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Copolymers of ε-caprolactone and quaternized ε-caprolactone as gene carriers

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Abstract

New copolymers of ε-caprolactone (CL) and 7-bromo-ε-caprolactone quaternized by pyridine (Py+CL) were investigated as non-viral vectors for gene delivery. Copolymers with two molar compositions (50 Py+CL/50 CL and 80 Py+CL/20 CL), each with a diblock or a random structure, were used to prepare nanoparticulate complexes with DNA. Average size and surface charge of the complexes and extent of the complexation were measured. The DNA condensation by the copolymers was analysed by a gel retardation assay. Cytotoxicity and transfection efficiency of the copolymers were also evaluated in HeLa cells and compared with polyethylenimine 50 kDa. The size of the polyplexes was approximately 200 nm. The zeta potential first increased with the copolymer/DNA charge ratio and became positive for charge ratios in the 2-4 range depending on the type of copolymer. DNA was completely condensed within the nanoparticles and the degree of interaction was very high. Cytotoxicity and transfection efficiency were found to be comparable to polyethylenimine 50 kDa. The experimental results suggest that the novel copolymers can be used as novel gene delivery vectors.

Keywords: Nanoparticles; Gene therapy; Polyplexes; Plasmid; Transfection

1. Introduction

Gene therapy is a promising technique for the treatment of both genetic and acquired diseases [1]. Since DNA is a large hydrophilic molecule with an overall negative charge, it does not pass easily in the cellular membranes [2]. Furthermore, plasmid DNA is very prone to nuclease degradation and is rapidly cleared from the bloodstream [3]. Therefore, a carrier is required to bring the plasmid into the target cell and to get an acceptable gene expression level. An ideal gene delivery vehicle should be stable in the bloodstream, should protect DNA against degradation by serum nucleases and should be small enough to extravasate. Among gene delivery systems, "polyplexes" are attractive, because of a spontaneous formation by electrostatic interactions between the positively charged groups of a polycation and the negatively charged phosphate groups of DNA. As result of this condensation, DNA is protected against the nuclease digestion and is more efficiently delivered into the cells [4]. Many cationic polymers have been explored as non-viral vectors. They include poly-L-lysine (PLL) [5-7], polyethylenimine (PEI) [8-12], dendrimers [13-16], chitosans [17-20] , poly(2-dimethylami-noethyl methacrylate) (pDMAEMA) [21] or poly-L-glutamic acid derivatives [22]. However, the transfection efficiency of these polymers is low compared to the viral vectors and many cationic polymers are relatively toxic and/or not biodegradable.

This paper aims at evaluating a new nanoparticulate delivery system consisting of poly(ε-caprolactone) (PCL) bearing pendant pyridinium groups, as a non-viral gene delivery vector. Copolymers of ε-caprolactone (CL) and γ-bromo-ε-caprolactone (BrCL) were prepared with a statistical and a blocky structure, and the bromide pendant groups were quaternized by pyridine. These copolymers fulfill requirements for transfection of plasmid DNA: (i) They are positively charged under physiological conditions and they can bind and condense DNA with formation of small-sized particles [14] ; (ii) The copolymers are biodegradable. They are hydrolyzed in α-hydroxy acids, easily eliminated by the usual metabolic pathways [23].

Capacity of the novel quaternized copolymers to be effective in the transfection of plasmid DNA, was first evaluated by measurement of the size and surface charge of the nanoparticles. DNA condensation by the copolymers was analysed by a Picogreen® assay and a gel retardation assay. The cytotoxicity of the copolymers and copolymer/DNA complexes with various charge ratios was determined by a MTT assay. Finally, the transfection efficiency of polyplexes was evaluated with a luciferase gene reporter and compared to polyethylenimine 50 kDa, a conventional polymer used in gene therapy.

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2. Materials and methods

2.1. Materials

Plasmid pGL2CMVLuc (6.233 kb) encoding luciferase gene reporter and under the control of a human CMV promoter was obtained from Gibco® BRL (Carlsbad, CA, USA). 7-Oxabicy-clo[2.2.1]heptane (Sigma-Aldrich, St. Louis, MO, USA), 48% (aqueous) HBr (Sigma-Aldrich, St. Louis, MO, USA), pyr-idinium chlorochromate (Sigma-Aldrich, Bornem, Belgium) and 75% *m*-chloroperoxybenzoic acid (Sigma-Aldrich, Bornem, Belgium) were used as received. Br-CL was synthesized starting from 7-Oxabicyclo[2.2.1]heptane as reported elsewhere [24] and dried by repeated (three times) azeotropic distillation of toluene just before polymerization. CL (Sigma-Aldrich, Bornem, Belgium) was dried over calcium hydride for 48 h at room temperature and distilled under reduced pressure just before use. Toluene (ChemLab, Somme-Leuze, Belgium) was dried by refluxing over calcium hydride and distilled under nitrogen atmosphere. Aluminium isopropoxide [$Al(O^iPr)_3$] (Sigma-Aldrich, St. Louis, MO, USA) was twice sublimated and then dissolved in toluene under nitrogen.

