted for 10 min and a NaOH solution was added to quench the reaction and the solution was overnight stirred. The block copolymer was recovered by a water/CH_2Cl_2 extraction.

DNA retardation assay. DNA binding was studied by means of an agarose gel retardation assay. Plasmid DNA (1 μg) and increasing amounts of copolymers were each diluted in 25 μl of 150 mM NaCl and mixed. After 15 min of incubation, samples (20 μl) were electrophoresed through a 1% agarose gel using Tris–borate–EDTA buffer. The DNA was visualised after SYBR Safe (Invitrogen) staining.

Cell culture. Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) was supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% of foetal calf serum (FCS). The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, ATCC) was used for both, the transfection experiments and the MTT assays. C2C12, a subclone of the C2 mouse myoblast cell line, was obtained from ATCC. The cells were routinely propagated in DMEM supplemented with FCS (20%, *v*/*v*), penicillin (100 IU/ml) and streptomycin (100 μg/ml). For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay, 5×10^{e4} cells/well were put into 24-well plates, and differentiation into myotubes was induced for 9 days by using medium containing 5% horse serum.

In vitro transfection experiments. HepG2 cells plated in 24-well plates were transfected once confluency reached 50–80%. DNA complexes were generated as follows: 4 μg of plasmid DNA and increasing amounts of copolymer were each diluted in 100 μl of 150 mM NaCl and gently mixed. After 15 min of incubation the mixture was diluted with serum-free medium to a final volume of 0.8 ml and then 0.4 ml were transferred into each well of the duplicate. After incubation for 3 h at 37°C, the medium was removed and replaced with fresh one containing 10% FCS. Luciferase activity was measured 30 h after transfection as previously described (10).

Cell viability assay. Cytotoxicity was evaluated by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay. Briefly, HepG2 and C2C12 cells in 24-well plates were incubated in serum-free medium with increasing amounts of copolymers with 2 μg of plasmid/well. After about 3 h and 30 min, the cell culture medium was removed and replaced by serum-free DMEM containing 0.5 mg/ml MTT. After incubation at 37°C for 3–4 h, the medium was removed and 200 μl of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable cells. Absorbance was then measured at 570 nm. Untreated cells were used as control (100% viability).

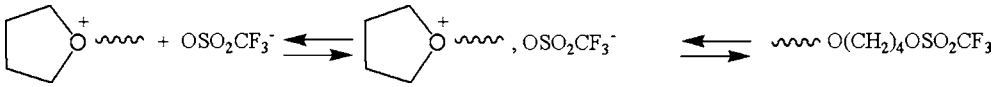

Fig. 1. Equilibrium between the various species.

In vivo experiments. Animal experiments were performed according to institutional guidelines for animal care and use. DNA complexes were formulated in 150 mM NaCl. Plasmid DNA and the polymer were each diluted in 20 µl and then gently mixed. After 15 min of incubation at room temperature, 35 µl of polymer/10 µg plasmid CMV-Luc solution were slowly injected into the tibialis anterior of female Balb/C mice (8 weeks old, Iffa-Credo). Mice were sacrificed 7 days post-administration. For the measurement of luciferase levels in muscle the following protocol was used: 500 µl of lysis buffer containing a cocktail of protease inhibitors (Sigma) were added to the collected organs. Each organ was then homogenized for about 30 s with an Ultra-turrax (Ika) and the homogenate was centrifuged for 10 min at 8,000×*g* at 4°C. A 5-µl aliquot of the supernatant was used for luciferase assay. Luciferase background was subtracted from each value and the transfection efficiency is expressed as total light units/10 s/mg protein and the values are the means of duplicates. Protein content was measured by using the BioRad protein assay (Bradford).

Kinetic of reporter gene expression. The 20-µg CMV-mSeAP plasmid in 35 µl of NaCl at 150 mM formulated or not with a copolymer were injected into the tibialis anterior (n=3). Before injection, at day 3 and at day 7, blood samples were collected and mSeAP was quantified at the end of the experiment. The background level of mSeAP (day 0) was subtracted from each value and the level at day 3 is expressed in percent as compared to day 7 (=100%). Detection of alkaline phosphatase activity was carried out with the Phospha-Light kit (Tropix Perkin-Elmer) following the manufacturer's instructions.

X-Gal staining. Cryosections (8 mm thickness) were prepared from frozen muscles. Muscle sections were fixed in 0.5% glutaraldehyde and incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

RESULTS

Synthesis of Poly(tetrahydrofuran-*b*-ethylene oxide) Block Copolymers

In order to be able to compare the behaviour of the new molecules with that of commercially available compounds, we tried to synthesize triblock copolymers having hydrophilic and hydrophobic blocks of the same size as the Pluronic L64 (pEO_{13}–pPO_{30}–pEO_{13} or expressed in MW for each block: pEO_{572}–pPO_{1740}–pEO_{572}).

