Tuning Macromolecular Structures of Synthetic Vectors for Gene Therapy

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Summary: The synthesis of triblocks poly(2-methyl-2-oxazoline-b-tetrahydrofurane-b-2-methyl-2-oxazoline) has been developed. It was shown that the technique of polymerization of the second block from the living species created on the two chain ends of poly(THF) is successful but makes the control of the size of the poly(THF) block difficult due a fast depolymerization upon the introduction of the second monomer. A purification technique was used to get rid of the possible homo-poly(2-methyl-2-oxazoline) formed. Various analytical techniques were used to characterize the behavior of the triblock and more particularly in the presence of DNA. Electrophoresis on agarose gels and neutron scattering, demonstrated that the neutral triblock does not appreciably interact with DNA. It was also shown that the triblock for which approximately half (47%) of the methyloxazoline units were transformed into ethylenimine units by hydrolysis gives only loose interactions with DNA. This result is assigned to the fact that charge density plays a major role in the interactions of positive polyelectrolytes with the negatively charged DNA. The triblock was shown being able to interact with bilayer lipid membranes mimicking cell membranes. The efficiency of the hydrolysed triblock was much higher, while the size of holes created in the membranes is not large enough to give passage to DNA.

Keywords: amphiphilic triblock copolymer; bilayer lipidic membranes; DNA-copolymer mixtures; gene therapy

Introduction

Research in the field of macromolecular design is of primary importance in the domain of synthetic vectors for gene therapy.[1] Heavy consequences are expected as well from the therapeutic point of view as from the pharmaceutical industry economy point of view. This therapy has a long way to go due to cytoxicity problems highlighted during clinical trials. Moreover DNA delivery into the cells relies on many constraints such as condensation, complexation, endocytosis through the bilayer lipid membrane, nuclear targeting and expression in the nucleus of an eukaryotic cell. The modification of the genetic information in cells by the introduction of DNA requires its protection by vectors against DNAses. Usually, DNA delivery systems can be classified in two types: viral vector-mediated systems and non-viral mediated systems (mainly synthetic ones). Although viral systems are by far the most effective for gene delivery, their safety, packaging and production problems make them less attractive than synthetic vectors. Thus, synthetic materials have become most desirable tools for gene delivery in both basic research units and clinical settings. In this context more particularly polymer based synthetic vectors, offer advantages such as relative simplicity of production, safety and versatility. However, up to now their low efficiency comparatively to virus based systems prevented their introduction in therapeutic treatments. Fundamental research

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was carried out aiming at understanding relationships between their structure and their transfection efficiency, as well as their toxicity. This research involved careful studies of polymer synthesis from the point of view of controlled architecture due to the need of high purity for these polymers to be used in physiological fluids.[1,2] Two classes of polymers were under investigation, the positive polyelectrolytes and the neutral amphiphilic block copolymers.

One of the preferred positive polyelectrolytes is polyethylenimine (PEI) which offers opportunity to treat cystic fibrosis or cancer. These synthetic vectors can be used when cells with negatively charged outer membrane are to be transfected. However, two forms of PEI are at hand, branched PEI (bPEI) and linear PEI (lPEI), the latter being largely more efficient than the former, but more toxic. It was suspected for a long time that the higher efficiency was due to the linear structure of lPEI, but the functional composition which was differing from each other prevented to reach definite conclusion. Thus, it was synthesized a linear PEI by polymer modification with the same functional content as bPEI. It was shown that this new polymer had an efficiency of the same order of magnitude as lPEI, much higher than that of bPEI.[1b] This result allowed concluding on the definite advantage of linear macrostructure for DNA transfection for positive polyelectrolytes.[3]

Block copolymers, which are the materials of choice of this research, found wide applications in many industrial domains. A new field seems to be on the verge to make profit of their outstanding properties. Indeed, neutral amphiphilic block copolymers have an important future in the treatment of myopathies by gene therapy, since the discovery of the potential of the triblock poly(ethylene oxide-b-propylene oxide-b-ethylene oxide).[4,5] However, it has been shown that the *in vitro* and *in vivo* efficiencies can be very different and not necessarily correlated for these polymers. Thus this research was dealing with the more pertinent *in vivo* experiments. Besides the real improvements in transfection efficiency which has already been observed, an important improvement is to be looked for in polymer labeling techniques for cell targeting. These polymeric synthetic vectors may have a future in the treatment not only of genetic diseases but also of cancer diseases.[6] There is still a large place for structure optimization of these polymers of therapeutic interest and high added value. The synthesis by cationic ring opening polymerization of the triblock poly(ethylene oxide-b-tetrahydrofurane-b-ethylene oxide) on which the poly(propylene oxide) block was replaced by a new more hydrophobic sequence of poly(tetrahydrofurane) allowed to study the in vivo transfection efficiency of this copolymer which was found to be the same as that of the one based on poly(propylene oxide).[2] This result allowed concluding that the chemical nature of the hydrophobic block was not of a prime importance. The synthesis of the triblock copolymer poly(2-methyl-2-oxazoline-b-propylene oxide-b-2-methyl-2-oxazoline) allowed to show that this triblock had approximately the same in vivo transfection efficiency as the copolymer based on poly(ethylene oxide).[2] One important question was the relative importance of the two types of sequence involved in the vector: can the relatively toxic poly(ethylene oxide) sequence be replaced by another hydrophilic block, and the poly(propylene oxide) one by another hydrophobic one ? Finally, the synthesis of the triblock poly(2-methyl-2-oxazoline-b-tetrahydrofurane-b-2-methyl-2-oxazoline), already synthesized by Goethals,[7] in which both types of sequence were new in the context of DNA transfer, was aiming at the demonstration that the most important parameter for this new type of synthetic DNA transfer agents is the amphiphilic character, the chemical structure only governing toxicity. Thus, careful synthesis by cationic ring opening polymerization of this amphiphilic block copolymer and the determination of its physico-chemical properties in physiological conditions was undertaken in order to answer this question.

Experimental Part

Chemicals

All the solvents used were dried by the following methods. Dichloromethane (DCM) was dried over calcium hydride and heated under reflux, under nitrogen for three hours and was then distilled and stored over molecular sieves 4 Å.

Tetrahydrofurane (THF) was dried over sodium and benzophenone, heated under reflux and used freshly distilled. Triflic anhydride (Tf_2O), tris borate, boric acid and EDTA (Aldrich) were used as received.

2-Methyl-2-oxazoline was distilled over calcium hydride.

NMR Experiments

^1H and ^{13}C NMR spectra were measured at 300 MHz and 75 MHz with a Bruker spectrometer (Avance 300); tetramethylsilane was used as an internal standard.

