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Micellar-type complexes of tailor-made synthetic block copolymers containing the *HIV-1 tat* DNA for vaccine application

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Abstract

A novel class of cationic block copolymers constituted by a neutral hydrophilic poly(ethylene glycol) (PEG) block and a positively charged poly(dimethylamino)ethyl methacrylate block was prepared for delivery of DNA. These block copolymers spontaneously assemble with DNA to give in aqueous medium micellar-like structures. Five of these novel block copolymers (K1–5), differing in the length of both the PEG chain and the linear charge density of the poly(dimethylamino)ethyl methacrylate block, were prepared and analyzed for gene delivery, gene expression and safety. All five block copolymers protected DNA from *DNAse* I digestion and delivered the DNA into the cell. However, only three of them (K1, K2 and K5) released the DNA at level allowing efficient gene expression into cells. No toxic effects of both the copolymers alone or their DNA complexes were observed in vitro or in mice. In addition, copolymers were scarcely immunogenic. These results indicate that this novel class of cationic block copolymers is safe and possesses the biological characteristics required for DNA delivery, thus, representing promising vehicles for DNA vaccination.

Keywords: Block copolymers; *HIV-1 tat* gene delivery and expression; Toxicity; Safety

1. Introduction

Vaccination against tumors and intracellular pathogens requires a cell-mediated immunity. This has let to the development of new forms of vaccination using direct injection of DNA or mRNA in the muscle or skin [1–5] and to the use of live expression vectors [6]. An important advantage of these vaccination methods over subunit proteins, polysaccharide conjugates, or inactivated virus vaccines is that in vivo-synthesized antigens can enter both major histocompatibility complex (MHC) class I and class II antigen processing pathways and can drive a broad range of specific and long-lasting immune responses, including cytotoxic T lymphocytes (CTL) [4,5]. In fact, DNA immunization has been found to induce protection in different models of viral diseases [7–13], to elicit antitumor activities [14] or to inhibit allergic responses [15,16].

Several studies have shown that, although muscle cells express the genes delivered by intramuscular injection of the DNA and can present antigens to immune cells, the immune response is not initiated by the transfected muscle cells, but by bone marrow-derived dendritic cells presenting the antigen released or secreted from the transfected muscle cells [17]. The magnitude and nature of these immune responses can be improved by a variety of strategies, including the use of DNA delivery systems [5,17–20]. Undoubtedly, the most effective systems for delivery and expression of DNA are represented by viral vectors [6,21]. However, these systems show several disadvantages including toxicity, immunogenicity, restricted cell targeting, limited DNA carrying capacity, limitation by pre-existing immune responses, production and packaging problems, high production costs, as well as different regulatory issues for approval for human use [21].

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Synthetic non-viral vectors for the delivery of plasmid DNA is an expanding research field since they are versatile, safe and easy to produce [22,23]. In addition, they favour and increase the uptake of the DNA by antigen-presenting cells, thus, allowing antigens synthesized intracellularly to be readily accessible to the antigen processing machinery that loads peptides onto the MHC class I molecules to induce CTL [24–29]. Cationic carriers with block or graft copolymer architecture, consisting of a polycation linked to a non-ionic water soluble polymer like poly(ethylene glycol) (PEG), represent a promising class of synthetic gene delivery vectors. These systems are specifically designed to self-assemble with DNA by electrostatic interactions.

Several block copolymers have recently been described including PEG-*b*-poly(L-lysine) [30,31], PEG-*g*-poly(L-lysine) [32], PEG-*b*-polyspermine [33], poly-*N*-(2-hydroxypropyl)-methacrylamide-*b*-poly(trimethylammoniiummethyl methacrylate chloride) [30] and poly(ethylene oxide)-*g*-poly(ethyleneimine) [34]. However, improvements of transfection efficiency of the plasmid DNA were reported only in a few cases [34].

We recently described a novel class of block copolymers, constituted by a neutral, hydrophilic PEG block of variable length, and a positively charged poly(dimethylamino)ethyl methacrylate block. These copolymers were partly or fully alkylated to give cationic block copolymers [35] which are able to spontaneously assemble with DNA to give in aqueous medium micellar structures, named polyion complex micelles. The supramolecular structure of these complexes is a core–shell-type micelle, in which the hydrophobic core consists of DNA, linked via electrostatic interactions to the charged part of the block copolymer, and the outer shell is constituted by the non-charged hydrophilic PEG block of the copolymer. Moreover, these novel block copolymers and their complexes with DNA possess physico-chemical characteristics appropriate for in vivo applications [35].

