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Polynorbornene polycationic polymers as gene transfer agents Influence of the counterion for in vitro transfection

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Abstract

Polycationic derivatives of polynorbornene with different non-cytotoxic counterions, have been prepared by organometallic polymerization of methyleneammonium norbornene and subsequent exchange of the counterion. In this paper the effect of the counterion on the polycationic polymer binding onto plasmid DNA was studied via different ethidium bromide assays, heparin displacement and protection against degradation by DNAse. According to the nature of the counterions and consequently the size of the polymer particles, their complexation with the DNA led to aggregates with variable binding affinity for the plasmid. The relative transfection efficiency of each polyplex was compared, on the basis of reporter gene expression, in cells in culture. The nature of the counterion was seen to affect gene delivery. The order of transfection efficiency of the counterions studied at equivalent charge ratios (NH_3^+/PO_4^-) is lactobionate, acetate, chloride. The results obtained with the polynorbornene methyleneammonium lactobionate and acetate are particularly encouraging.

Keywords: Polynorbornene; Cationic polymer; Counterion; Transfection; DNA complexation

1. Introduction

Gene therapy has been considered as an attractive concept for a broad variety of biomedical applications. Non-viral systems are essentially based on DNA complexation into particles by electrostatic interaction between polyanionic and cationic lipids (Behr et al., 1989; Schwartz et al., 1995; Felgner et al., 1995; Remy et al., 1995) or polymers (Hansma et al., 1998; Midoux et al., 1993; Felgner, 1996; Boussif et al., 1995; Tang et al., 1996). Delivery vehicles involving non-viral methods are advantageous in that they are non-immunogenic and safer (Boussif et al., 1995; Tang et al., 1996; Bragonzi et al., 1999; de Smedt et al., 2000; Reschel

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et al., 2002; Mahato et al., 2003). A great research effort is currently dedicated to optimizing the transfection efficiency of non-viral vectors, such as polycationic polymers (Jones et al., 2000; Dubruel et al., 2003). These compounds encompass a wide range of characteristics. They self assemble with anionic DNA through electrostatic interactions leading to the formation of complexes. The ability of cationic molecules to condense DNA indicates that a major factor governing the complexation of DNA is charge neutralization between the negatively charged nucleic acid phosphate groups and the positively charge groups of the cations (Bloomfield, 1996, 1997; Stevens, 2001), the condensed particles produced serving as a platform for enhancing the cellular delivery of DNA. The condensation of DNA, involving a dramatic decrease in the volume occupied by the DNA molecule, is of immense biological importance. Nanoparticles formed by the condensation of DNA in the presence of polycationic polymers are essential for the transport of plasmid DNA through the cell membrane (Zuber et al., 2001; Luo and Saltzman, 2000; Junghans et al., 2001; Liu et al., 2001) and in nuclear trafficking mechanisms (Pollard et al., 1998). Therefore, the structural, physico-chemical and energetic aspects of the condensation process continue to receive great attention. For example, it was demonstrated in the case of a cationic lipid-mediated transfection that the nature of the counterion accompanying the ammonium group could affect the transfection efficiency (Aberle et al., 1996). In this context, we have synthesized a family of polycationic polynorbornenes with different counterions (chloride, acetate and lactobionate). As we have described in previous papers (Serres et al., 1999; Noel et al., 2000; Franceschi et al., 2002), some of these polymers spontaneously form small, stable latexes in water. For example, in the case of lactobionate counterions, the complexation with DNA leads to very small aggregates with a diameter of around 10–20 nm.

Complexation of DNA in very small particles is fundamental for efficient transfection, so we evaluated DNA delivery with these new polymers into cultured cells. In addition, the purpose of this paper was to compare both the influence of the counterion on the percentage of transfection and its relationship with the physico-chemical properties of the different complexes.

2. Materials and methods

2.1. Materials

All the solvents and chemicals were reagent grade and were used without further purification. Ethidium bromide and agarose were purchased from Sigma. Foetal calf serum, Dulbecco's modified Eagle's minimum essential medium (MEM) and trypsin-EDTA 0.25% were purchased from Eurobio. Streptomycin and penicillin were purchased from Gibco, L-glutamine from Intermed and D-glucose from Gibco. The plasmid Maxi Kit was purchased from Qiagen (Chatsworth, CA).

2.2. Synthesis of polynorbornenes methyleneammonium

The polynorbornene methyleneammonium chloride was obtained following a procedure described previously (Serres et al., 1999; Noel et al., 2000; Franceschi et al., 2002). The polynorbornenes methyleneammonium acetate and lactobionate were obtained by neutralization of the polynorbornene methyleneammonium chloride with NaOH (Fig. 1). Then, it was treated with either an acetic acid or a lactobionic acid in methanol or methanol/water (85/15, v/v) solution. Titration with $NaHCO_3$ solution showed that the degree of protonation was respectively 100, 70 and 50% for polymers chloride, acetate and lactobionate. Moreover the pK_a of a methylamine norbornane unit was evaluated to 10.6.

2.3. Amplification and purification of DNA

A 4.7 kbp plasmid (pEGFP-C1, CLONTECH), carrying the green fluorescent protein (GFP) gene under the control of the cytomegalovirus promoter, was used. The plasmid was prepared from transformed *Escherichia coli* by using a Maxiprep DNA purification system (Qiagen) and the purified plasmid was diluted in sterilized water. Its purity was confirmed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining, and DNA concentration was then measured by UV absorption at $\lambda = 260$ nm.