HeLa (human cervic carcinoma) cells were acquired from ATCC (American Type Culture Collection, Manassas, VA, USA). Ampicillin, ethidium bromide, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (MTT) and PEI 50 kDa polymer were purchased from Sigma-Aldrich (St. Louis, MO, USA). PEI 50 kDa was used as 10 mM aqueous stock solution [9 mg of the 50% (wt/vol) commercial solution diluted in 10 ml of water]. The solution was neutralized with HCl and filtered (Millipore, 0.2 μm). Agarose, restriction enzyme *Hin*dIII, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin mixtures were from Gibco® BRL (Carlsbad, CA, USA). DNA molecular weight marker II (0.12-23.1 kbp) was acquired from Roche (Indianapolis, IN, USA). The Endofree plasmid kit was obtained from Qiagen (Hilden, Germany), the Picogreen® assay kit from Molecular Probes (Leiden, The Netherlands), the Micro BCA Protein Assay Kit from Pierce (Rockford, USA) and the Luciferase Assay System from Promega (Madison, USA). Ultrapure water was used throughout and all other chemicals were of analytical grade.

2.2. Preparation of plasmid DNA

The plasmid pGL2CMVLuc was propagated in DH5α *Escherichia coli* competent cells (using 50 mg/ml ampicillin as selective antibiotic), extracted by the alkali lysis technique and purified by column chromatography with a Endofree plasmid kit according to the manufacturer's instructions. The purity and integrity of the plasmid DNA were assessed by UV spectroscopy ($A_{260\ nm}/A_{280\ nm}$ ratio) and by agarose gel electrophoresis. The concentration of the plasmid was determined by UV spectroscopy (1 OD = 50 μg/ml at 260 nm) [5].

2.3. Synthesis and characterization of statistical and diblock copolymers of CL and BrCL

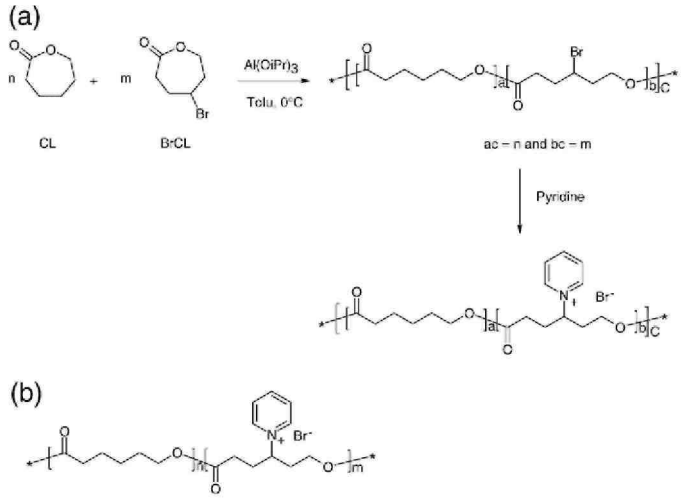
Synthesis and characterization of the statistical and diblock copolymers were reported elsewhere [23, 24]. Both diblock and random copolymers of two molar compositions (20 mol% CL and 50 mol% CL) were synthesized and quaternized by pyridine (Fig. 1).

2.3.1. Polymerization technique [24]

Random copolymerization was carried out in a previously flamed glass reactor in toluene at 0 °C. BrCL, CL, toluene and the initiator [$Al(O^iPr)_3$ in toluene] were successively added to the reactor through a rubber septum with a syringe or a stainless steel capillary. After polymerization (2.5 h), an excess of 1 N HCL was added, and the copolymer was recovered by precipitation in cold methanol.

Block copolymers were prepared by sequential polymerization of CL and BrCL initiated by $Al(O^iPr)_3$ in toluene at 0 °C. After 45 min, an aliquot of the first PCL block was picked out, deactivated by HC1 and precipitated in cold methanol for analysis by size exclusion chromatography and proton NMR. After the complete reaction of the second comonomer (BrCL; 2.5 h), an excess of 1 N HC1 was added and the copolymer was precipitated in cold methanol.

***Fig. 1**. (a) Synthesis and chemical structure of random copolymers; (b) Chemical structure of diblock copolymers.*



2.3.2. Quaternization reaction [23]

In a representative experiment, 0.5 g of copolymer was dissolved in 5 ml of pyridine and the mixture was heated under stirring at the desired temperature. At the end of the reaction, the excess of pyridine was eliminated under reduced pressure, and the polymer was purified by repeated precipitation from THF to heptane.

2.3.3. Characterization [24]

The copolymer composition and degree of polymerization [defined as the number of monomeric units per chain] were determined by proton NMR. Proton NMR spectra were recorded in $CDCl_3$ with a Bruker AM 250 (250 MHz) apparatus at 25 °C. Apparent molecular weight and polydispersity were determined by size exclusion chromatography in THF with a Waters chromatograph connected to a Waters 410 differential refractometer. Four 5 µm Waters columns of increasing pore size (100, 1000, 10^5 and 10^6 Å) were used and calibrated with polystyrene standards.