1D and 2D spectra were treated with Bruker-WINNMR software.

Synthesis of HO-Poly(THF)-OH (Mn = 3000)

Dry THF (m = 17.09 g, FW = 72) was stirred at –9 °C under nitrogen then dry Tf_2O (m = 2.12 g, FW = 282, d = 1.677, V = 1.26 mL) was added. The reaction mixture was let under continuous stirring over 14 min. When an α,ω-dihydroxy polymer was targeted, 5 M NaOH aq (V = 5 mL) was added and the resulting solution stayed overnight at room temperature. Concentration under vacuum was performed and the crude material was extracted with DCM then the organic phase was dried over Na_2SO_4 and filtered. Evaporation of the solvent afforded a pale yellow oil which was dried under vacuum overnight, to give a white solid corresponding to HO-PTHF-OH.

^1H, ^{13}C- NMR and SEC were performed to characterize the polymer obtained. Common NMR signals: δ_H (300 MHz, *d6*-DMSO) 1.44 (4H, br s, $2 \times CH_2$); 3.28 (4H, br s, $2 \times CH_2$, $–CH_2–O–CH_2–$) and 4.32 (OH, br).

Synthesis of Poly(MeOx)

Dry CH_3CN, (10 mL) was stirred with 20 g of MeOx (d = 1.005) and 1,41 g of methyl tosylate. The polymerization was carried out at 80 °C for 24 hours. Precipitation in ethyl ether and drying under vacuum allowed to collect 10.9 g of pMeOx. The protonic spectrum is shown on Figure 1. Assuming one tosylate function per chain, a polymerization degree of 30 is calculated. It is clear that these DPn calculations are relying on the assumption of one tosylate group per chain.

Synthesis of PMeOx-PTHF-PMeOx triblock[2]

With continuous stirring, dry Tf_2O (m = 0.745 g, FW = 282, d = 1.677, V = 0.44 mL) was added to a solution of dry THF (m = 6 g, FW = 72, d = 0.89, V = 6.74 mL, n = 0.0727 mol) at −9 °C under nitrogen. After 12 min, dry MeOx (FW = 85.11, d = 1.005, V = 0.610 mL, n = 7.2 mmol, 2 units) was added to terminate the polymerization of THF. The reaction medium was left overnight at −9°C to allow complete quenching. Unreacted THF was removed by evaporating and replaced by 46 mL of dry CH_3CN. This polymeric precursor solution was used as a macroininiator for the 2-methyl-2-oxazoline polymerization. After being dissolved in dry acetonitrile, 2-methyl-2-oxazoline was added so that it corresponded to a concentration of 2 M. In general, the polymerization was carried out in the temperature range of 70 °C to 85 °C for 2–3 hours. The medium is then quenched by 4 mL of sodium hydroxide, dried under vacuum, dissolved in CH_2Cl_2, precipitated in ether, filtered off, washed with Et_2O and dried in vacuum overnight.

To determine the purity of the material obtained, ^1H and ^{13}C NMR were performed in $CDCl_3$: δ_H (300 MHz, $CDCl_3$) 1.63 (4H, $2 \times –CH_2–$, PTHF), 2.16 (3H, (–N–$COCH_3$, MeOx starting monomer), 2.26 (3H, –N–CO–CH_3, PMeOx), 3.42 (4H ,$2 \times CH_2$, $–CH_2–NCOMe–CH_2–$, MeOx starting monomer), 3.44 (4H ,$2 \times CH_2$, $(–CH_2)_2N–CO–$, PMeOx), and 3.47 (4H, $–CH_2–O–CH_2–$, PTHF).

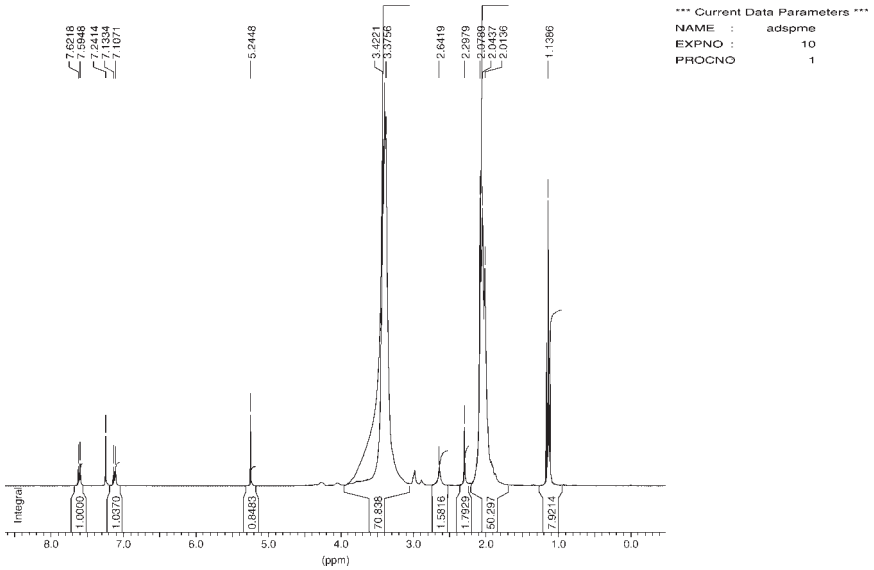

Figure 1.
^{1}H NMR spectrum of poly(2-Methyl-2-oxazoline) in $CDCl_3$. The methyl group of residual ether is shown at 1.14 ppm, the methyl substituent of tosylate aromatic ring is shown at 2.30 ppm, and the aromatic ring is shown at 7.1 and 7.6 ppm.

Δ_C (75 MHz, $CDCl_3$) 21.68 (CH_3 PMeOx), 26.97 (CH_2 PTHF), 47.06 (CH_2 PMeOx), 47.31 (CH_2 PMeOx), 71.08 ($0CH_2$ PTHF) and 171.8 (CO PMeOx).

To increase the purity of the compound further precipitation in Et_2O was performed to eliminate mainly unreacted 2-methyl-2-oxazoline. When necessary, homo-polymethyloxazoline was eliminated by emulsion phase separation (see below).

Controlled Hydrolysis of PMeOx-PTHF-PMeOx Triblock

A special study was devoted to the hydrolysis of the poly(2-methyl-2-oxazoline). It was found that the hydrolysis of pMeOx in aqueous HCl 0.0155 M for 6 hours at room temperature did not involve any detectable hydrolysis of monomer units, while after workup the tosylate content was largely decreased. An exposure to aqueous HCl 0.6M heated under reflux for 3 hours, followed by sodium hydroxide neutralisation, dissolution in CH_2Cl_2, drying under vacuum and dissolution in methanol, was found necessary to give a hydrolysis ratio of the monomer units of 47%.