2.4. Zeta potentials

Observations were performed on a Malvern Instrument Zetasizer 3000. The zeta potentials of

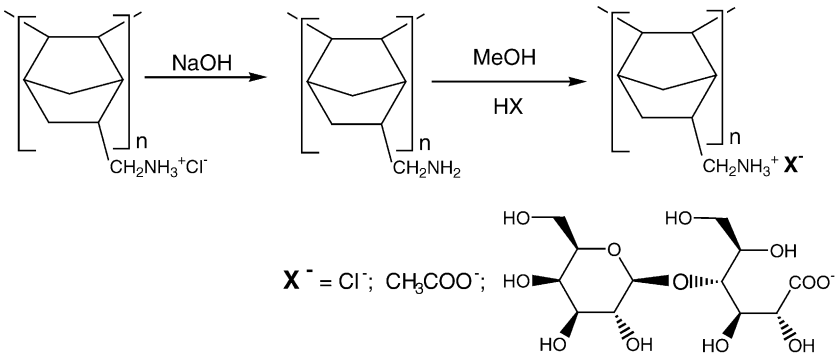

Fig. 1. Polynorbornene methyleneammonium polymers.

DNA–polymer complexes formed with variable amounts of polynorbornene methyleneammonium polymers, were determined using an electrophoretic light scattering technique. Measurements were made on complexes in water. Increasing amounts of polymer were mixed to a supercoiled plasmid DNA solution (15 μg DNA; 2.5 ml H_2O). For each measurements 10 μl of polymer solution were added successively to the DNA solution. The concentration of polymer solution was chosen according to NH_3^+/PO_4^- ratio of 1; the added volume of polymer (50 μl) solution was kept small comparatively to the DNA solution. For each formulation, mean particle electrophoretic mobility was measured in a thermostatically controlled microelectrophoresis cell equilibrated at 25 °C at a frequency of 1000 Hz.

2.5. Electron microscopy measurements

The DNA–polymer complexes were studied by transmission electron microscopy. For this study, the DNA was used at a final concentration of 10 μM in nucleotide units. TEM grids were glow-discharged (110 mV, 60 s) before deposit of the complexes. A drop of sample solution was left on the carbon-coated copper grids for 1 min. Samples were negatively stained with a phosphotungstate solution (1% w/w; pH 7). Observations were performed at 60 kV with a Philips EM 301 transmission electron microscope.

2.6. Ethidium bromide spectrofluorimetric assays

Ethidium bromide (EtBr) intercalation between DNA base pairs was determined through fluorescence spectroscopy (at excitation and emission wavelengths of 530 and 590 nm, respectively) using a PTI QM1 Photon Technology Instrument Quanta Master 1 Spectrometer.

Firstly, the ability of EtBr to intercalate into DNA, complexed by polymers, was investigated. For the dye exclusion assay, the plasmid pEGFP-C1 (10 μg) in sterilized water was mixed with cationic polymers in 500 μl of phosphate buffer (5 mM; pH 7.4; 15 mM NaCl) at different NH_3^+/PO_4^- ratios varying from 0 to 4. After standing for 20 min at room temperature 20 μl of EtBr solution (20 μg/ml) and 1500 μl of phosphate buffer (5 mM; pH 7.4; 15 mM NaCl) were added to the polymer/DNA mixtures and measurements were carried out.

Secondly, the EtBr displacement from DNA by cationic polymers was studied. This investigation provides an assessment of the polycations binding onto DNA, by measuring changes in the fluorescence of the EtBr probe. The plasmid pEGFP-C1 (10 μg) in sterilized water was mixed with EtBr solution (5 μl; 500 μg/ml). Then, cationic polymers in 500 μl of phosphate buffer (5 mM; pH 7.4; 15 mM NaCl) were added to the plasmid solution in order to obtain polymer/DNA complexes at NH_3^+/PO_4^- ratios varying from 0 to 4. The samples were mixed gently and the fluorescence was measured after 20 min of incubation at room temperature.

The relative fluorescence was calculated as below:

$$\%\,\text{Relative fluorescence} = \frac{\text{fluorescence}_{(\text{obs})} - \text{fluorescence}_{(\text{EtBr})}}{\text{fluorescence}_{(\text{DNA+EtBr})} - \text{fluorescence}_{(\text{EtBr})}}$$

where $fluorescence_{(obs)}$ is the fluorescence of DNA + EtBr + polymer; $fluorescence_{(EtBr)}$ the fluorescence of EtBr alone; and $fluorescence_{(DNA+EtBr)}$ the fluorescence of DNA + EtBr.

2.7. Effect of heparin on complexes stabilities

The effect of heparin on the stability of complexes was evaluated by means of the change in fluorescence intensity obtained with the fluorescent probe EtBr. Heparin was added to the mixtures containing DNA complexes with polymers, chloride, acetate or lactobionate at NH_3^+/PO_4^- ratios varying from 0 to 4 to yield a final heparin concentration of 0.075 mg/ml. After EtBr addition (5 μl; 500 μg/ml), the fluorescence was measured as indicated above.

2.8. DNA retardation assay and sensitivity of the complexes to DNAse I

DNA binding was studied by means of agarose gel retardation assays. Supercoiled plasmid DNA (1 μg) in phosphate buffer (5 mM; pH 7.4; 15 mM NaCl) was mixed with increasing amounts of polymers. After 30 min incubation at room temperature, naked DNA and polymer/DNA complexes were submitted to digestion by DNAse I to a final concentration of 0.04 μg/ml per μg of DNA, in Tris–HCl buffer (50 mM; pH 7.4), $MgCl_2$ (10 mM), DTT (10 mM) and ATP (1 mM). After 15 min incubation at 37 °C, the reaction was stopped by adding 3 μl of EDTA (0.5 M). After addition of 10 μl of 20% heparin, samples (25 μl) were examined by 0.8% agarose gel electrophoresis.