2.4. Preparation of nanoparticles of quaternized copolymer/ DNA complexes

Nanoparticles were prepared by the solvent evaporation technique [25,26]. Briefly, a quaternized copolymer was dissolved in dichloromethane. The organic phase was then emulsified in water (for biophysical studies) or culture medium (for cytotoxicity and transfection studies) containing the plasmid DNA solution with a high-speed homogenizer Diax 900 (Heidolph Instruments, Germany) at 8000 rpm for 1 min. Finally, the emulsion was stirred at room temperature for 2 h in order to remove the organic solvent. For all formulations, plasmid DNA was used with a fixed concentration of 50 µg/ml (for biophysical studies) or 10 µg/ml (for cytotoxicity and transfection studies), while the copolymer quantity was varied to yield various copolymer/DNA charge ratios N/P, i.e. from 0.1 to 25. This charge ratio N/P is merely the ratio between the number of positive charges on the copolymer and the number of negative charges on DNA.

2.5. Size of the nanoparticles

The average particle size and size polydispersity of the quaternized copolymer/DNA complexes prepared in water were determined by photon correlation spectroscopy using a Malvern HPPS 3.3 Zetasizer or a Malvern Nano ZS (Malvern Instruments, UK). Scattered light was detected at an angle of 90° and a temperature of 25 °C. The viscosity (0.89 mPa s) and the refractive index (1.33) of distilled water at 25 °C were used for data analysis. The instrument was calibrated with standard latex particles (Malvern Instruments, UK). Experimental values were the average of at least 3 measurements.

2.6. Zeta potential of the nanoparticles

Zeta potential of nanoparticles formulated in water was measured with a Malvern Zetasizer 2000 or a Malvern Nano ZS (Malvern Instruments, UK) at 25 °C. Polystyrene nano-spheres (-50 mV ± 5 mV) were used as standards (Malvern Instruments, UK). Experimental data were the average of at least 5 measurements.

2.7. Interaction of quaternized copolymers and DNA

Interaction of plasmid DNA with quaternized copolymers was estimated from the difference between the total amount of DNA used in the nanoparticles preparation and the amount of DNA left in the supernatant solution after centrifugation of the nanoparticles at 15,000 rpm for 60 min [27]. The amount of free pGL2CMVLuc in the supernatant was determined from the fluorescence developed by the Picogreen® reagent and measured with a Packard Fluorocount Microplate Fluorometer (Packard BioScience Company, Meriden, USA). A calibration curve was set up with standard DNA solutions in the 25 ng/ml to 1 µg/ml concentration range (Molecular Probes, Leiden, The Netherlands). The lack of interference of nanoparticles, which could remain in the supernatant following the centrifugation process, and the absence of sedimentation of free DNA were demonstrated.

2.8. Gel retardation assay

Quaternized copolymers/DNA complexes were electrophor-esed on 1% (w/v) agarose gel in TBE buffer (Tris base 0.090 M, boric acid 0.89 M, EDTA 0.5 M, pH = 8) at 75 V for 3 h. The gel was stained with ethidium bromide (0.1 µg/ml) for 24 h and observed with a UV transilluminator in order to localize the plasmid DNA.

2.9. Cell culture

HeLa cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 100 IU/ml of penicillin G sodium and 100 µg/ml of streptomycin sulfate. The cells were maintained in an incubator supplied with 5-95% CO_2-O_2 at 37 °C.

2.10. Cell viability

Cytotoxicity of the quaternized copolymers and their complexes with DNA was evaluated for different charge ratios by the MTT assay [28]. Briefly, HeLa cells were seeded in 96-well microtiter plates at a density of 10 cells/well in 100 µl of DMEM with 10% FBS and antibiotics and grown overnight. DMEM (100 µl) containing quaternized copolymers at different concentrations (ranging from 0.025 µg/ml to 500 µg/ml) and complexes with DNA at various charge ratios was then added. The cells were incubated for 4 h, after which the metabolic activity of the cells was measured. The formulations were replaced with 100 µl of fresh DMEM containing 50 µg MTT and cells were incubated for additional 3 h. MTT containing medium was aspired off and 100 µl of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a BioRad microplate reader. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. Triton X-100 1% was used as positive control of cytotoxicity. Cytotoxicity of copolymers was compared with polyethylemine 50 kDa, a conventional polymer used in gene therapy, and poly(ε-caprolactone) polymer. The results were expressed as mean values±standard deviation of eight measurements.

2.11. In vitro transfection

For the transfection studies, HeLa cells were seeded in 96-well plate at a density of 10^4 cells/well in 100 µl of DMEM supplemented with 10% FBS. After incubation for 24 h, the medium was removed and replaced with copolymers/DNA complexes prepared in serum-free medium at various charge ratios ranging from 0.1 to 10. The total amount of plasmid DNA loaded was maintained constant at 1 µg/well. Following 2 h of incubation with complexes, 100 µl of medium supplemented with 20% FBS was added in each well. After 4 h treatment with polyplexes, medium was replaced with fresh medium containing serum and cells were incubated for an additional 44 h to allow protein expression. Transfection experiments with 50/50 diblock copolymer were also performed in the presence of 10% serum. Cytotoxicity and luciferase expression were evaluated in two independent 96-wells plates. Experiments were carried out in triplicates for transfection. Six determinations were done for cytotoxicity. Two independent experiments were performed with similar results.