THF Polymerization

Polymerization of THF initiated by triflic anhydride was studied in details by Smith and Hubin (11), and the proposed mechanism suggests the occurrence of a sulfonic ester on one side of the growing pTHF chain and a ionic (oxonium) group on the other end of the growing polymer chain (free ion or ion pair; Fig. 1). Buyle *et al.* (12) reported the interconversion

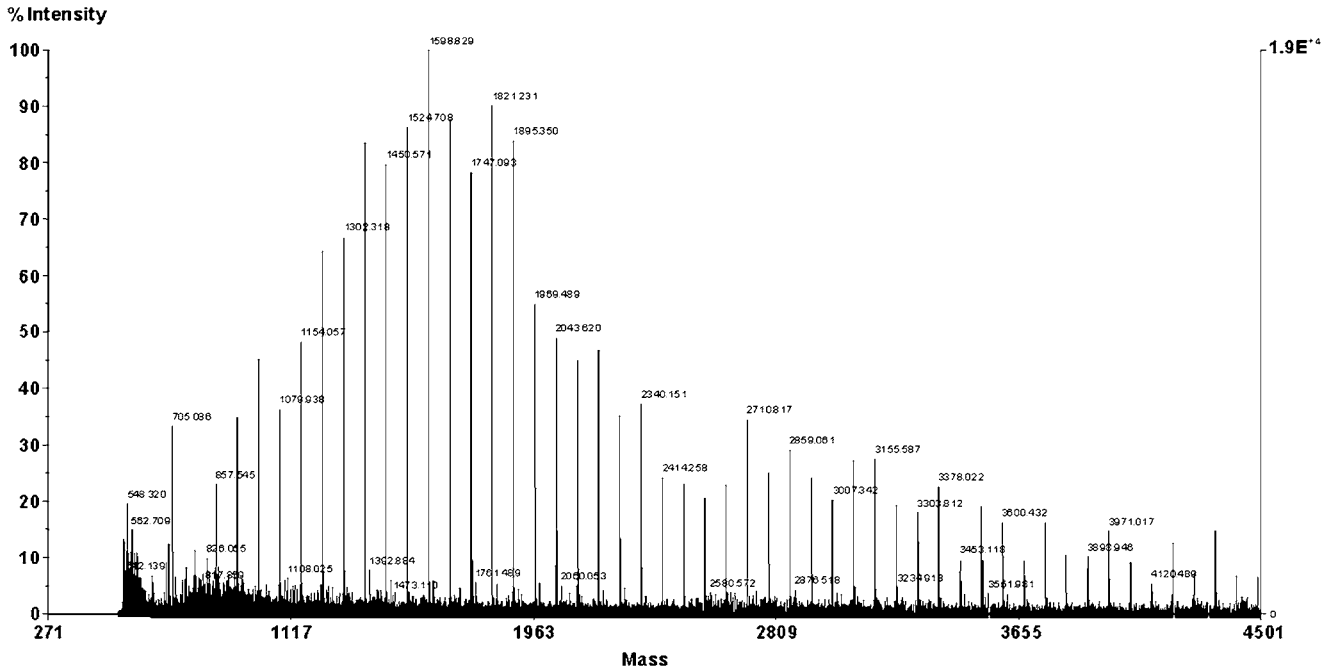

Fig. 2. MALDI-TOF spectrum of pTHF (nTHF=204 mmol, n triflic anh.=6.5 mmol, polymerization time is set to 12 min, T°C=−9°C, matrix: ditranol; [polymer]/[NaAc]=1/50; laser intensity, 3,200 ua for three values of laser shots).