2.9. Cell culture

Chinese hamster ovary (CHO) cells were used. The cells were grown at 37 °C in monolayers in a 5% CO_2 incubator in Eagle's minimum essential medium (MEM0111, Eurobio, France) supplemented with glucose (3.5 g/l), tryptose phosphate (2.95 g/l), vitamins and 8% foetal calf serum. Antibiotics penicillin (100 units/l) and streptomycin (100 μg/ml) and glutamine (0.585 ng/ml) are added extemporally.

2.10. In vitro transfection

Cells were seeded at 10^5 cells per 35 mm petri dish 12 h prior to transfection. The following day, the medium was drawn off and replaced with 1 ml of culture medium with 2% of foetal calf serum and 100 μl of complexes. The complexes consist of 5 μg of pEGFP-C1 plasmid in phosphate buffer (5 mM; pH 7.4; 15 mM NaCl) and polymers at various NH_3^+/PO_4^- ratios. The complexes were allowed to remain at room temperature for 1 h prior to addition to the cells. The cells were then incubated for 6h at 37 °C in 5% CO_2. Following this incubation, the transfection mixture was removed and the cells were overlaid with 2 ml of culture medium supplemented with 8% of foetal calf serum. The cells were cultured for an additional 48 h period and were then harvested by trypsinization and analysed by flow cytometry (flow cytometer, Becton Dickinson). This method permits the evaluation of the percentage of fluorescent cells (i.e. percentage of GFP expressing cells). Each transfection experiment was done in triplicate.

2.11. Cell viability

Polynorbornene cytotoxicity was evaluated in the concentration range corresponding to the different NH_3^+/PO_4^- ratios used for the transfection. The same protocol was used as for cell transfection. For the viability tests, only polymers were added to the cells (100 μl). After 6 h incubation, the medium was supplemented with 8% of foetal calf serum and the cells were cultured for an additional 48 h. Viability was measured by quantifying the ability of cells to grow over more than one generation (at 24 and 48 h) by crystal violet staining (Gabriel and Teissie, 1995).

3. Results

3.1. Interaction between the polymer and DNA

3.1.1. Zeta potential

The zeta potentials of these complexes were determined by a laser Doppler micro-electrophoresis. The particles of complex were formed by increasing the NH_3^+/PO_4^- ratio (Fig. 2).

3.1.1.1. Polynorbornene methyleneammonium chloride. The zeta potential of DNA (−40 mV) increases with the amount of polymer. The zeta potential from the resulting complex changes from a negative to a positive charge for an NH_3^+/PO_4^- ratio of 1.1 corresponding to

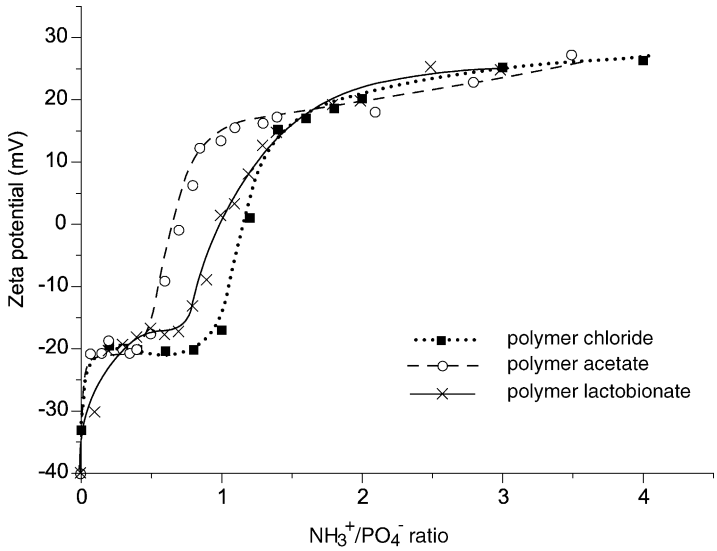

Fig. 2. Zeta potential of polynorbornene methyleneammonium polymers.

a zeta potential close to zero. For this polymer an important stage was observed around −20 mV, so there is no change in zeta potential and this is significant of a slow complexation. Beyond, we observed a very sharp change of slope around neutralization indicating an abrupt complexation of DNA.

3.1.1.2. Polynorbornene methyleneammonium acetate. The zeta potential of the complex changed from negative to positive as the NH_3^+/PO_4^- ratio was increased. For this polymer neutralization (zeta of 0) appears at an NH_3^+/PO_4^- ratio of 0.7. The stage of neutralization is in this case shorter than for chloride polynorbornene indicating a greater affinity for DNA; with progressive complexation even at low NH_3^+/PO_4^- ratios.

3.1.1.3. Polynorbornene methyleneammonium lactobionate. As for the other polymers, the curve shows a sigmoid variation of the resulting complex from a negative charge (DNA alone) to a positive charge as the quantity of polymer is increased. For this polymer the isoelectric point (zeta of 0) corresponds to an NH_3^+/PO_4^- ratio of 1. Here, we observed an intermediate situation between the chloride and the acetate. Complexation starts sooner than with chloride but also we observed a sharp change of slope around neutralization.

3.1.2. Transmission electron microscopy

By transmission electron microscopy polynorbornene/DNA complexes were characterized at different NH_3^+/PO_4^- ratios varying from 0.5 to 3 with no significant change of size or shape for each polymers (Fig. 3). The chlorhydrate polynorbornene/DNA complexes appeared to be relatively dense with toroidal morphology. The size of these particles varied between 80 and 150 nm. The acetate polynorbornene/DNA complexes are spherical. The diameter of these structures is approximately 50–70 nm. For the lactobionate polynorbornene/DNA complexes, the particles are smaller and the measured diameter are 10–20 nm.