2.11.1. Detection of luciferase activity

The luciferase assay was carried out according to the manufacturer's instructions using a Luciferase Assay System. Briefly, culture medium was removed, cells were washed twice with PBS and 200 µl of lysis buffer was added to each well. To measure the luciferase content, 100 µl of luciferase reagent was automatically injected into 20 µl of cell lysate and the luminescence was integrated over 10 s with a Glomax microplate luminometer (Turner Biosystems, Sunnyvale, USA). Total recovered cellular protein content of the cells was determined using a Micro BCA assay kit. Bovine serum albumin (BSA) was used for calibration. Thirty microliters of the samples from luciferase detection protocol were placed in individual wells of a 96-well plate and diluted with PBS to 150 µl. BCA reagent (150 µl) was added into each well and mixed well. The plate was allowed to incubate for 2 h at 37 °C and absorbance was measured at 570 ran with a BioRad microplate reader. The amount of protein was read from the standard curve. Results of luciferase expression were expressed as relative light units (RLU)/mg protein. At least three determinations were performed for each sample. In all transfection experiments, naked DNA as well as untreated cultures were used as controls. The transfection efficiency of polyplexes was compared with polyethylenimine 50 kDa/DNA polyplexes formulated at a charge ratio N/P of 9 according to Boussif et al. [31],

2.11.2. Evaluation of cytotoxicity

The cellular viability was tested 48 h after transfection using a MTT colorimetric assay, as described previously. Briefly culture medium was replaced with fresh culture medium containing MTT and cells were incubated for 1 h at 37 °C in CO_2 incubator. MTT containing medium was replaced with 100 µl of DMSO. Absorbance was measured at 570 nm using a BioRad microplate reader.

2.12. Statistics

Statistical significance of the results was evaluated by Student's *t*-test. To compare the mean and standard deviations of more than two groups, Tukey's procedure was used. Software JMP 5.1 was used for statistical analysis.

3. Results and discussion

The aim of this study was to evaluate novel quaternized copolymers as non-viral gene delivery system. To this end, copolymers composed of ε-caprolactone monomers (CL) and 7-bromo-ε-caprolactone monomers quaternized by reaction with pyridine (Py+CL) were used to generate DNA nanoparticulate systems. Copolymers with diblock or random structure, each of them available with two monomer ratios (50 Py+CL/50 CL and 80 Py+CL/20 CL), were investigated.

3.1. Synthesis and characterization of the copolymers

The copolymers were prepared by ring-opening polymerization of CL and BrCL initiated by $Al(O^iPr)_3$. A mixture of the two comonomers with the appropriate composition was used for the synthesis of the statistical copolymers, as schematized in Fig. 1.

For diblock copolymers, CL was first polymerized under the aforementioned conditions, for 45 min, followed by the addition and polymerization of BrCL for 2.5 h and deactivation of the growing chains by HC1.

Upon reaction with pyridine, the bromide functions were quaternized.

The quaternization yield was 100 and 85% for the statistical copolymers containing 50 mol% and 80 mol% BrCL, respectively. It was 75% for the polyBrCL block of the diblock copolymers.

The molecular characteristic features of the copolymers used in this work, i.e. the quaternized poly(CL-co-BrCL) and poly (CL-δ-BrCL) copolymers, are reported in Table 1. They have quite comparable average molecular weight (12,500-13,000) and polydispersity (~ 1.25). The experimental molar composition of both the statistical and block copolymers is close to the expected values, thus 20 and 50 mol% CL.

3.2. Preparation of nanoparticles of quaternized copolymer/ DNA complexes

Polymers being not water soluble, nanoparticulate systems were formulated at different charge ratios copolymers/DNA using the solvent evaporation technique.

The formulation of nanoparticulate systems did not require the use of surfactant which are known to be cytotoxic for cells.

Nanoparticles present several advantages for the delivery of plasmid DNA : (i) Nanoparticles, because of their subcellular size, are actually endocytosed by the cells which could result in higher cellular uptake of the entrapped DNA [32] ; (ii) Since DNA is encapsulated within a polymeric matrix, it would be protected from extracellular and intracellular nuclease degradation [33].

***Table 1**: Molecular characteristic features of the polymers used in this study*

Copolymers	Molecular weight[a]	Polydispersity[a]	DP[c]	mol% of cationic units[b]
Poly(CL-co-Py+CL) 50/50	12,500	1.23	79	48
Poly(CL-co-Py+CL) 20/80	13,000	1.24	74	78
Poly(CL-b-Py+CL) 50/50	12,300	1.26	69	49
Poly(CL-b-Py+CL) 20/80	13,000	1.27	67	79
Poly(CL)	15,400	1,15	-	-
Poly(CL-*b*-PEG)	14,900 CIV 4600 PEG	1,15	-	-

[a]Apparent molecular weight and polydispersity were determined by size exclusion chromatography (polystyrene calibration).
[b]Determined by proton NMR.
[c]DP: Degree of polymerization, determined by proton NMR.

3.3. Size and zeta potential of the nanoparticles

Capacity of the quaternized copolymers to be effective in the transfection of plasmid DNA, was first evaluated by measuring the size and surface charge of the nanoparticles. For efficient endocytosis and gene transfer, the complex must be small and compact [34,35]. The surface charge of the DNA delivery systems is known to be one of the major factors influencing their biodistribution [36] and transfection efficiency [37].