Copolymers were hydrolysed under the following procedure over a varying reaction period: 2 g of triblock (0.684 mmol, 1 eq, 24 $NCOCH_3$ units) were dissolved in water (26 mL). Then, a solution of conc HCl (1.37 mL, 16.44 mmol, 24 eq, $C_{H30+} = 0.6$ M) was added to the mixture and let under reflux for the required duration (usually in the range 1–6 h). A solution of 5N NaOH (50 mL) was introduced and the resulting mixture was stirred over a period of 1 h. The solution was concentrated under reduced pressure to afford a white solid. Acetonitrile was added to the solution and then filtration occurred. The solvent was then evaporated and a small volume of acetonitrile was reintroduced before precipitation in cold Et_2O. The precipitate was dried under *vacuum* and ^{1}H NMR revealed a hydrolysis rate of 29.4% in 86% yield for a 1 hour hydrolysis duration.

Δ_H (300 MHz, H_20) 1.59 (CH_2 PTHF), 2.03–2.11 ($NCOCH_3$ PMeOx), 2.67–2.77 (CH_2–NH–CH_2) and 3.48 (CH_2–O–CH_2 PTHF and CH_2–$NCOCH_3$–CH_2 PMeOx).

δ_C (75 MHz, H_20) 19.8, 24.55, 42.26, 43.31, 44.31, 44.86, 45.49, 46.77, 47.9, 49.08, 69.28 and 173.6.

Interaction with Bilayer Lipid Membranes (Electrical Measurements)

Bilayers lipid membranes are made at room temperature by using the original method of Rudin and Mueller[8] a film of a 1% solution of diphytanoylphosphatidylcholine-Lecithine (Avanti) in decane is spread across a 150 μm wide hole drilled in a polysulfone wall separating the two compartments of a commercial chamber (Warner). Each compartment contains 1 ml of 1M KCl, 5 mM HEPES (at pH 7.5). Voltage is applied between the two compartments thanks to two Ag electrodes covered with AgCl, and can be varied from −150 to +150 mV. In order to determine the behavior of the polymers towards a lipidic bilayer, it is necessary that the membrane is thin enough to make sure that an insertion of the polymer macromolecule, leading to pore formation, is detected. For instance, after thinning of the decane film and formation of a planar bilayer, the channels are formed by adding for instance 0.15 nanomole of a macromolecule from a mother solution in one compartment. The number of channels in the membrane is controlled eventually by dilution and perfusion. Whatever the type of experiments, the electrical current corresponding to a flow of ions passing through a pore for a given applied electrical potential is measured with two Ag/AgCl reversible electrodes in contact with the electrolyte on each side of the lipid membrane. One electrode is connected to ground, the other one to the headstage of a patch-clamp amplifier used in ''voltage-clamp, whole cell'' mode (Axopatch 200B, Axon Instruments, BLM 120 from BioLogic was also used). The amplifier is connected to a data acquisition system (Digidata 1322A, Axon Instruments) and to a computer. The data presented are filtered at 10 kHz.

Experiments were carried out in order to determine the behavior of the polymers towards lipid membranes mimicking cell membranes. The polymer was dissolved in a solution of KCl 1M-Hepes 5mM (pH = 7.5), at a concentration of 10 mg/mL. 100 μL of this solution were added to the cell. The current intensity was scanned and the membrane capacity (before pore formation) allowed the determination of the membrane thickness.

Small Angle Neutron Scattering and Dynamic Light Scattering Experiments

Taking into account that some of the polymers were positively charged polyelectrolytes (after hydrolysis), it was of interest to check the behavior of such materials in water in physiological conditions. The scattering experiments were performed at *Laboratoire Léon Brillouin* on spectrometer PACE (Saclay, France). The range of scattering vector are 1.9×10^{-2} Å$^{-1} < q < 2 \times 10^{-1}$ Å^{-1}. The samples were held in 2 mmor 1 mm quartz cells. Initial data treatment was carried out following Cotton.[9]

In the dilute regime, at small q values, whatever the shape of the macromolecules, Guinier analysis leads to express $I(q)$ as $\ln I(q) = \ln I_0 - \frac{R_g^2}{3} q^2$ where R_g^2 is the square radius of gyration of macromolecules. This expression allows us to determine the radius of gyration. At higher q values, measurements become sensitive to the shape of the molecules. A power law could represent the variation of the scattering intensity as a function of q, $I(q) \sim q^{-a}$. Theoretically, the more compact the scattering bodies, the higher the a coefficient values: $a = 1$ is expected for a rigid rod, $a = 2$ for an ideal chain, and $a = 1{,}7$ for a chain with excluded volume.

Dynamic Light Scattering Measurements data were obtained with a Brookhaven light scattering apparatus, equipped with a Spectra Physics argon-krypton laser ($\lambda = 514.5$ nm) and BI9000AT correlator. The beam was focused onto the sample cell through a temperature controlled chamber filled with decaline matching the refractive index of the glass holder. The samples were filtered through 0.2 μm filters at room temperature.

Gel Retardation Assay

Polymer triblocks with varying hydrolysis percentage were combined to 146 bp DNA

(C = 1 mg/mL and 8.8 10^{-2} μg/mL) from calf thymus and let for incubation over a period of 10 min. Then, 5X Tris-boric acid-EDTA (TBE 5X) and charged buffer were added.

The samples were then electrophoresed on a 1% (w/v) agarose gel pretreated with 0.8 mL of ethidium bromide in TBE 1X buffer at 100 V for 1h. Then, the gel was analyzed on a UV transilluminator to show the location of DNA.

To handle DNA with a well define size, DNA from calf thymus was extracted as follows: Chromatin was extracted in low ionic strength buffer after micrococcal nuclease digestion of nuclei.[10] After removal of linker histones, 146 bp DNA was obtained by controlled digestion with micrococcal nuclease. After precipitation in cold 2-propanol, DNA pellets were dried under vacuum and stored at −80 °C. The pellets were solubilized in an adequate buffers 10 mM Tris, 1 mM EDTA, and 150 mM NaCl with the pH adjusted to 7 before using them.

Results and Discussion

Synthesis of Polymers and Copolymers

Synthesis of the Central Poly(THF) Block

PTHF prepolymer was obtained by ring opening cationic polymerization using Tf_2O as bifunctional initiator and literature has described its polymerization as an equilibrated ''living process''. The quench of the reaction was performed using 2-methyl-2-oxazoline causing a ring opening of the terminal oxonium species, forming a bifunctional unit with oxazolinium group at each end. Experiments were achieved at the equilibrium, in bulk or in solution to control more accurately the length of the hydrophobic segment. The poor nucleophilic behavior of the oxazoline molecule can affect the termination process since it is less reactive than sodium hydroxide or methanol due to the N=C bond and therefore not giving the expected degree of polymerization (D_{Pn}). Actually, it has been shown that the D_{Pn} of PTHF is higher when the reaction is quenched by NaOH compared to 2-methyl-2-oxazoline, due to depolymerization during quench process. Thus, it is preferable to target a higher D_{Pn} prior to the addition of oxazoline quench.