3.1.3. Ethidium bromide displacement assay

Ethidium bromide (EtBr) displacement is a simple and reproducible method to monitor the interaction of DNA with the polymers (Geall and Blagbrough, 2000). This method was used to assess the ability of the different polymers to bind to DNA. Sequential addition of these polymers to DNA/EtBr complex in solution caused a decrease in selective fluorescence, because of

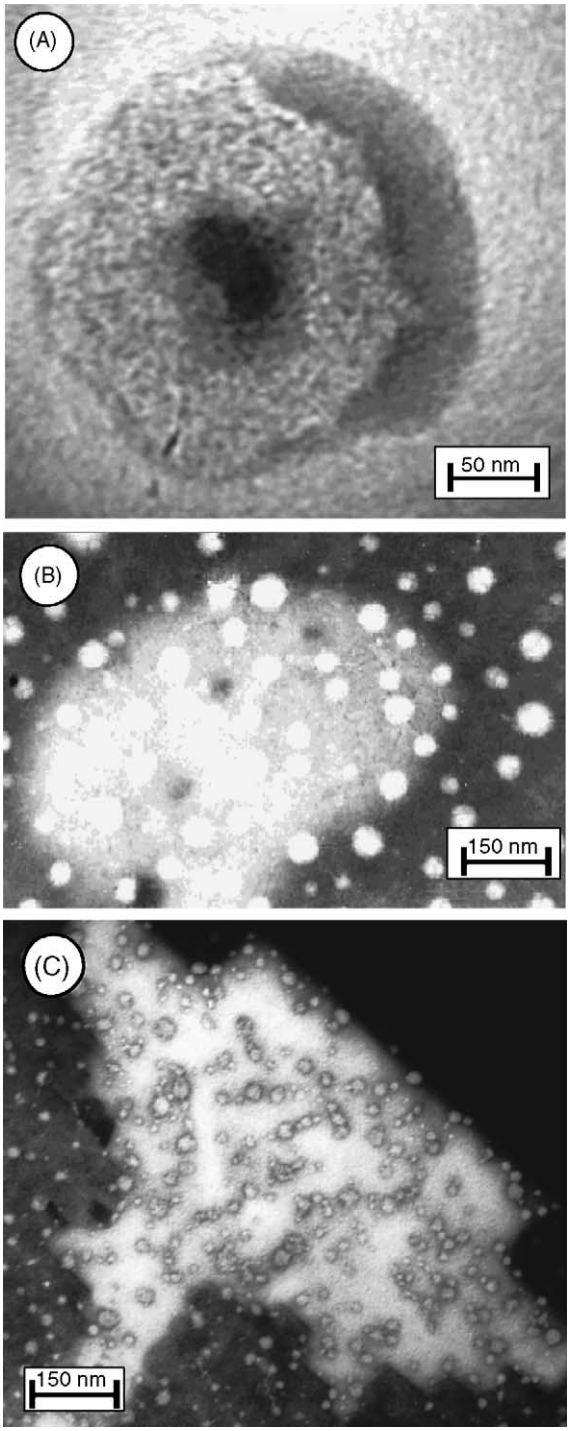

Fig. 3. Transmission electron micrographs of DNA/polymers complexes: (A) chloride polymer; (B) acetate polymer; (C) lactobionate polymer.

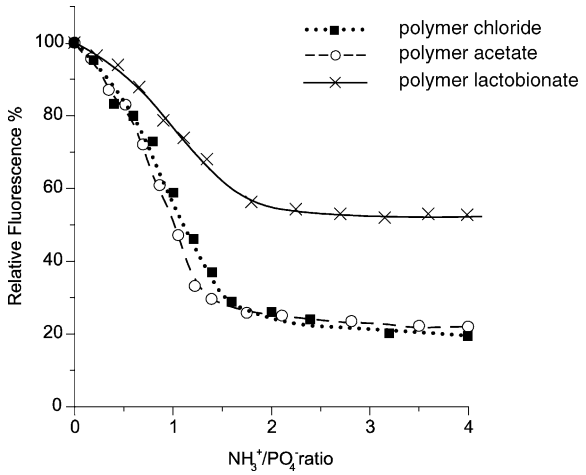

Fig. 4. Assay of EtBr displacement by polynorbornene methyleneammonium polymers. Each point is expressed as percentage of the maximum fluorescence obtained with naked DNA.

the displacement of the fluorescent dye by the polymer (Fig. 4).

3.1.3.1. Polynorbornene methyleneammonium chloride. For this polymer the displacement profile can be divided into two regions. In the first region, between NH_3^+/PO_4^- ratios of 0 and 1.5, a substantial fall in fluorescence was observed. At ratios higher than 1.5, values tailed off at relative fluorescence percentage around 25.

3.1.3.2. Polynorbornene methyleneammonium acetate. In this case, the displacement profile is the same as for polynorbornene methyleneammonium chloride and it can be divided into two regions. Between NH_3^+/PO_4^- ratios of 0 and 1.5, fluorescence decreased gradually to finally reach, for ratios higher than 1.5, a value corresponding to 25% of the relative fluorescence. This may correspond to the maximal condensation of DNA by polymers.