The average particle size and polydispersity index of copolymers/DNA complexes were measured by photon correlation spectroscopy (Fig. 2). For all the copolymers, it was found that particle size depends on the structure of the polymer and the charge ratio. Complexes with a size between 150 and 400 nm were formed at low charge ratios depending on the type of copolymer. Larger particles with a high polydispersity were formed at a charge ratio of 2 for the two types of copolymers (random and block) with a 50/50 composition and at a charge ratio of 4 when the composition was 20/80. At these charge ratios, the surface charge of the complexes was close to neutrality, as confirmed by zeta potential data (Fig. 3). This observation is in agreement with previous studies which indicated that aggregation between the complex particles occurs at a near neutral surface charge, at which charge-to-charge repulsion between complex particles becomes minimal [6,29]. Nanoparticles with a size of around 200 nm were produced above the charge ratio of 4. No further change of the size of the complexes was observed between the charge ratios of 4 and 25.

The results of zeta potential studies are shown in Fig. 3. As expected, the copolymers alone possessed a positive zeta potential value (e.g., 80.3±3.0 mV for poly(CL-co-Py+CL) 50/50 copolymer). For all the copolymers investigated, zeta potential values progressively increased with the charge ratio of copolymer/DNA complexes. Complete shielding of negative charges of plasmid DNA occurred at the charge ratio of 2 for the random and block copolymers of a 50/50 composition and at a charge ratio of 4 when the molar composition is changed to 20/80. At the charge ratio of 25, the surface zeta potential value reached a saturation level (e.g., 53.9± 1.1 mV for poly(CL-co-Py+CL) 50/50 copolymer).

Size and surface charge of copolymer/DNA complexes formulated by addition of DNA after the formation of cationic nanoparticles were also determined. Similar sizes were observed. However, zeta potential values

obtained (40.3 ± 0.7 mV) were slightly lower than these of the complexes prepared by adding DNA during the formation of nanoparticles (46.5 ±2.7 mV). Data could suggest that DNA could be partially encapsulated in nanoparticulate systems using the second method of preparation. This could be explained by electrostatic interactions between quaternized caprolactone present in the core of nanoparticles and DNA.

Fig. 2*. Particle size for the quaternized copolymers/DNA complexes at various charge ratios N/P. Values are the average of three measurements±standard deviation.*

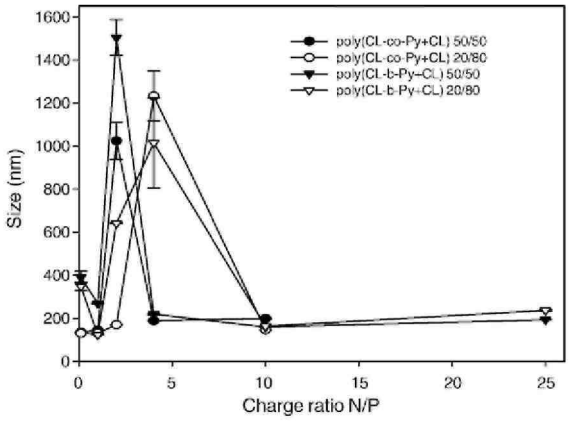


Fig. 3. *Zeta potential values of copolymers/DNA complexes at various charge ratios N/P. Values are the means of five measurements± standard deviation.*