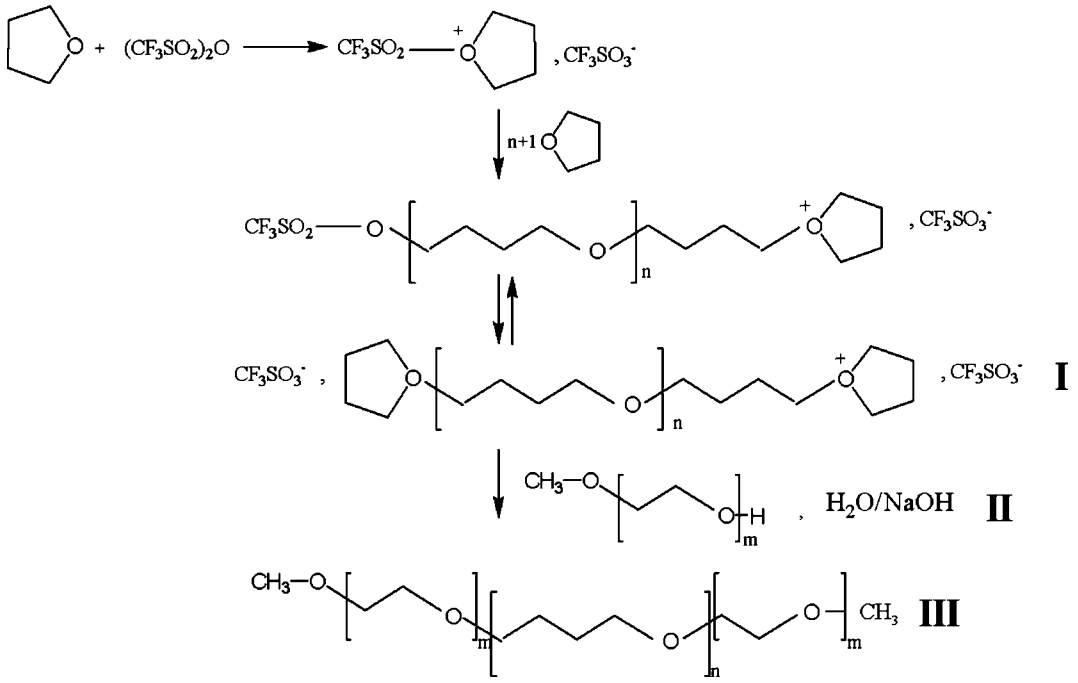

Fig. 3. Scheme of bulk polymerization of THF quenched by pEO-monoOH to form a triblock copolymer pEO_k–*block*–$pTHF_m$–*block*–pEO_k.

between the macroester and the macroion pair leading to a coexistence of both growing species depending on the polarity of the solvent. This last assumption is now commonly accepted to explain the various results concerning the THF polymerization. D'Haesse and Goethals (13) observed a linear relationship between molecular weight and conversion, at conversion lower than 20%. These authors suggest that a predictable molecular weight can be achieved in this conversion range. At higher conversion, macrocycles formation and transfer reaction were reported. However, quantification of cyclic compounds by Pruckmayr (14) showed that side-reactions were getting significant when thermodynamic is controlling the polymerization (i.e. large reaction time).

In order to synthesize a well defined triblock copolymer, we first investigated the experimental conditions that allow the synthesis of pTHF of controlled molecular weight with limited side reactions. We started with the bulk THF polymerization initiated with triflic anhydride ([triflic anhydride]=0.38 mol l^{-1}) at room temperature and we observed a gelification within 1 min. The temperature was then decreased in order to slow down the polymerization kinetic and a molecular weight close to 2,000 was achieved in a reproducible manner at −9°C within 12 min. The polymer yield was 70% and viscosity of the solution was compatible with subsequent polymer modification. In the same way, a polymer pTHF of molecular weight 1,000 g mol^{-1} was achieved at −15°C within 15 min ([triflic anh.]=0.37 mol l^{-1}). MALDI-ToF experiments (Fig. 2) confirmed the absence of cyclic compounds which is consistent with the fact that thermodynamic equilibrium is not reached and confirmed the polydispersity of 1.3. Such reaction conditions allow a high macroion concentration which is an advantage for subsequent coupling reactions.

Copolymer Synthesis

Smith and Hubin (11) reported that the growing macroion resulted from THF polymerization can quantitatively react with methanol. Goethals *et al.* (13) used this reaction to telechelically functionalize growing pTHF by hydroxy derivatives and to synthesize triblock copolymers ABA, having the structure pEO_k–*block*–$pTHF_m$–*block*–pEO_k. Fig. 3 summarizes the polymerization of pTHF initiated by triflic anhydride and quenched with α-hydroxy-ω-methylpoly(ethylene oxide) (pEO-monoOH).

In a first attempt, we synthesized a pEO_k–*block*–$pTHF_m$–*block*–pEO_k triblock copolymer using the experimental conditions (nTHF=1.2 mol; *n* triflic anh.=2.3 mmol; room temperature) described by Goethals *et al.* (9) who synthesized a block copolymer with a pTHF block molecular

Table I. Bulk Polymerization of THF Quenched by pEO-monoOH

Exp.	*n* THF (mmol)	*n* anh. triflic (mmol)	*n* PEO (mmol)	Mn OE (g mol^{-1})	Mn THF (g mol^{-1})	Mn diblock (g mol^{-1})	Mn triblock (g mol^{-1})	% triblock/ % diblock
CP1	198.1	6.3	12.0	660	2,380	/	3,700	100/0
CP1-b	213.9	6.6	13.5	450	1,850	2300	2,760	84/16

Polymerization time is set to 12 min, T=−9°C, time of coupling is 10 min at room temperature with pEO solution of 2.5 mol/l (in toluene). Mn of various molecules as determined from ^{13}C NMR.