3.1.3.3. Polynorbornene methyleneammonium lactobionate. For this polymer, there is also a gradual decrease in fluorescence between NH_3^+/PO_4^- ratios of 0 and 1.5. At ratios higher than 1.5, a plateau was reached but in this case, with a relative fluorescence percentage of around 50. Compared to the other two polymers, this highly value could be explained by a partial displacement of the EtBr from the DNA. It is not clear how to

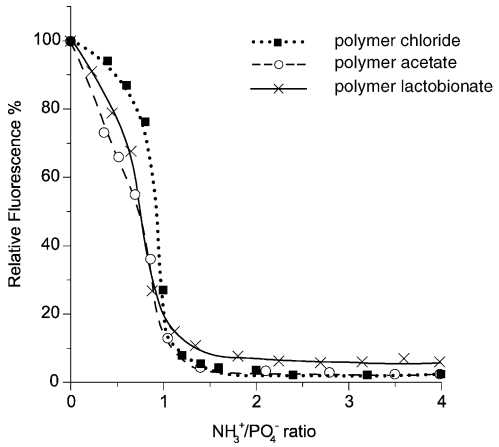

Fig. 5. Assay of EtBr exclusion by polynorbornene methyleneammonium polymers. Each point is expressed as percentage of the maximum fluorescence obtained with naked DNA.

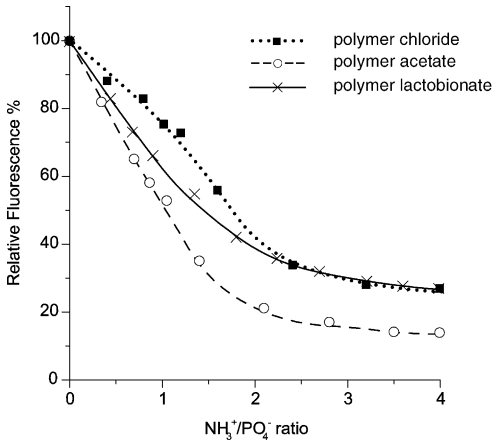

Fig. 6. Effect of heparin on the fluorescence intensity of DNA. The values are expressed as a percentage of the maximum fluorescence obtained with naked DNA.

explain such a difference of affinity because the intercalation of EtBr into DNA modifies the size and the topology of the plasmid (Pope et al., 1999) by disrupting the electrostatic and hydrophobic interactions, and could therefore affect complexation with this polymer. To better understand the binding of these polymers with DNA, EtBr exclusion assay had to be performed.

3.1.4. Ethidium bromide exclusion

The ability of the polymers to bind DNA was monitored by titration assay at different NH_3^+/PO_4^- ratios (Fig. 5). A solution of EtBr was added to the complexes and the fluorescence of the solutions was measured. For all three polymers the exclusion profile was quite similar and can be divided into two regions. In the first region, between 0 and 1 NH_3^+/PO_4^- ratio, an important fall in the fluorescence was observed. The relative fluorescence percentage tailed off at around 5 for ratios higher than 1. As the ratio of cationic polymer to DNA was increased, the EtBr fluorescence decreases, indicating that less DNA was accessible to the EtBr. An important observation is that in this experiment the polymer lactobionate appeared to have the same binding ability as the other two polymers.

3.1.5. Influence of heparin on the stability of complexes

The effect of heparin on the stability of the complexes was evaluated by means of the change in relative fluorescence of EtBr (Fig. 6). Some polyanions, such as heparin, are able to release DNA from complexes with cations (Moret et al., 2001), as a consequence of competitive interaction. The fluorescence of the intercalated dye was measured by titration assay at different NH_3^+/PO_4^- ratios. By this procedure, we evaluated the binding strength of the DNA for the different polymers. For all the polymers, we observed a strong complexation of DNA, not easily displaced by heparin. The displacement curves can be divided for all the polymers into two regions for NH_3^+/PO_4^- ratios between 0 and 2. In the region below NH_3^+/PO_4^- ratio of 2, a rapid decrease in the relative fluorescence of the dye indicates an increasing interaction between the DNA and the polymer with low displacement by the heparin. Above an NH_3^+/PO_4^- ratio of 2, there is a slow decrease in the fluorescence. Compared to the other complexes, the polymer acetate/DNA complex was the least easily displaced by heparin.

3.1.6. Stability of the complexes towards DNAse I

In order to investigate the protective effect of complexes on the degradation of DNA by DNAse I, naked DNA and DNA complexes were incubated for 15 min with the enzyme. After addition of heparin to release DNA from the complexes, agarose gel electrophoresis was carried out. In these conditions, degradation of naked DNA was complete. However, partial degradation can change the shape of DNA. In its natural state, circular DNA is supercoiled. Breaking a single bond

in one strand will result in a relaxed circular DNA. Breaking both strands at the same position will result in the formation of a linear molecule. Supercoiled DNA, relaxed circular DNA and linear DNA of the same molecular weight will migrate at different rates through the gel. The order of migration from the well is usually: relaxed circular DNA, linear DNA and supercoiled DNA. The agarose gel retardation assays performed in the absence of DNAses treatment, but with or without heparin, are in good agreement with the zeta potential curves (Fig. 2) and the fluorescence studies (Figs. 5 and 6). However, Fig. 7 shows that both DNA protection against DNAse and DNA release by heparin from complexes, were different for the polymers, chloride, acetate and lactobionate.

With the polymer chloride, DNA release from complexes was total, whatever the NH_3^+/PO_4^- ratio investigated. Total degradation of DNA was observed for NH_3^+/PO_4^- ratios lower than 1. For ratios higher than 1, the amount of damaged DNA decreased, the linear form totally disappeared and DNA was essentially recovered in circular and supercoiled forms. These data indicate that polymer chloride might partially protect DNA against DNAse at NH_3^+/PO_4^- ratios higher than 1 (Fig. 7).

With polymer acetate at NH_3^+/PO_4^- ratio higher than 1, DNA was complexed and only small amounts were released from the complexes by heparin (Fig. 7). These data suggest a strong interaction between the polymer and DNA. After DNAse I action, no DNA migration was observed but the strong fluorescence in the well at NH_3^+/PO_4^- ratios higher than 1 suggest the presence of degraded DNA still complexed by the polymer, which could be more easily intercalated by the ethidium bromide.