Gated ^{13}C NMR revealed being a useful tool for polymer analysis. Terminal hydroxy function of poly(THF) gives a signal at 61 ppm. Being present on the spectra of copolymers, this peak witnesses the presence of free poly(THF) chain ends. This can be the case when homopoly(THF) or diblock is present in the analysed sample.

Typical ^{1}H NMR signals in *d6*-DMSO for this type of polymer are as follows: 1.44 ppm (CH_2); 3.28 ppm (CH_2–O–CH_2–) and 4.32 ppm (OH), whereas in ^{13}C NMR, signals at 26.7 ppm (CH_2), and 69.09 ppm (CH_2–O–CH_2), are the most common.

NMR estimation of the monomer unit content has been found to be a good technique for molar mass determination. Since the end chains (oxazolinium) are not always apparent in the spectrum, hydrolysis of the end chains could be considered transforming the cyclic end structure into a primary alcohol and also, covalent bonds with the counter ion may occur. Therefore, to determine the Mn, those signals have to be checked.

Synthesis of a Block Copolymer Poly(2-methyl-2-oxazoline-b-tetrahydrofurane-b-2-methyl-2-oxazoline)

It was decided to synthesize block copolymers which could mimic the poloxamer which already revealed to be useful as a transfer agent, namely Pluronic 6400.[4,11] According to the structure of the latter, the targeted structure was constituted of a central block of poly(tetrahydrofurane) (pTHF), and two side blocks of poly(2-methyl-2-oxazoline) mimicking the poly (ethylene oxide) (pOE) blocks of the Pluronic 6400 of around 600 Da molar mass. This model compound contains around 40% (w/w) of poly(ethylene oxide) with a number average molar mass close to 2900 Da. Thus, the goal was a triblock

copolymer p(2-MeOX-b-THF-2-MeOX). If it is admitted that for the sake of comparison, the poly(2-MeOX) block must have the same polymerization degree as the POE blocks in the Pluronic triblock the mass distribution of the blocks should be 1160-1800-1160 Da,. This point must not be taken for granted. Recipes could be found in the literature and needed to be adapted to the present study.[7]

Study of the Polymerization of 2-methyl-2-oxazoline in the Presence of THF

The block copolymerization technique involved the possibility of inducing the polymerization of the second block, i.e. the polymerization of 2-methyl-2-oxazoline in the presence of residual THF monomer. A short study was carried out in order to determine the effect of the presence of THF monomer on the polymerization of 2-methyl-2-oxazoline. Thus a mixture of 5g of MeOX, 5 g of THF and 276 mg of methyl iodide was allowed to react at room temperature for 48 hours. After quenching by 3 mL of aqueous sodium hydroxide 30%, extraction by a 50/50 (v/v) methylene dichloride/water mixture, evaporation of the organic phase, dissolution in CH_3CN and precipitation in ether, only 600 mg of p(MeOx) were recovered, showing that the MeOx polymerization was severely inhibited by the presence of THF monomer. In another assay, the same mixture of 2-methyl-2- oxazoline (5 g) and methyl iodide (276 mg) was left at room temperature for 24 hours. The same workup procedure gave 4.8 g of a polymer which was analysed by NMR spectroscopy. The collected polymer was pure poly(2-methyl-2-oxazoline). On the contrary the same mixture of 2-methyl-2- oxazoline (5 g) and methyl iodide (276 mg) was left at room temperature for one hour, then 5 g of THF were added, and the system was left at room temperature for 24 hours. The same workup procedure gave 1.9 g of a polymer which was analysed by NMR spectroscopy. The spectrum is shown on Figure 2: the collected polymer was pure poly(2-methyl-2-oxazoline), as it can be concluded by the absence of any peak corresponding

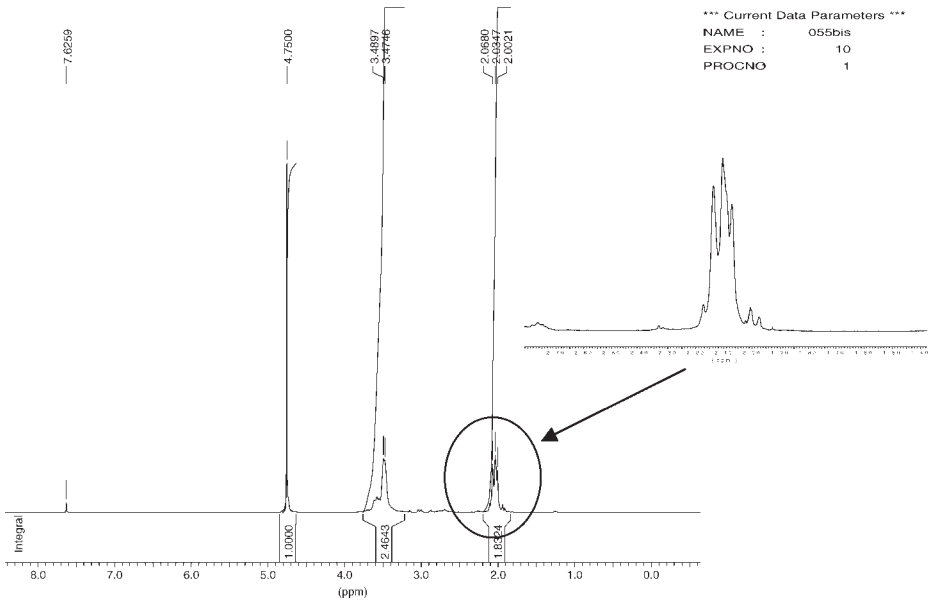

Figure 2.
^{1}H NMR spectrum of poly(2-methyl-2-oxazoline) polymerized in the presence of THF introduced 1 hour after mixing the monomer with methyl iodide initiator. The polymer is pure poly(2-methyl-2-oxazoline), as shown on the insert by the absence of a peak around 1.6 ppm.

to pTHF units. These results show that the presence of THF inhibited the initiation of MeOX polymerization by methyl iodide. The second and the third polymerization assays showed that once the initiation is obtained, the polymerization is undergoing on the initiated macromolecules but slower than when THF is absent. From these results it is possible to conclude that it is better to eliminate unreacted THF from the THF polymerization medium after quench by MeOX before the introduction of MeOX for the synthesis of the second block. Thanks to the intensity of the protons linked to the terminal functions, it is possible to calculate the molar mass of the oligomers. In one example, the poly(THF) quenched by MeOX was found to contain around 11 THF monomer units and aproximately 4 oxazoline units due to the quench. The calculation gives a molar mass Mn = 1200 Da. The same sample analysed by SEC corresponded to a molar mass of 1865. This difference can be assigned to several reasons: a part of the oxazolinium species, or of the terminal hydroxylic groups, (or both) are not linked with the oligomers. This situation could happen if the medium before quench contains free triflic acid. A second reason is provided by SEC which is not in the validity domain for the oligomers of the lowest polymerization degree, or with an improper universal calibration (or both).