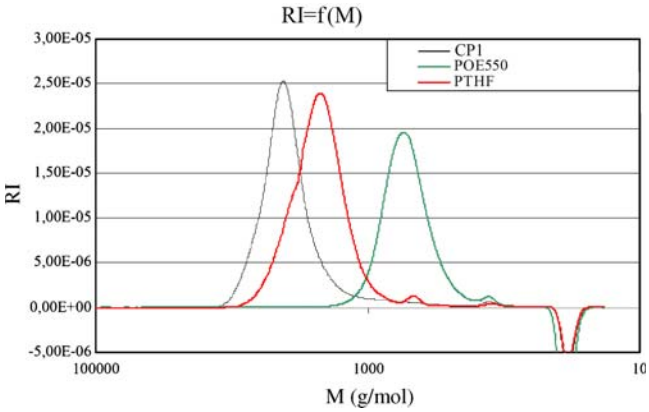

Fig. 4. SEC chromatogram of copolymer CP1, of free pEO (Mn= 550 g mol^{-1}) and of sampled pTHF.

weight close to 20,000 g mol^{-1}. After 15 min, pEO-monoOH in toluene (0.24 mol l^{-1}) was added to the polymerization mixture to form triblock copolymer (n_{pEO-OH}=2 $n_{triflic}$). Coupling reaction was allowed for 10 min as suggested by Goethals *et al.* (9). The polymer was precipitated in solution of cold water with NaOH. Coupling reaction between the telechelic oxonium functions of the growing pTHF and the hydroxy functions of the pEOmonoOH is evidenced by an increase of the molecular weight of the final copolymer and no signal (^{13}C NMR analysis) attributed to the HO–*C*H$_2$–*C*H$_2$–CH$_2$–CH$_2$– functions of pTHF was evidenced at 30.10 and 62.34 ppm, confirming the absence of both, pTHF and diblock pTHF–*block*–pEO. Our results are thus consistent with data published by Goethals *et al.* (9). However, decreasing the molecular weight of the pTHF central block in the same conditions conducted to an incomplete coupling reaction, i.e. formation of triblock mixed with diblock copolymer, as witnessed by the presence of the two signals at 30.10 and 62.34 ppm attributed to hydroxytelechelic pTHF. Using inverse gated ^{13}C NMR, it is also possible to determine the ratio of undesirable molecules present with the triblock. In order to improve the rate of coupling between the growing pTHF chains and the pEO-monoOH, the reaction procedure was changed as follows. The ratio [THF]/[triflic anh.] was decreased from 500 to 30 (Table I).

Fig. 4 reports the SEC analysis of the pEO, the sampled pTHF and the triblock copolymer. As expected, CP1 do not show any peak related to the presence of pEO or pTHF. This observation is confirmed by the ^{13}C NMR characterization since no signals attributed to the presence of telechelic hydroxyl functions can be detected (Fig. 5). CP1-b was synthesized in same experimental conditions as CP1 (Table I). One can see that during the second synthesis, diblock copolymer was present (as evidenced with NMR) in a low content. Also, the size of the pEO and pTHF blocks slightly differed with that of CP1, indicating that it is not trivial to obtain exactly the same compound as in the first run. Using NMR, we calculated the molecular weight of the pTHF block of CP1. The results indicate a pTHF chain of 2,350 g mol^{-1} (i.e. 33 IU of THF) which is very close to the targeted value (30 IU). The molecular weight of the pEO block is 660 g mol^{-1} (15 IU) which is also near the value we expected (13 IU). Taken together, the optimized conditions allowed for the synthesis of a copolymer which is close to Pluronic L64.

In order to synthesize a triblock copolymer having a lower degree of polymerization of the pTHF block, the polymerization temperature was decreased to −15°C, and the polymerization time set to 15 min. Again, formation of pure triblock copolymer could be obtained, despite the low coupling temperature (Table II). In the first protocol we used a short coupling reaction time and incomplete reaction was observed as witnessed by the presence of diblock copolymer in the reaction media (CP2-a). Pure triblock (CP2) was synthesized with a coupling reaction time of 150 min. By using NMR techniques, the average molecular weight of each block could be determined: pEO470–pTHF640–pEO470 (or expressed in monomer units, pEO11–pTHF9–pEO11; Table II).