Interestingly, the DNA complexed with the polymer lactobionate displayed complexes with retarded migration over a range of NH_3^+/PO_4^- ratios from 1 to 4 (Fig. 7). When the complexes were only treated by heparin, without DNAse I action, total decomplexation and migration of supercoiled DNA was observed, in agreement with the results of fluorescence (Fig. 6). The absence of DNA released by the heparin at high NH_3^+/PO_4^- ratios indicates the absence of supercoiled DNA after DNAse I treatment. Moreover, the high fluorescence in the well suggest an increase of EtBr intercalated DNA but modified by the DNAse to a degraded form still complexed by this lactobionate

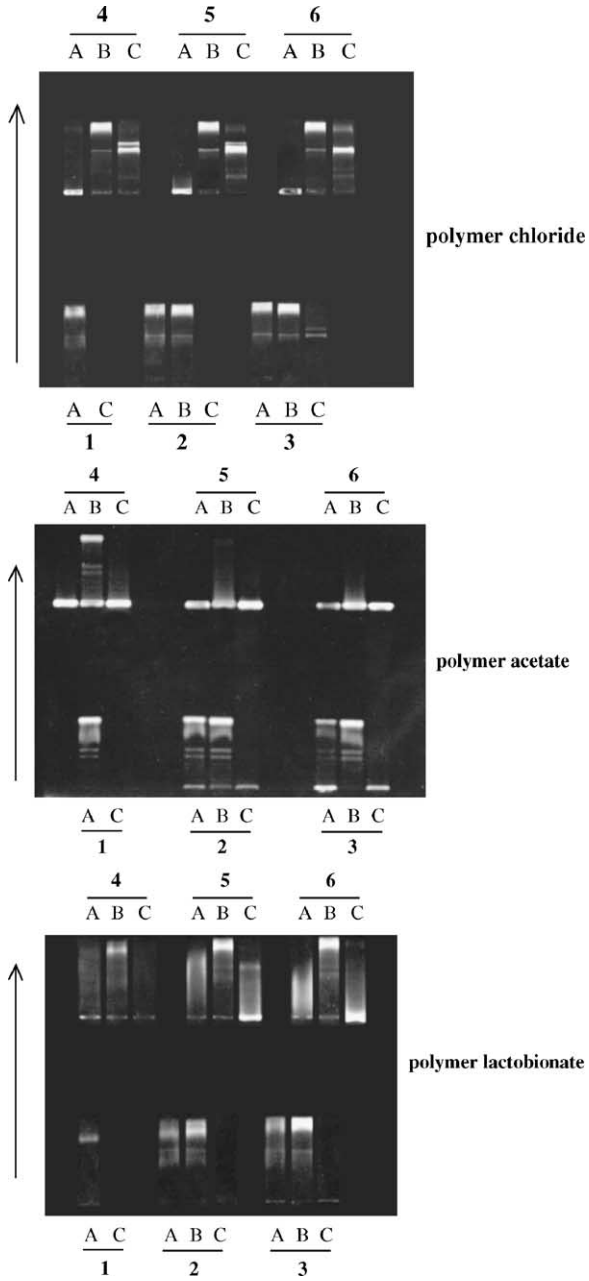

Fig. 7. Agarose electrophoresis of: (A) polynorbornene polymers/DNA complexes; (B) polynorbornene polymers/DNA complexes in the presence of heparin at different NH_3^+/PO_4^- ratios; (C) polynorbonene polymers/DNA complexes at different NH_3^+/PO_4^- ratios, in the presence of DNase and heparin. (1) Naked DNA; (2) $NH_3^+/PO_4^- = 0.2$; (3) $NH_3^+/PO_4^- = 0.5$; (4) $NH_3^+/PO_4^- = 1$; (5) $NH_3^+/PO_4^- = 2$; (6) $NH_3^+/PO_4^- = 4$.

polymer. This polymer appeared to form a metastable complex with the supercoiled DNA, allowing its partial degradation by the DNAses and displacement by heparin.

3.2. Evaluation of the transfection efficiency of the polyplexes

Gene delivery compositions comprising polymer and pEGFP-C1 plasmid DNA prepared in an NH_3^+/PO_4^- charge ratio between 0 and 4 were used to investigate in vitro delivery and expression of pEGFP-C1 plasmid DNA in CHO cells. The plasmid is a positive control vector for monitoring the transfection efficiencies. The advantage of using Green Fluorescent Protein as a reporter gene resides in the fact that it is possible to get access to the percentage of cells expressing the activity. Transfected cells were measured for pEGFP-C1 activity by cytofluorimetric analysis. As for polymer based gene delivery, the transfected DNA follows a trafficking route in endosomes and lysosomes; the experiments were therefore performed with and without chloroquine, the lysosome-disrupting agent. In order to increase the cellular uptake of DNA complexes by spontaneous endocytosis mediated by anionic cell surface proteoglycans (Ruponen et al., 1999), high NH_3^+/PO_4^- ratios, four-fold those required for neutralization, were chosen to increase the positive charge on the complexes. The transfection percentage was calculated from the number of fluorescent cells in a selected population.

The cytotoxicity of the polymers was tested at the concentrations used during cell transfection (Fig. 8). In these conditions, the polymers did not show any toxic effects on the cells. As shown in Fig. 9, the polymer lactobionate displayed the highest transfection efficiency, which was maximal for an NH_3^+/PO_4^- ratio of 4. At this NH_3^+/PO_4^- ratio and in the absence of chloroquine, 8, 30, 33% of the cells were transfected by chloride, acetate and lactobionate/DNA complexes respectively. Chloride complexes displayed the lowest efficiency. Addition of chloroquine only slightly increased the percentage of cells transfected.