Synthesis and Purification of a Block Copolymer Poly(2-methyl-2-oxazoline-b-tetrahydrofurane-b-2-methyl-2-oxazoline)

The difficulty of such synthesis is to avoid the homopolymerization of 2-methyl-2-oxazoline. Two chemical processes can be at the origin of its formation. If the system contains small amount of triflic acid, this species can be an initiator for such a polymerization. Transfer can also be at the origin of homopolymer production. Indeed, an investigation using ^{13}C NMR spectroscopy revealed that poly(THF) terminated by an OH group has the α-carbon resonating around 62.3 ppm ($CDCl_3$), and the content of this carbon using the gated NMR technique can be quantitatively

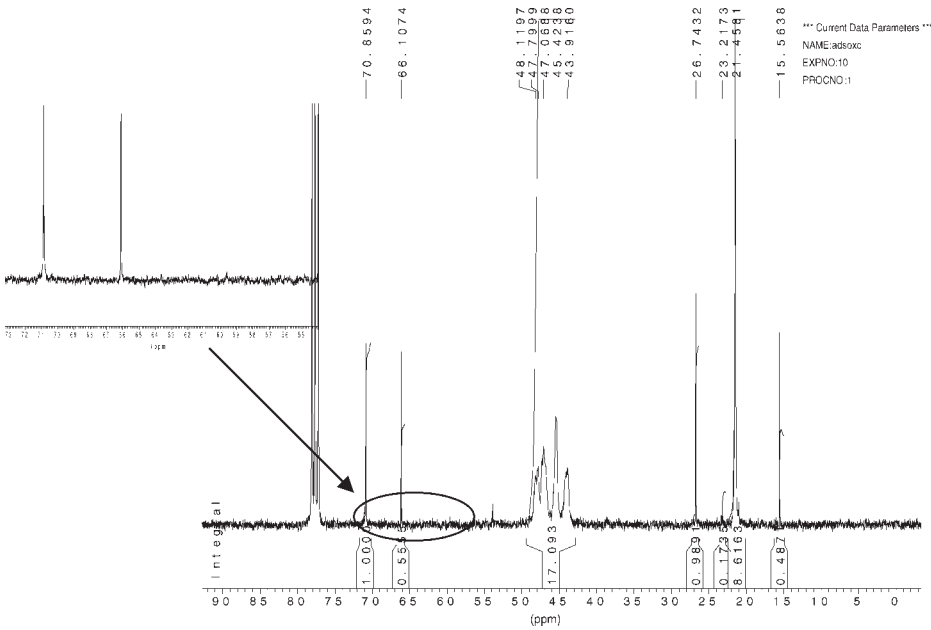

Figure 3.
Gated ^{13}C NMR spectrum of crude block copolymer poly(MeOXZ-b-THF-b-MeOXZ) in $CDCl_3$. It can be seen in the insert the absence of any peak around 62.3 ppm which shows the absence of pTHF-OH chain ends.

measured, allowing the determination of molar mass by the comparison with the intensity of the carbons of the monomer units. In the case of this synthesis in our conditions, this carbon has always been absent (Figure 3). This observation entails some consequence. It means that the transformation of the THF active species into an oxazolinium active species was complete, within the accuracy of these experiments. The gated ^{13}C NMR spectrum of this polymer is shown (Figure 3). In order to separate homopoly(2-methyl-2-oxazoline) from the block copolymer it was decided to use its amphiphilic properties. The polymer was dissolved in chloroform, and water was added. After vigorous stirring, the system decanted into three phases. The upper one is an aqueous solution of mainly poly(2-methyl-2-oxazoline), as shown by the analysis of the residue after evaporation (see below). Its ^{13}C NMR spectrum is shown on Figure 4.

This fraction of the block copolymer which was soluble in water after emulsification in a $CHCl_3$/water mixture contained only about 1.5% in weight of poly(THF), while the emulsified fraction contained about 7.6% in weight of poly(THF) (Figure 5). The lower one is a chloroformic phase practically pure, and the intermediate white phase is an emulsion strongly aggregating with time. After separation, this white phase was dried under vacuum giving a polymer which was a practically pure block copolymer. Its gated ^{13}C NMR spectrum of the emulsified fraction of the block copolymer after workup is shown on Figure 6. This spectrum allowed to calculate a monomer unit ratio THF/MeOX of around 7.8% and an average number molar mass of 5500 Da, using the intensity of the peak at 22 ppm which corresponds to a β-carbon of the first THF monomer unit neighboring a MeOX unit. NMR analysis was carried out on the crude polymer, on the polymer collected in the aqueous phase after drying under vacuum, and on the emulsion after drying under vacuum. The results are shown on Table 1.

On Table 1, it can be seen that the extraction of the high molar mass by the water phase from the aggregated emulsion is successful in that the polymer extracted by water is richer in poly(MeOXZ) than the crude polymer. While being small, the THF unit content of this polymer is clearly seen on the spectrum, (Figure 4). The molar mass of the crude polymer can be overestimated, because of the uncertainty linked with the determination of the carbon atom content for the block junction one (carbon at 22 ppm). A molar mass determination for the crude polymer by SEC

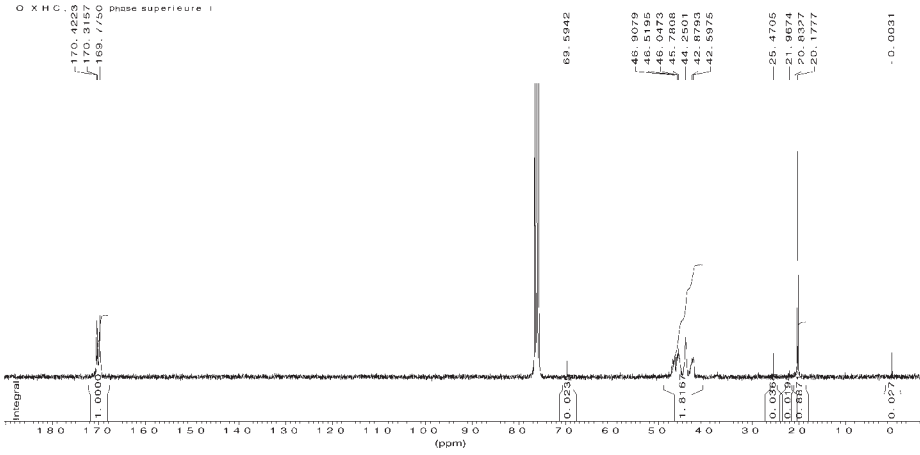

Figure 4.
Gated ^{13}C NMR spectrum of the fraction of the block copolymer soluble in water after extraction of the emulsified phase.