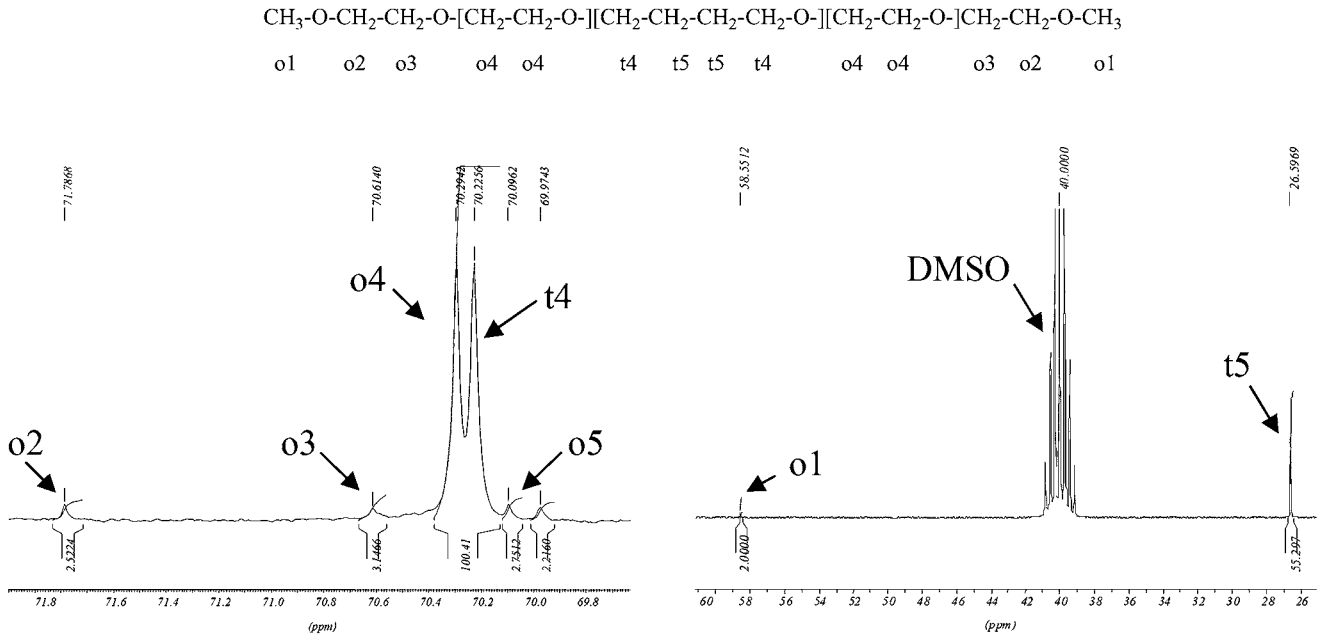

Fig. 5. ^{13}C-NMR spectrum (300 MHz, T=300°K) of **CP1** in DMSO-d6 (O5 corresponds to the ethylene oxide unit linked to the pTHF block).

Table II. Bulk Polymerization of THF Quenched by pEO-monoOH

Exp.	*n* THF (mmol)	*n* anh. triflic (mmol)	*n* PEO (mmol)	Time of coupling (min)	Mn OE (g mol^{-1})	Mn THF (*g* mol^{-1})	Mn diblock (g mol^{-1})	Mn triblock (g mol^{-1})	% triblock/ % diblock
CP2-a	235.8	7.5	10.9	10	440	1,750	2,190	2,630	70/30
CP2	234.0	5.9	12.9	150	470	640	/	1,580	100/0

Polymerization time is set to 15 min, T=−15°C, V solvent of pEO-monoOH=5 ml of toluene. Mn of various molecules as determined from ^{13}C NMR.

DNA Binding Assay and *In Vitro* Transfection Experiments

We started our activity study by investigating whether the copolymers are able to interact with plasmid DNA. Therefore, we mixed increasing amounts of copolymer CP1 or CP2 [from 0.01 to 0.2% (*w*/*v*)] with 1 μg of DNA and we tested whether the presence of the polymers alters the migration of DNA toward the cathode during agarose gel electrophoresis. The results show that none of the two compounds was able to change the migration profile of DNA, even at the highest concentration tested (data not shown). Next, we evaluated the transfection efficiency *in vitro* of both polymers. Concentrations of copolymers ranging between 0.01% and 0.35% (*w*/*v*) were tested on HepG2 cells with 2 μg/well of a plasmid encoding the *luciferase* gene. The expression levels of luciferase 30 h after transfection indicate that neither CP1 nor CP2 could promote gene transfer (not shown). Taken together, these results are in good agreement with reports using commercially available pEO–pPO–pEO poloxamers or pMeOXZ–pPO–pMeOXZ copolymers (15). Indeed, studies conducted for example with Pluronic L64 could not demonstrate an interaction between the copolymer and DNA (8) nor was the polymer able to mediate gene transfer *in vitro* (8).