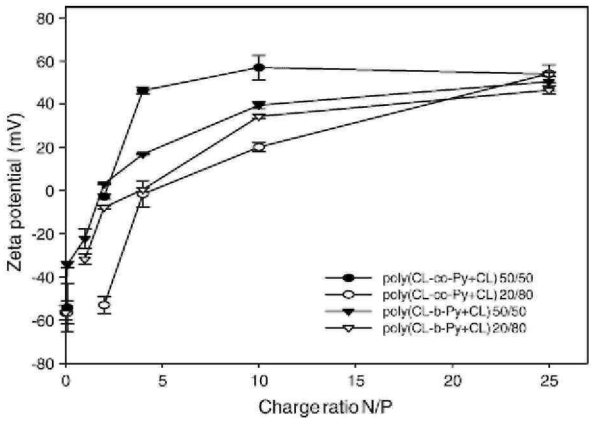


Fig. 4. *Degree of interaction between copolymers and plasmid DNA.*

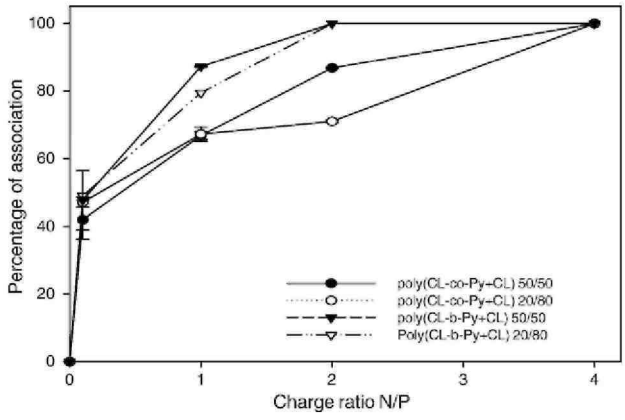
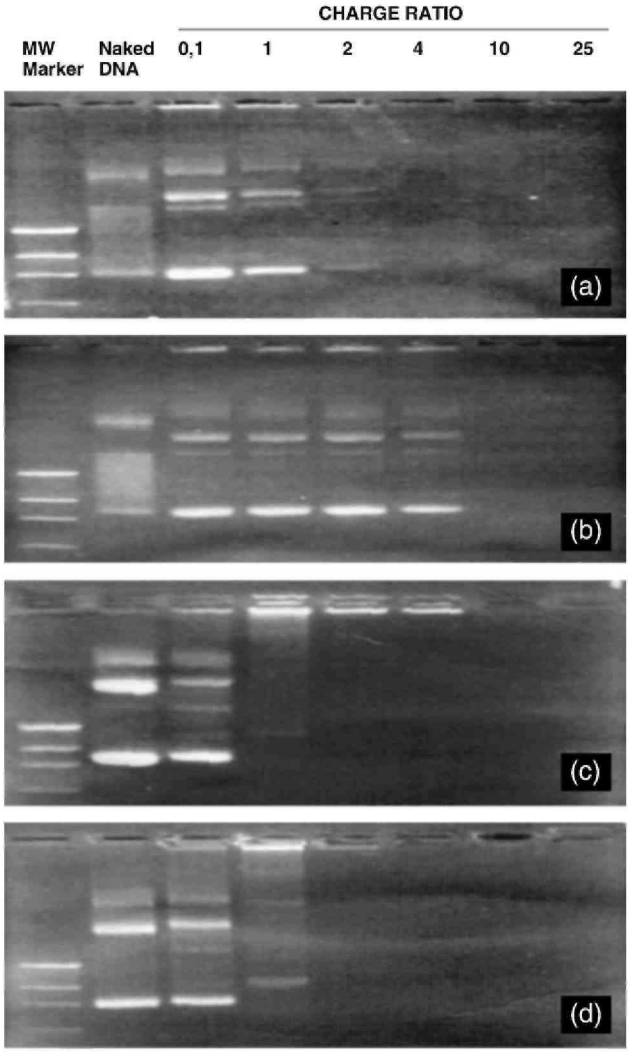


Fig. 5. *Agarose gel electrophoresis of copolymers/DNA complexes. Panel (a) poly(CL-co-Py+CL) 50/50; (b) poly(CL-co-Py+CL) 20/80; (c) poly(CL-b-Py$^+$CL) 50/50; (d) poly(CL-b-Py$^+$CL) 20/80. Lane 1: DNA molecular weight marker; lane 2: plasmid DNA alone (0.5 µg); lanes 3 through 8: DNA (0,5 µg) with progressively increasing proportions of copolymers.*



3.4. Degree of interaction copolymers/DNA

A gene carrier should bind sufficient amounts of plasmid DNA to ensure reliable delivery of DNA into the cells. In order to evaluate the degree of binding of the quaternized copolymers to plasmid DNA, a Picogreen® assay was performed (Fig. 4). Picogreen® reagent is an ultrasensitive fluorescent nucleic acid stain for quantifying DNA in solution. This method was found to be highly reproducible and very sensitive for monitoring complex formation. The degree of interaction between quaternized copolymers and plasmid DNA progressively increased according to the charge ratio copolymer/DNA for all the copolymers investigated. The binding of copolymer to plasmid DNA was complete from a charge ratio of 4 for the random poly (CL-co-Py+CL) copolymers and from a charge ratio of 2 for the block poly(CL-*b*-Py+CL) copolymers. Therefore, the blocky structure is more favorable than the statistical one for interaction with DNA.

3.5. Gel retardation assay

Complex formation between the quaternized copolymers and plasmid DNA was qualitatively confirmed by a gel retardation assay (Fig. 5). DNA molecular weight marker (0.12-23.1 kbp) in lane 1 was used to evaluate the molecular weight of the fluorescent bands. The three fluorescent bands in lane 2 corresponded to the various topological forms (supercoiled, open circular and linear forms) of free plasmid DNA. Plasmid DNA was combined with the copolymers at various charge ratios (lanes 3 to 8). The electrophoretic mobility of plasmid

DNA was retarded with increasing amount of copolymer and even remained at the top of the gel at charge ratios ranging from 2 to 10 according to the type of copolymer used. Complete retardation of plasmid DNA occurred from a charge of 4 for the 50/50 random copolymer, of 10 for the 20/80 random one and of 2 for the block copolymers. The results of gel retardation assay confirmed these obtained using the Picogreen® assay. Indeed, for block copolymers, complete condensation of plasmid DNA occurred at lower charge ratio than random copolymers.

3.6. Cellular viability

The cytotoxicity of quaternized copolymers and their complexes with plasmid DNA was evaluated on HeLa cell line by a MTT assay. Copolymers were applied to the cells at concentrations ranging from 25 ng/ml to 500 µg/ml. The effect of copolymers on the cell viability was compared with poly(ε-caprolactone) polymer and with polyethylenimine 50 kDa, which is a commercially available in vitro transfecting reagent first introduced by Boussif et al. [31]. Polyethylenimine (PEI) is one of the most successful and widely studied gene delivery polymers and is used as a reference when compared with other newly designed polymers [38]. PEI offers a significantly more efficient protection against nuclease degradation than other polycations due to its higher charge density and more efficient complexation [39]. The high transfection efficiency of PEI can be attributed to the buffering effect or the "proton sponge effect" of the polymer caused by the presence of amino groups in the molecule [40].