Good results, even in the absence of chloroquine, were obtained with both polymer lactobionate and polymer acetate.

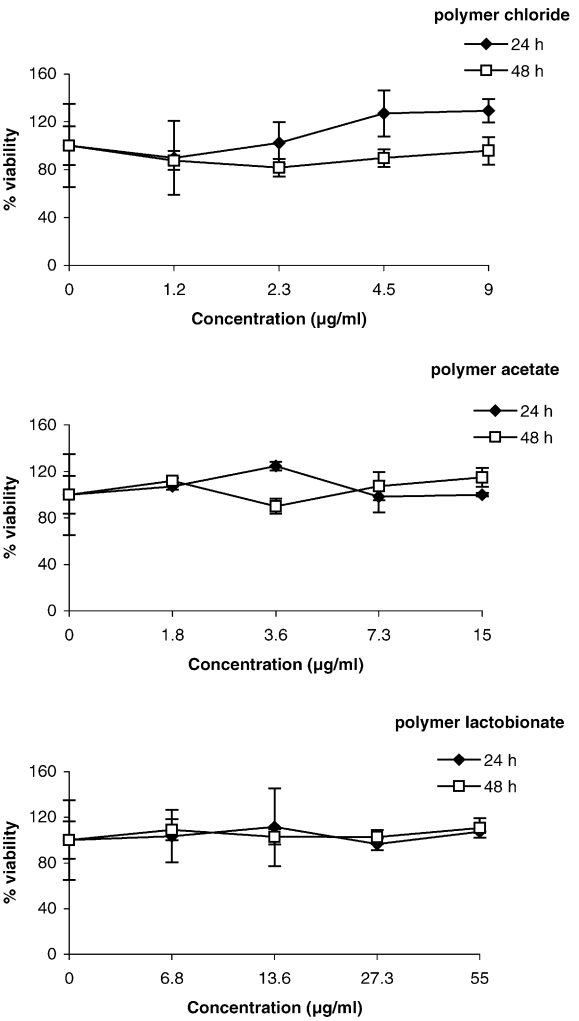

Fig. 8. Cytotoxicity of the polymers as a function of their concentration and time.

4. Discussion

According to results previously described (Serres et al., 1999; Noel et al., 2000; Franceschi et al., 2002), the nature of the counterion has an important influence on the conformation of the polymers in solution (stretched or latex), and also on the size and the stability of the complexes with DNA. Unlike polylysine or PEI, the DNA is here not only complexed by electrostatic interactions, but also by the hydrophobic effect and packing of the norbornane units (Puech et al., 1997, 2000). Consequently, we obtained different results in transfection

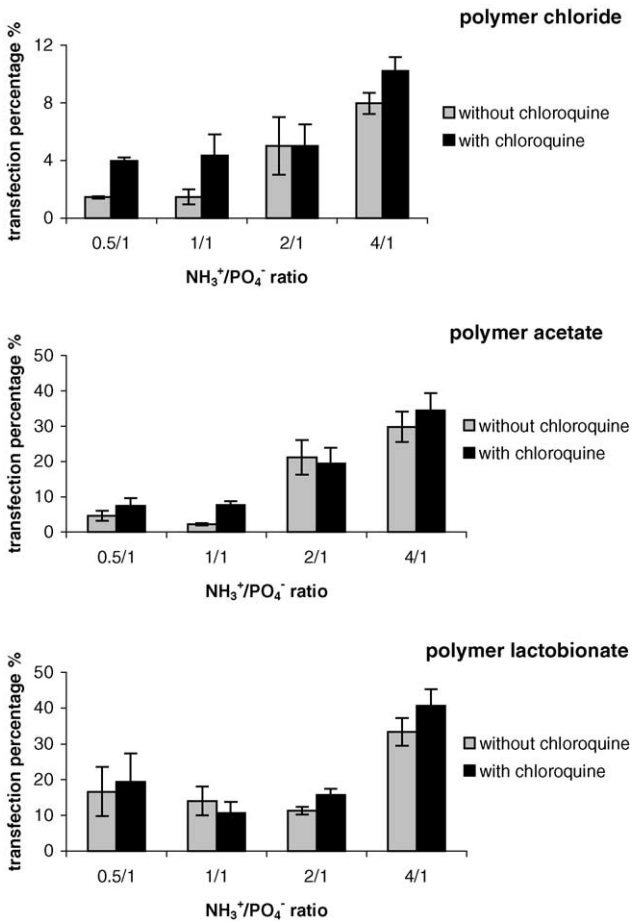

Fig. 9. Transfection efficiencies of methyleneammonium polynorbornene polymers into CHO cell lines. The cells were incubated with polynorbornene polymers/DNA complex containing 5 μg of plasmid DNA and polynorbornene polymers at different NH_3^+/PO_4^- ratios. Cells were harvested and GFP activity determined 48 h after transfection as described in the experimental part.

of DNA into cell cultures according to the counterion used.

The polymer with the chloride counterion is a fully quarternized polymer. Analysis by transmission electron microscopy (TEM) does not show the presence of latex particles, but only stretched chains of polymer (Serres et al., 1999). For this polymer, the interactions with DNA led to weakly complexed aggregates with large toroidal or spherical morphologies. Moreover, all the binding experiments with DNA seem to show that this polymer possesses a moderate affinity for DNA, leading to aggregates displaced by heparin. Chloride is a counterion strongly bonded to the ammonium group, leading to a shielding of the electrostatics repulsions between the units and the formation of large aggregates. Concerning this polymer, the complexation with DNA involves essentially electrostatic interactions and gives rise to large aggregates easily displaced by the heparin. The moderate protection of genes by the polymer, was also confirmed by its enhanced stabilization against degradation by DNAses I only at high NH_3^+/PO_4^- ratios (>2).