In Vivo Gene Transfer

Next, in order to evaluate whether copolymers CP1 and CP2 were able to increase gene expression in muscle, 10 μg of plasmid DNA encoding the *luciferase* gene were mixed with increasing concentrations of polymer and injected into the tibialis anterior of Balb/c mice. The muscles were harvested 7 days after injection, and the luciferase activity was measured. The results show that addition of the polymers significantly increases the level of luciferase (Fig. 6A) as compared to naked DNA. Indeed, the average values of light units were 3.3×10^{e6}, 32.2×10^{e6} and 31.9×10^{e6} for naked DNA, 0.025% CP1 (*w*/*v*) and 0.025% CP2 (*w*/*v*), respectively. Thus, the two polymers increase by a factor of ten the expression level 1 week after the injection. These results are roughly comparable to those obtained with the optimal concentration of L64 (Fig. 6B) since the mean luciferase values obtained with naked DNA and DNA formulated with 0.1% of L64 were 1.9×10^{e6} and 43×10^{e6} light units, respectively. One can note that CP2 and L64 seem to give more reproducible results than CP1.

To evaluate whether the kinetic of gene expression is different between the three polymers, we used as reporter gene a murine secreted alkaline phosphatase (mSeAP) (16)

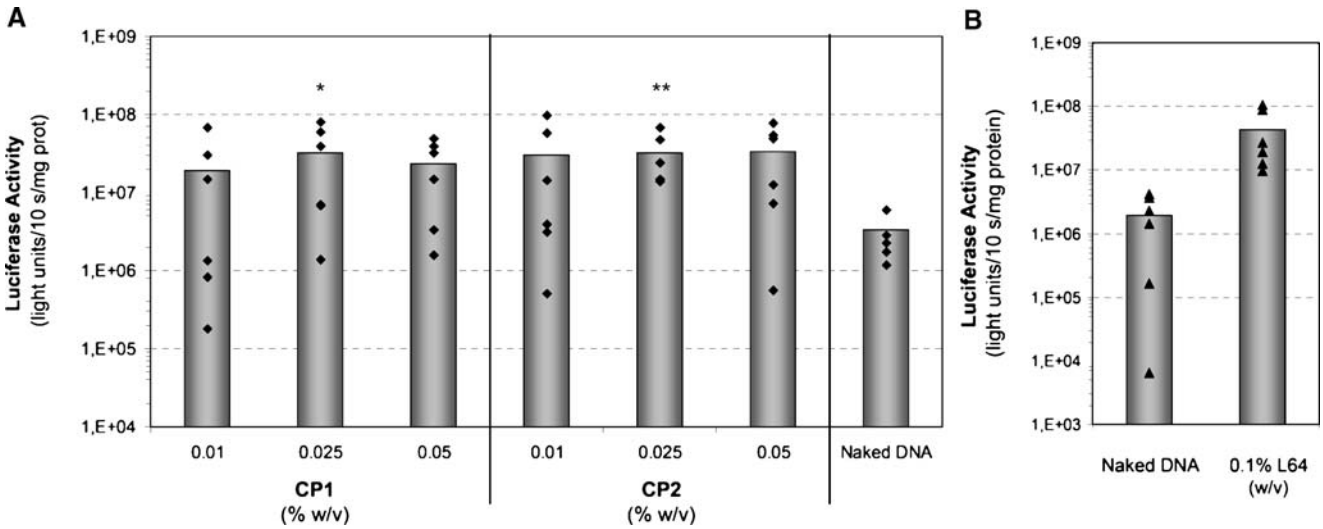

Fig. 6. Evaluation of the *in vivo* efficiency of the copolymers. Complexes were prepared in 150 mM NaCl with a CMV-Luc plasmid mixed or not with a copolymer. (A) The three following concentrations were tested for compound **CP1** and **CP2**: 0.01, 0.025, and 0.05% (w/v). (B) The Pluronic L64 was used at 0.1% (w/v). 35 μl of the transfection solution containing 10 μg of CMV-Luc plasmid was slowly injected into the tibialis muscle (n=6) and 7 days later, the luciferase levels were measured from the processed muscles. The grey bars indicate the average luciferase activity per condition. (A) Differences between naked DNA and DNA + copolymer were determined using the Student's paired t test: *$p \leq 0.05$; **$p < 0.01$.