Fig. 6. *Effect of copolymers on the viability of HeLa cells. The MTT dye reduction assay was performed 4 h after incubation of the cells with various concentrations of the copolymers (n = 8).*

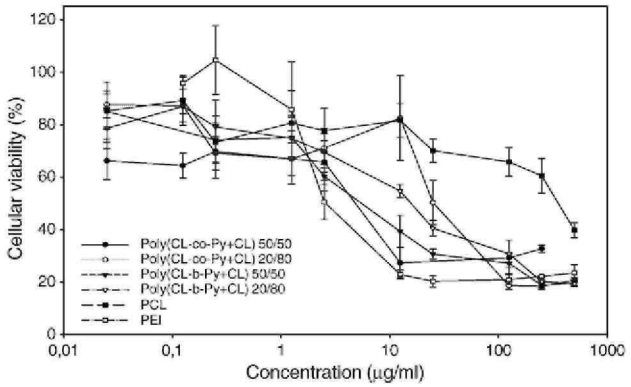
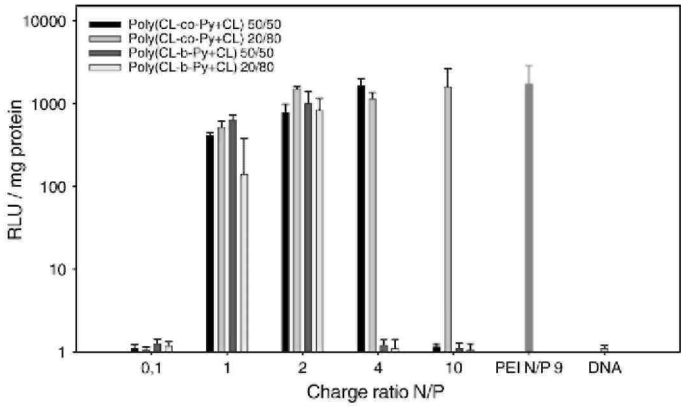


Fig. 7*. Transfection efficiency of copolymers/DNA complexes in HeLa cells. HeLa cells were seeded at a density of 10 cells/well in 96-well plate and transfection was performed at a dose of 1 µg of DNA for all groups and analysed at 48 h after transfection. The luciferase expression in relative light units (RLU) is normalized to mg of cellular protein (n = 3).*



As shown in Fig. 6, a dose-dependent cytotoxicity was observed with increasing concentration of copolymers. The cytotoxicity of copolymers was mainly due to the presence of pyridinium groups since poly(ε-caprolactone) polymer alone was much less cytotoxic. Biocompatibility is thought to be influenced by different properties of the polymers such as (i) molecular weight, (ii) charge density and type of the cationic functionalities, (iii) structure and sequence (block, random, linear, branched) and (iv) conformational flexibility [41], Polycation/DNA complexes are usually found to be less cytotoxic than uncomplexed polycations [42]. The effect of polycations alone on cell viability was compared with the effect of their complexes with DNA. Polymer cytotoxicity was not reduced when complexed with plasmid DNA (data not shown). All quaternized copolymers were less cytotoxic than polyethylenimine 50 kDa polymer. Poly(CL-co-Py+CL) 20/80 was found to be the less cytotoxic polymer of all the polymers tested.

3.7. Transfection efficiency

Copolymers/DNA complexes were assessed for in vitro transfection efficiency in HeLa cells and compared to commercially polyethylenimine 50 kDa transfection reagent. Polyethylenimine/DNA complexes were formulated at a charge ratio N/P of 9 at which an optimal transfection efficiency was previously obtained [31]. Naked DNA as well as untreated cultures were used as controls.

As shown in Fig. 7, the transfection efficiency was dependent on the type of copolymer used and the charge ratio. For all quaternized copolymers, luciferase activity close to background levels was observed at a charge ratio N/P of 0.1. The amount of luciferase protein expressed increased between a charge ratio of 1 and 2 for poly(CL-*b*-Py+CL) copolymers and 4 for poly(CL-co-Py+CL) 50/50 and 10 for poly(CL-co-Py+CL) 20/80. However, at N/P ratios of 4 and above for poly (CL-*b*-Py+CL) copolymers and of 10 for poly(CL-co-Py+CL) 50/50, transfection efficiency strongly decreased and luciferase levels comparable to naked DNA were observed. Polyplexes prepared at relatively low N/P ratios do not have the optimal physicochemical characteristics for maximal cellular association and internalization, whereas at high N/P ratios transfection levels drop due to toxicity of the free, non-DNA associated, cationic polymer. Poly(CL-co-Py+CL) 20/ 80 copolymer maintained high levels of luciferase expression until charge ratio of 10, probably due to its lower cytotoxicity compared to other copolymers investigated. Transfection efficiency of copolymers/DNA complexes was comparable to polyethylenimine 50 kDa polymer at charge ratios N/P ranging from 1 to 10 according to the type of copolymer used ($p = 0.45$). The maximum transfection efficiency was obtained with poly(CL-co-Py+CL) 20/80 polyplexes formulated at a charge ratio of 10.