The polynorbornene acetate or lactobionate were obtained by reaction of acetic acid or lactobionic acid with the polynorbornene methyleneamine in methanol or methanol/water solution. In these conditions the reaction lead to a rapid precipitation of the polymer exchanged. In the case of the reaction with acetic acid

the polymer exchanged is maintained in solution for a longer time than the lactobionate and consequently we obtain a good degree of protonation (70%).

Furthermore, reactivity on polymers is often difficult due to their limited solubility, resulting in partially folded chains not easily accessible to the reagents (Swift et al., 1997).

With acetate counterions the polymer is able to produce latex particles (Noel et al., 2000) with packed cores formed by the non-quarternized norbornane units, surrounded by hydrophilic moieties (ammoniums). Unlike the polymer chloride, complexation of the polynorbornene acetate with DNA led only to small spherical aggregates. Furthermore, all the physicochemical experiments seem to indicate a high affinity of the polymer acetate for the DNA.

Acetate counterion is fairly bonded to the ammonium group leading to the strengthening of the electrostatic repulsions between the ammoniums, but at the same time this effect increase the hydrophobic interactions between the norbornane units. In this case the aggregates formed could not grow as larger than the chloride polymer, leading to small particles. For this polymer, the interactions with DNA are both electrostatic and hydrophobics, strengthening the stability of the complexes. Also, this strong interaction could explain that this complex is not easily displaced by heparin.

In lactobionic acid case, because of water solution and the more hydrophilic nature of lactobionate ion, a rapid precipitation of the polymer exchanged occurred, leading to a lower degree of protonation than the acetate.

Indeed, only half of the methyleneamino groups are quarternized. For this polymer, electron microscopy shows very small spherical particles with a diameter of approximately 20–40 nm for the majority of the population (Franceschi et al., 2002). These latex particles consisted of a packed core, formed by the non-quarternized norbornane units, surrounded by the methyleneammonium units and a shell of hydrated lactobionate counterions.

The interactions of the polymer lactobionate with the DNA led to very small aggregates with a diameter of around 10–20 nm. Lactobionate counterion is a sugar weakly bonded to the ammonium group, and also this counterion promotes the water organization by a kosmotropic effect (Koynova et al., 1989; Epand and Bryszewska, 1988). This effect leads to the ammonium group dehydration increasing their repulsion and promoting a greater hydrophobic packing of the norbornane units. The conjunction of these two effects gives rise to very small but metastable particles because of the important repulsion between the ammonium groups. Consequently, the complexation of the DNA by this polymer involves an important hydrophobic contribution, but this hydrophobic effect could be also strongly decreased if the DNA is already intercalated by the EtBr (Fig. 4). Finally, the metastable nature of these complexes could explain that the DNA is easily displaced by the heparin. The transfection ability of the polynorbornene polymer has been assessed to study the effect of the lactobionate counterion which, like acetate showed a high transfection efficiency even in the absence of chloroquine, in agreement with physico-chemical studies showing that both polymer lactobionate and polymer acetate provide small complexes. These two characteristics may contribute strongly to the efficiency of the polymers. The transfection efficiency for all the polymers increased with the NH_3^+/PO_4^- ratio. This effect is possibly due to an increasing interaction of the different polymers/DNA complexes with the cell surface, thanks to the positive charge on the complexes. Similar observations have been reported for other types of polycationic polymers (Rungsardthong et al., 2001; Choi et al., 1998). The cationic charge of the complexes is an important parameter, but the percentage of transfection is also strongly affected by the size of the polymer/DNA complexes. For the same charge, we have a better diffusion of the small particles and lower probability of the presence of multiple DNA molecules in a same aggregate. Like this, its not surprising that a lactobionate polymer/DNA complex even at a charge ratio of 0.5 could be more efficient than a more larger aggregate of chloride polymer/DNA at a charge ratio of 4 (Fig. 9). Concerning the lactobionate polymer, the percentage of transfection seems to show a threshold at a charge ratio of 4, after that we observe an important increase of the transfection efficiency. This phenomenon could be related to a sufficient concentration of lactobionate polymer before the counterions could acts directly on the transfection process. Some sugars could interact with some lipids of the membrane (Koynova et al., 1989; Epand and Bryszewska, 1988), favoring their destabilisation. The same process could occur with

lactobionate counterions and contribute to an increase of the penetration of the complexes.

5. Conclusion

By zeta potential measurements, we studied the formation of polymer/DNA complexes. Polymer/DNA binding was also studied by ethidium bromide assays, heparin displacement and protection against DNAse I degradation. All of these experiments pointed out the importance of the counterion on the behaviour of the polymer in solution. The size and the hydrophilicity of the counterion influence the morphology of polymer aggregations in water and consequently their interaction with DNA. According to the counterion, the contribution to the complexation of the electrostatic and hydrophobic interactions can be modulated, and the binding strength with the DNA is in the following order, acetate, lactobionate and chloride. Moreover, changing the nature of the counterion of a same polymer, seem to be an original way to modulate the size of the complexes and their affinity toward DNA. Finally, the relative efficiency at equivalent charge ratio (NH_3^+/PO_4^-) of each polyplex was compared on the basis of GFP reporter gene expression in cells in culture, and the results obtained with the lactobionate and acetate counterions are encouraging. Work is now in progress for a better understanding of the cellular action mechanism of these compounds.

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