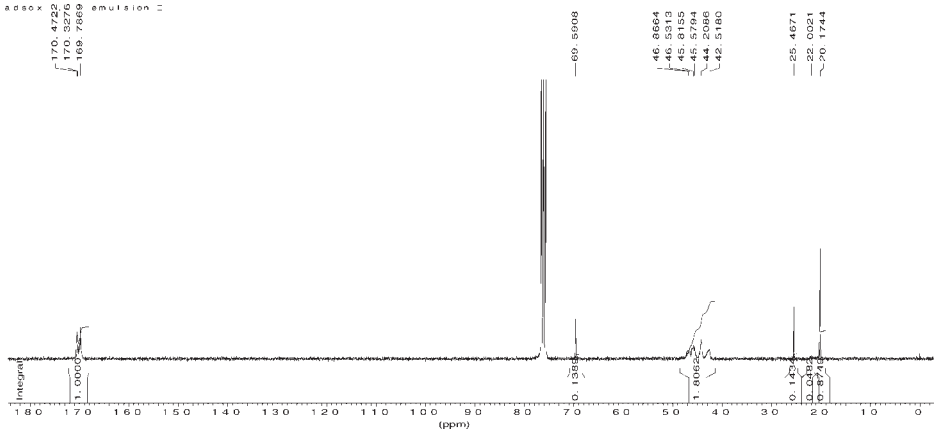

Figure 5.
Gated ^{13}C NMR spectrum of the emulsified fraction of the block copolymer after separation of the emulsified phase, drying and dissolution in $CDCl_3$.

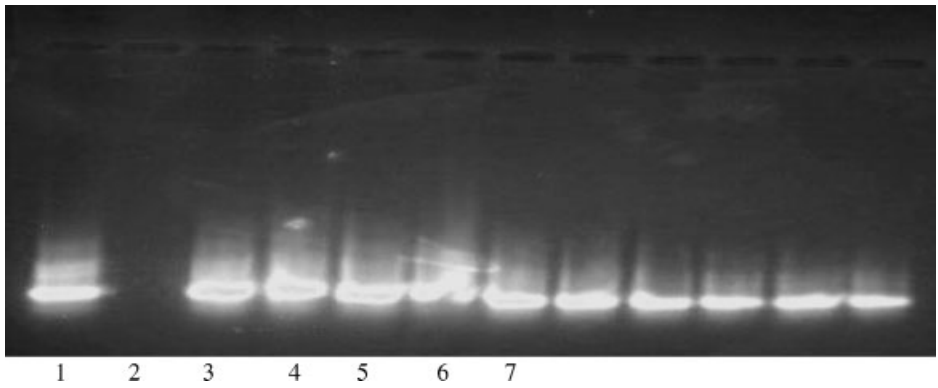

Figure 6.
Electrophoresis of triblock1/DNA mixture, showing that the block copolymer does not appreciably interact with DNA even at high concentration (well 1: DNA alone). Each well was filled with a mixture of 5 μL of DNA (1 mg/mL), 3 μL of TBE5X, 2 μL of charge buffer and 10 μL of a solution of polymer (pH = 8): well N°3 Co = 225 mg/mL, well N°4 Co/2, well N°5 Co/3, well N°6 Co/4, etc. up to well N° 12 Co/10.

gave a value of 4900 Da, which is in agreement with the above conclusions. Finally, the block copolymer collected in the emulsion phase, called triblock 1 in the following, which corresponds to a central p(THF) block of DPn of around 4.6 between two poly(MeOX) side chains each of DPn of around 30.5 was used to check its

Table 1.
^{13}C NMR analysis of poly(MeOXZ-b-THF-b-MeOXZ) after emulsion phase separation.[a)]

	Monomer units ratio MeOXZ/THF	DPn of the p(MeOX) block.	Molar mass of the polymer (Mn/Da).
Crude block copolymer	17	60	10800 Mn SEC = 4900
Polymer collected in the aqueous phase*	65,4	43	7500
Block copolymer in the emulsion	13	30,5	5500

[a)]The peak at 22 ppm was used allowing molar mass determination.

behavior in various situations useful to understand its transfection efficiency.

Study of the Behavior of the Block Copolymer Triblock 1 in Physiological Conditions

Study of DNA Complexation by Electrophoresis

It was first verified that that the uncharged polymer triblock 1 did not complex DNA. High polymer to DNA ratio did not prevent DNA migration in electrophoresis on agarose gels. Various composition ratios (w/w) polymer/DNA were used in electrophoresis experiments in a ratio range 8–16. For none of these mixtures any DNA migration retardation was noticed. This behavior is similar to that of poloxamer PE 6400 which was shown to be a good vector for DNA transfection on muscle tissues.[11] It is to be noticed that polymer hydrolysis by HCl 0.6M gave a polymer 47% of its p(MeOXZ) units of which were hydrolysed. This polymer was also used for the sake of comparison.

Keeping in mind these results, the complexation ability of triblock 1 was examined with much higher polymer concentration. A concentration of 225 mg/mL for the mother solution was used and the results are shown on Figure 6.

It can be seen that even at the highest concentration (well N°3), at a ratio of 450 (w polymer triblock 1/w DNA) a moderate retardation is only visible, if any, and is completely absent (well N°7) for a weight ratio of 90 or less.

When the polymer was hydrolysed at a ratio of 47% of its oxazoline monomer units, the complexation was more efficient. It can be seen that complete complexation could be obtained and the migration of DNA is reversed between wells 4 and 5 (Figure 7). Thus, complexation and DNA charge neutralisation could be obtained for a weight ratio (47% hydrolysed) triblock 1/DNA between 55 and 73. It easy to calculate that if charge neutralisation would happen at a one-to-one charge ratio, the weight ratio polymer/DNA would be of the order of 0.5. These results show that the block copolymer, while positively charged, does not strongly interact with DNA.

By comparison with the effect of polyethylenimine which is a strong DNA complexing agent, these results indicate that for such a complexation the charge density on the polymer plays an important role.