The transfection efficiency of a polymeric carrier is profoundly influenced by the cytotoxicity of the cationic polymers to be used [29,30]. The effect of copolymer/DNA complexes on the viability of HeLa cells was evaluated 48 h after transfection experiment using a MTT colorimetric assay (Fig. 8). Results of transfection efficiency of quaternized copolymers could be correlated to their cytotoxicity. At charge ratios N/P of 1 and 2, transfection efficiencies of the different polyplexes were similar ($p = 0.19$ and 0.12) and, statistically, the cellular viability values for all polyplexes were not significantly different at these charge ratios (p=0.33 and 0.23) . However, at N/P ratio of 4 and above for poly(CL-*b*-Py+CL) copolymers and 10 for poly(CL-co-Py+CL) 50/50 copolymer, the amount of polymer used surpassed the cut-off concentration at which cells could be viable, and transfection efficiencies were in the same range as the transfection efficiency of naked DNA. Consequently, low cytotoxicity of copolymers is a pre-requisite for effective gene transfection.

Fig. 9 shows the transfection efficiency of diblock 50/50 copolymer/DNA complexes in the absence and presence of serum in HeLa cells. The results indicated that the transfection efficiency of polyplexes decreased in presence of serum in culture medium. However, in contrast to PEI/DNA complexes, good transfection efficiencies were observed at charge ratios N/ P of 1 and 2.

As for PEI, an increase in the size of polyplexes in culture medium was observed and could promote efficient in vitro transfection activities (data not shown).

To sterically stabilize copolymers/DNA complexes, a poly (CL-*b*-PEG) copolymer (14,900-4600 Da) was associated with polyplexes during the formation of nanoparticles to create a hydrophilic corona on the surface of the complexes. PEG has widely been used in the polymeric gene carriers because of its excellent characteristics: (i) it reduces the cytotoxicity of the polymer/DNA complexes; (ii) PEG increases the water-solubility of the polyplexes; (iii) PEG shields excess positive charges of polymer/DNA complexes, resulting in the reduction of interaction between polyplexes and blood components; (iv) PEG can be used as a spacer between a targeting ligand and a polymeric carrier, which facilitates the access of the ligand to its receptor [43]. Poly(CL-*b*-PEG) copolymer was combined with poly(CL-*b*-Py+CL) 50/50 polyplexes at a proportion of 1/3 poly(CL-*b*-

PEG)/poly(CL-*b*-Py+CL) 50/50 copolymer. When associated with PEG, the transfection efficiency of diblock 50/ 50 polyplexes was significantly reduced (twofold decrease) at charge ratios of 1 and 2 *(p*<0.001*).* It could be due to the steric stabilization and the reduction of non specific interactions imparted by the PEG chains on the surface of copolymer/DNA complexes (data not shown).

Fig. 8. *Cytotoxicity of copolymers/DNA complexes to HeLa cells. The MTT dye reduction assay was performed 48 h after incubation of the cells with the same amount of the preparations as in the transfection trial (n=6).*

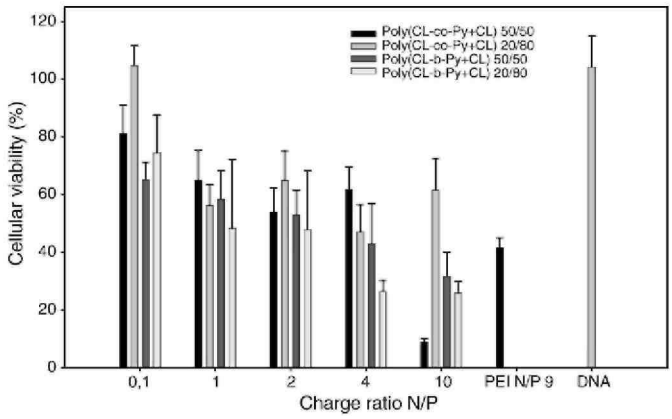
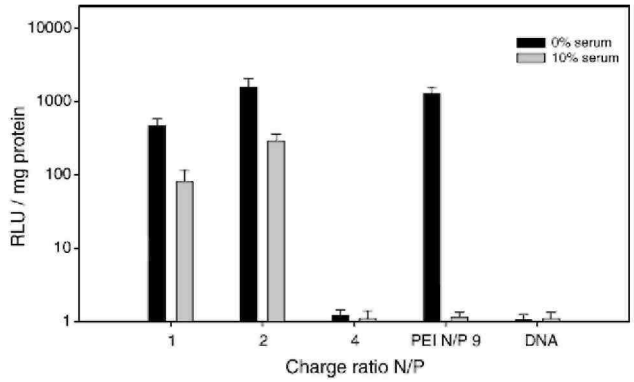


Fig. 9*. Effect of serum on transfection efficiency of diblock 50/50 copolymer/ DNA complexes in HeLa cells (n = 3).*



4. Conclusion

Copolymers consisting of poly(ε-caprolactone) (PCL) with pendant pyridinium groups could be used as effective gene delivery vectors. All the quaternized copolymers formed nanoparticles with a high interaction with plasmid DNA. The chemical composition of the polymers has an impact on the physicochemical properties of the polyplexes. Cytotoxicity and transfection efficiency on cultured HeLa cells were comparable to polyethylenimine 50 kDa, a polycation commonly used for gene delivery applications. These novel copolymers are currently investigated for in vivo gene transfection.

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