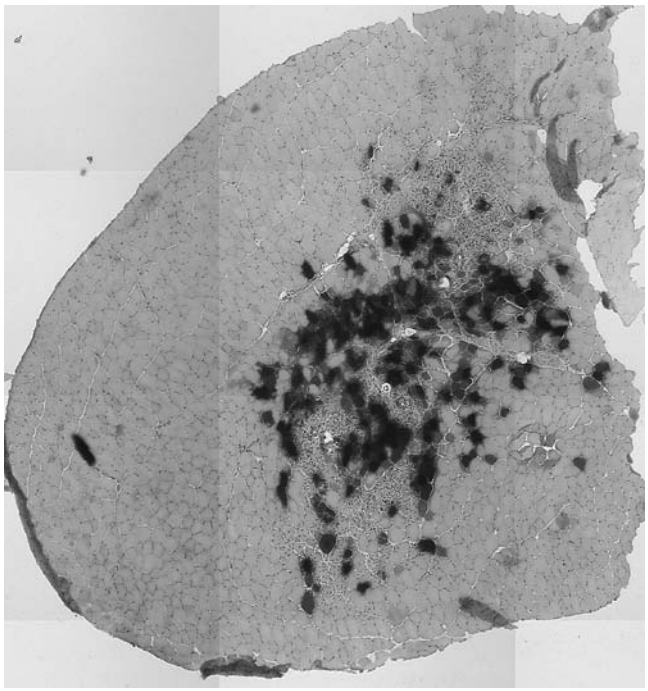

Fig. 7. Transfection efficiency *in vivo*. 20 μg of a plasmid encoding the *LacZ* gene under the CMV promoter was formulated with 0.025% of **CP1** in 150 mM NaCl and injected into the tibialis. 7 days after administration, the treated muscle was isolated, and expression of the transgene was revealed after X-Gal staining.

which allowed us, by collecting blood samples, to monitor the level of expression at day 3 and 7 after injection. The results indicate that there are no major differences in the kinetic of transgene expression between the different conditions that were tested (i.e. DNA +/− copolymer; Table III). Next, we determined the efficiency of gene transfer by using as reporter gene *lacZ*. The X-Gal staining of the muscle, which was injected with 20 μg CMV-LacZ/0.025% CP1/35 μl NaCl 150 mM 7 days before, revealed that a significant amount of fibers expressed the transgene (Fig. 7). Notably, an important infiltration of mononuclear cells can be seen, which is expected since the product of the *LacZ* gene is known to be highly immunogenic.

Finally, we evaluated the toxicity of copolymer CP1 and CP2 in the presence of DNA using HepG2 and C2C12 cells (Fig. 8). The MTT assay indicates that none of the two copolymers is cytotoxic at concentrations between 0.01% and 0.35% for HepG2 cells and between 0.01% and 0.1% for C2C12 myotubes. By contrast, L64 displayed a significant toxicity at 0.35% and 0.1% on HepG2 and C2C12 cells, respectively.

Table III. Kinetic of Reporter Gene Expression

Formulation	Level of mSeAP (in %) at day 3 compared to day 7
0.025% CP1	41
0.025% CP2	35
0.1% L64	44
Naked DNA	33

DISCUSSION

It has been recently reported that amphiphilic triblock copolymers arranged in a pEO_k–*block*–pPO_m–*block*–pEO_k structure are able to facilitate gene delivery in the skeletal muscle. In the present study, we investigated the effect of a chemical change of the central block on the transfection activity. As hydrophobic block we chose polytetrahydrofuran chains because its local dynamic is higher than that of the pPO. This parameter may be important if destabilization of the lipid bilayer by amphiphilic A–B–A bloc copolymer plays a role in the gene transfer mechanism (17). In our first attempts of synthesis we used the experimental conditions previously reported by Goethals *et al.* (9). However, while they were described to allow the synthesis of a copolymer with a long pTHF chain (i.e. molecular weight of around 20,000) they did not work well for the synthesis of shorter pTHF blocks. After optimization of the synthesis conditions, we were able to obtain two new copolymers having respectively the structure pEO15–pTHF33–pEO15 (CP1) and pEO11–pTHF9–pEO11 (CP2).

Gel retardation assays were unable to detect interactions between the copolymers and plasmid DNA. This is consistent with the results obtained by Gau-Racine and colleagues with Pluronic L64 (17). Furthermore, neither CP1 nor CP2 could mediate gene transfer *in vitro* on HepG2 cells. This again is in good agreement with the results obtained with L64 by Pitard and co-workers (8). Notably, these authors found by using radiolabeled DNA that L64 was unable to enhance plasmid cell uptake *in vitro*.