Determination of Triblock 1-DNA Interactions by Neutron Scattering

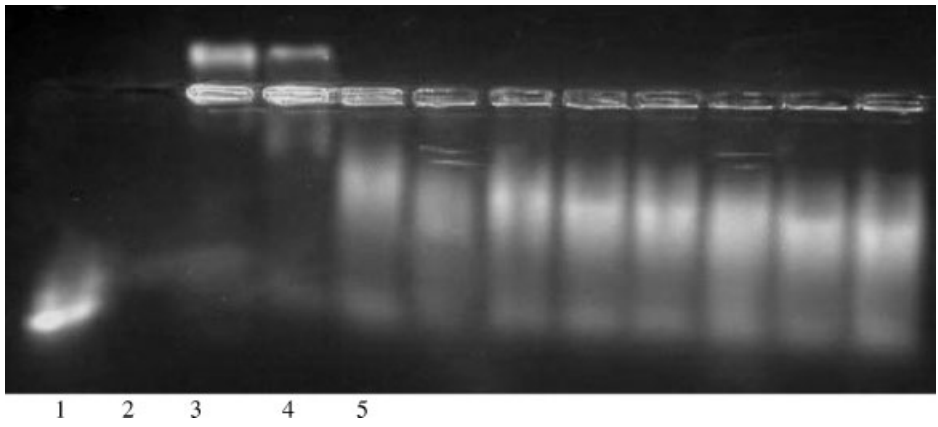

Figure 7.
Electrophoresis of 47% hydrolysed triblock 1/DNA mixture. DNA charge neutralisation is observed for a polymer concentration between that of wells 4 and 5. Each well was filled with a mixture of 5 μL of DNA (1 mg/mL), 3 μL of TBE5X, 2 μL of charge buffer and a few μL of a solution (90 mg/mL) of polymer (pH = 8): well N°3 v = 2 μL, well N°4 v = 3μL, well N°5 v = 4μL, well N°6 v = 5 μL, etc. up to well N° 12 v = 11 μL.

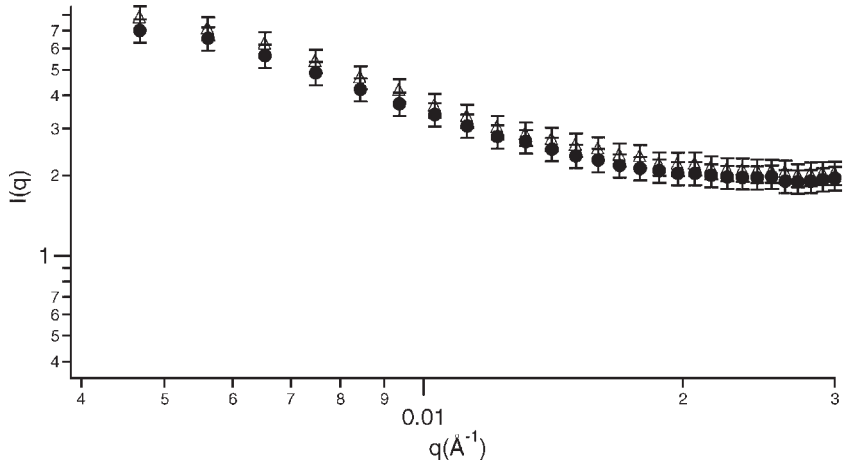

Figure 8.
Neutron intensity scattered at small angles by solutions of 5 mg/ml of 47% hydrolysed triblock 1 in the presence (triangles) and in the absence (circle) of DNA (146 bp at 5 mg/ml).

The weakness of the interactions between DNA and the hydrolysed triblock was a little surprising. It was decided to check this result by small angle neutron scattering. The intensity of the scattered neutrons by the polymer was measured in the absence and in the presence of DNA (146 bp). Results are shown on Figure 8 which shows that the intensity scattered by the polymer solutions in the absence and in the presence of DNA are practically identical, confirming the weakness of the interactions between DNA and the positively charged triblock 1.

Determination of the Formation of Mesophases by the Hydrolysed Triblock 1

The question which was addressed was the following: since hydrolysis of the methyl-oxazoline units increases the hydrophilicity, does the hydrolysed triblock form mesophases? It can be taken for granted that the non hydrolysed triblock forms mesophases, since this formation was used to purify the

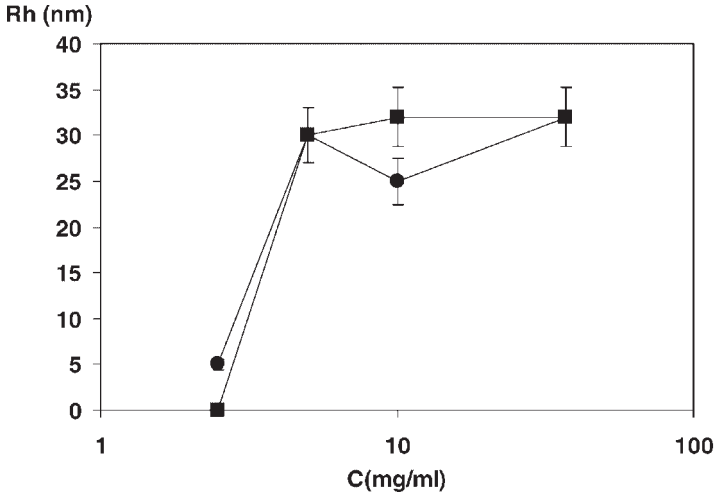

Figure 9.
Variation of the hydrodynamic radius Rh as a function of the 47%-hydrolysed triblock concentration for two temperatures 22 °C (squares) and 37 °C(circles).

material, as explained above. In the case of the 47%-hydrolysed one, this question deserved a particular study. Dynamic light scattering was used for this investigation. The critical micelle concentration (cmc) was determined at two temperatures (22 and 37 °C), following the measurement of the hydrodynamic radius as a function of the polymer concentration. The results are shown on Figure 9. It is clear that the hydrolysed polymer triblock 1 forms still mesophases: cmc was independent of temperature in the range of the experiments (22–37 °C), and is found to be between 2.5 and 5 mg/mL. This result is important in that it shows that, despite a higher hydrophilicity due to the transformation of methyl-oxazoline units into ethylenimine ones which can be cationized at least partly in physiological conditions, there is still formation of mesophases which could interact with the hydrophobic part of DNA. Of course, the positive charges on the hydrolysed triblock 1 can increase the interaction with the negatively charged DNA. This point has been evidenced with the electrophoresis experiments above. However, it has been shown that this effect on DNA complexation was rather modest.

Electrophysiological Measurements Using Model Membranes

The behavior of the triblock 1 towards a lipid bilayer mimicking a cell membrane was determined by electrophysiological measurements. The bilayer was formed of a membrane of diphytanoyle-lecithine between two compartments filled with 1 mL of an aqueous solution of KCl 1M-Hepes 5 mM (pH = 7.5). The resistance of the membrane was of the order of one Giga-Ohm. The membrane capacity was 84 pF corresponding to a thickness of 3.7 nm. It was observed that 15 minutes after polymer mixing (10 μL of an aqueous solution of KCl 1M-Hepes 5 mM of the polymer at 10 mg/mL) short conductivity jumps were observed (Figure 10, right plot). It is possible to analyse the structure and distribution of the interactions of the polymer molecules with the membrane. However, after this first step, the formation

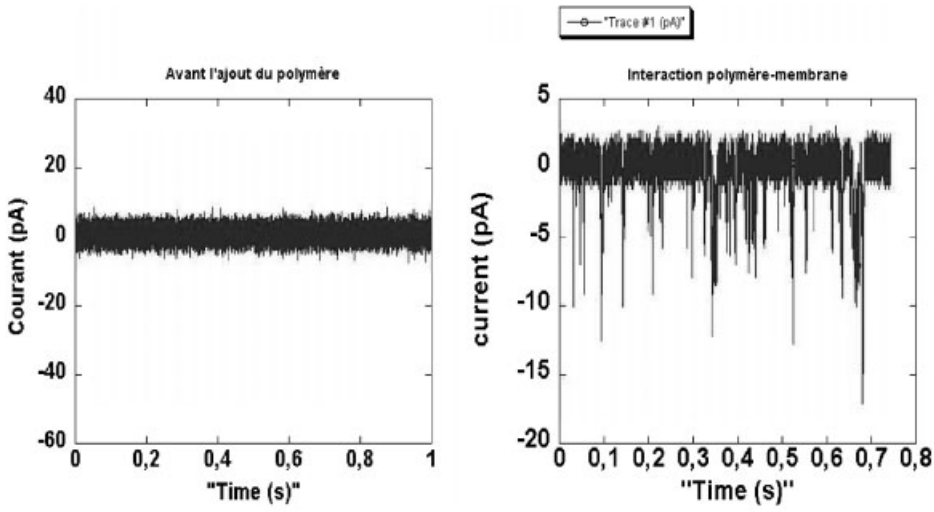

Figure 10.
Interaction of triblock 1 with a membrane of diphytanoylphosphatidylcholine-Lecithine between two compartments containing 1 ml of 1M KCl, 5 mM HEPES (at pH 7.5) under electric field (100 mV). 100 μL of its solution (at a concentration of 10 mg/mL) were added to the cell (right plot). The left plot shows a stable current intensity (0 pA) before the introduction of triblock 1. The right plot shows the intensity jumps due to triblock 1/membrane interactions.

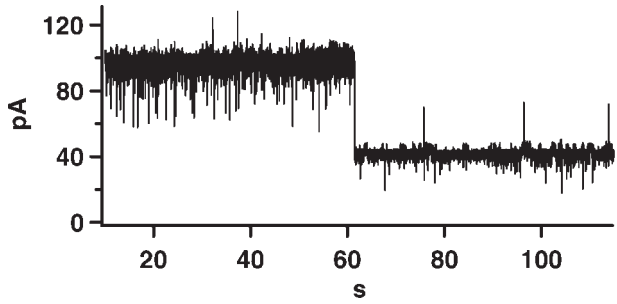

Figure 11.
Interaction of hydrolysed triblock 1 with a membrane of diphytanoylphosphatidylcholine-Lecithine between two compartments containing 1 ml of 1M KCl, 5 mM HEPES (at pH 7.5) under electric field (75 mV). 100 μL of triblock I solution (at a concentration of 10 mg/mL) were added to the cell (right plot). This plot shows between 0 and 60 sec a stationary current intensity close to 90 pA due to the presence of 2 permanent holes in the bilayer lipid membrane, while after 60 sec one hole closed and the current corresponded two only one hole (appr. 45 pA).

of a more permanent hole was observed (Figure 11). For a voltage of −100 mV, a current intensity of 20 pA was measured. Using the geometry of the system and the known characteristics of the solutions, it can be calculated that this intensity corresponds to a hole of around 0.7 nm diameter.

When the hydrolysed triblock 1 was used, it was possible to see that it interacted with the membrane with formation of holes with duration of the order of one second. A second regime was also observed with the formation of more stable holes. From the frequency of hole formation and from the stability of the holes in the membranes, it was shown that in our conditions, the hydrolysed triblock 1 was approximately 8.10^4 times more efficient than the non hydrolysed triblock 1 to get included in the membranes.

In all cases, when DNA was added in the compartment containing the polymer, no DNA crossing was observed. This is assigned to the size of holes which is not enough large to offer a way for DNA. This was confirmed by the fact that once a hole was created, the introduction of DNA in the polymer compartment induced hole closure.

Conclusion

A strategy for the synthesis of triblocks poly(2-methyl-2-oxazoline-b-tetrahydrofurane-b-2-methyl-2-oxazoline) has been developed. It was shown that the technique of polymerization of the second block from the living species created on the two chain ends of poly(THF) was successful but made difficult the control of the size of the poly(THF) block due a fast depolymerization upon the introduction of the second monomer. A purification technique was used to get rid of the possible homopoly(2-methyl-2-oxazoline) formed. NMR analysis was also used to characterize the triblock and thanks to carbon atoms at the junction of the two blocks, a determination of the average number molar mass was possible. Various analytical techniques were used to characterize the behavior of a chosen polymer, corresponding to a poly(THF) central block (DPn = 4.6) between two side blocks of poly(MeOX) (DPn = 30.5), in conditions close to its possible use as a transfection agent, and more particularly in the presence of DNA. These techniques, electrophoresis and neutron scattering, demonstrated that the above neutral triblock does not appreciably interact with DNA. Thus, the behavior of this triblock is similar to that of the poloxamer PE6400.[11] More surprisingly, it was also shown that the triblock for which approximately half (47%) of the methyloxazoline units were transformed into ethylenimine units by hydrolysis gave only loose interactions with DNA, despite being positively charged.

This result is assigned to the fact that charge density plays a major role in the interactions of positive polyelectrolytes with the negatively charged DNA. The triblock was shown being able to interact with bilayer lipid membranes mimicking cell membranes, but the efficiency of the 47% hydrolysed triblock is much higher, while the size of holes created in the membranes is not large enough to give passage to DNA.

Altogether, this study is a basis allowing the determination of the parameters governing DNA transfection with this type of polymers. In the next study, the structure of the triblock will be varied having in view an efficient tool for DNA transfection.

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