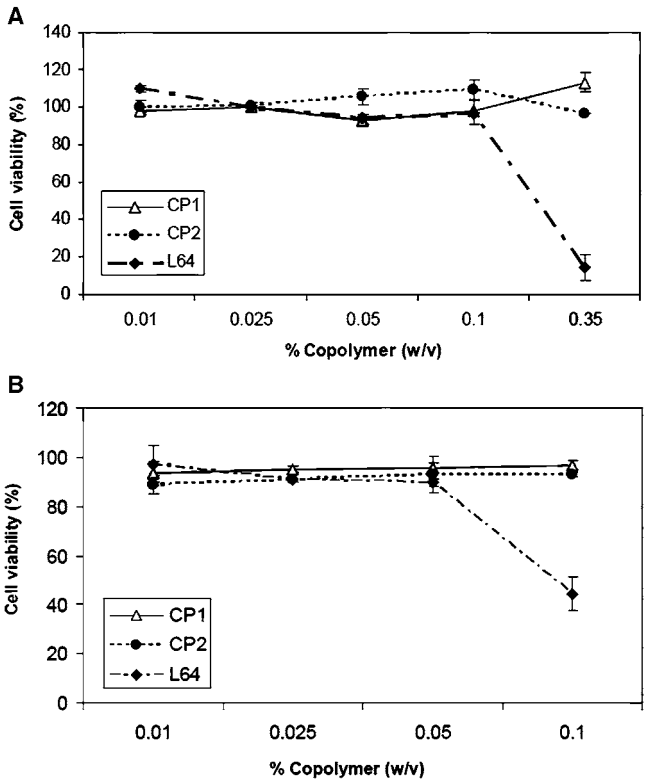

Fig. 8. Study of the cell viability. The MTT assay was performed as described in the experimental section using either HepG2 cells (A) or C2C12 myotubes (B). Each experiment was done in duplicate and the values used for A&B are the averages of the duplicates.

Next, we evaluated the *in vivo* transfection activity of both compounds by co-administering 10 µg of CMV-Luc and increasing amounts of polymer. Interestingly, we found that both pEO–pTHF–pEO triblock copolymers were able to increase muscle gene transfer in a very similar manner than the Pluronic L64 (Fig. 6A and B).

These results, together with the recent report by Brissault *et al.* (15) which shows that the hydrophilic pEO blocks can be replaced by polymethyloxazoline (pMeOXZ–pPO–pMeOXZ) without inducing an alteration of the gene transfer properties, clearly indicate that the capacity to enhance DNA transfection is not restricted to pEO_k–*block*–pPO_m–*block*–pEO_k chemical arrangements. Notably, Brissault and colleagues found that the pMeOXZ–pPO–pMeOXZ copolymer enhanced the luciferase levels by twenty-fold as compared to naked DNA (15) while we improved the expression levels by tenfold by using polytetrahydrofuran derivatives under the same experimental conditions. This may indicate that not all modifications enable a maximal enhancement, although it may be too premature to really conclude about this point since the degree of polymerization between the two types of polymers (i.e. pMeOXZ–pPO–pMeOXZ and pEO–pTHF–pEO) is not the same.

Altogether, the present results and others (15) indicate that there is a significant flexibility in terms of design of new amphiphilic triblock for improving transfection and/or safety properties. This structural flexibility appears intriguing, and raises the question of how these polymers promote transgene expression. One hypothesis is that the amphiphilic copolymers transiently permeabilize cell membranes, and thus allow enhanced DNA delivery to muscle cells (17). Another possibility is that A–B–A copolymers increase the transcription of genes, in part through activation of stress signaling pathways (18,19). Whether it is the first or the second mechanism—or a mix of both—by which the copolymers promote skeletal muscle gene transfer remains to be determined.

Following the encouraging results obtained with Pluronics and derivatives in muscle, it is important now to study the mechanism by which they act, evaluate their safety, and compare their activity to other delivery systems, including physical methods such as electrotransfer (20), ultra-sound (21) and intra-arterial injection (22). Concerning the latter point, Pitard and co-workers already showed that L64 allows for gene transfer levels similar to those obtained with electrotransfer (8). Interestingly, in contrast to electroporation, the muscle does not seem to be damaged by these compounds. While this point is in favour of Pluronics as compared to electrotransfer, the chemical-mediated transfection seems to generate less reproducible results than the physical method. In any case, for clinical applications, we need a better understanding of the molecular requirements for successful gene delivery and this, in turn, will allow rational design of new vectors.

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