A key issue for the development of new gene delivery systems is the efficiency of gene delivery, which is dependent on the uptake, protection of nucleic acid from extra and intracellular degradation, and efficient release and expression of nucleic acid from the nucleic acid–polymer complexes. Thus, we analyzed the capability of these new block copolymers to deliver, release and allow expression of plasmid DNA, as well as their safety in vitro and in vivo.

2. Materials and methods

2.1. Plasmids

In these studies three plasmids were used. The plasmid pCV-Tat, expressing the *HIV-1 tat* gene, and the empty plasmid pCV-0 were previously described [36]. The plasmid pCV-Tat–EGFP, expressing a Tat–EGFP hybrid protein under the transcriptional control of the adenovirus major late promoter, was constructed as follows. Briefly, the *tat–EGFP* fusion gene, where the full-length cDNA (258 bp) of the *HIV-1* (BH10 clone) *tat* gene is upstream and in frame with the enhanced green fluorescent protein (*EGFP*) gene, was amplified in a DNA thermal cycler (Perkin-Elmer Cetus, Perkin-Elmer, Forster City, CA) from the plasmid *ptat–EGFP* [37] using primers 5′-GGGCTGCAGATGGAGCCAGTAGATCCTAGA-3′ (forward), mapping at the 5′ end of the *tat* gene (nucleotides 5864–5884 on *HXB2 HIV-1* genome, gene bank accession number K03455M384332), and 5′-GGGCTGCAGTTACTTGTACAGCTCGT-3′ (reverse), mapping at the 3′ end of the *EGFP* gene (nucleotides 1394–1381 on pEGFP-N3 plasmid DNA, gene bank accession number U57609). Both primers contained the *Pst* I restriction enzyme site. The PCR product was purified with the QIAquick PCR purification kit (Qiagen, Santa Clarita, CA), digested with *Pst* I and cloned into the plasmid pCV-0. The recombinant plasmid was sequenced with a commercial kit (Dye Terminator Cycle Sequencing, Perkin-Elmer, Forster City, CA) on an ABI 373 DNA automated sequencer (Applied Biosystem, Perkin-Elmer Biosystem). Plasmid DNAs were purified onto two CsCl gradients, according to standard procedures [38], and resuspended in sterile phosphate-buffered saline (PBS) with calcium and magnesium.

2.2. Copolymers and assembly of complexes at different charge ratios

Cationic block copolymers, characterized by a PEG block of variable length and a poly(dimethylaminoethyl) methacrylate block, either partly or fully alkylated, were synthesized and characterized as described previously [35]. Five polymers (K1–5) were examined in this study. K1 (M_n, 28,000) and K2 (M_n, 91,000) copolymers are fully methylated with methyl iodide and possess 90 and 300 positive charged groups per molecule, respectively. K3 (M_n, 56,400) and K5 (M_n, 48,200) are partly methylated (55 and 30%) and possess 127 and 70 positive charged groups per molecule, respectively. K4 (M_n, 80,600) is fully alkylated with bromobutane and possesses 230 positive charged groups per molecule. For calculation of the charge ratio an average mass per charge of 330 Da was used for DNA. The mass per charge for all the cationic copolymers was calculated from the degree of polymerization obtained by the ^{1}H-HNMR spectra. Complexes were prepared in buffer solution by mixing the appropriate block copolymer solution to DNA at various concentrations, and left for 30 min at room temperature before use. After incubation, complexes were used directly without further processing. The molar ratio of copolymer quaternary ammonium positive groups to DNA phosphate negative groups (N:P ratio) in the complexes ranged between 0.5 and 5.0.

2.3. Particle size and ζ-potential measurements

For dynamic light scattering measurements, the pCV-Tat plasmid was diluted in pure water to a concentration of

20 μg/ml. Then, the appropriate quantity of the polymer solution was added to 1.5 ml of DNA solution. To compare the complexing behavior of the various block copolymers, the complexes were prepared at defined N:P ratio ranging from 0.5 to 1.6. After 30 min incubation to allow complex formation, *Z*-average particle size and polydispersity index (PI) of the complexes were determined by dynamic light scattering (DLS) at 25 °C with a Zetasizer 3000 HS system (Malvern, UK) using a 10 mV He–Ne laser and PCS software for Windows (version 1.34, Malvern, UK). For the data analysis, the viscosity and refractive indexes of pure water at 25 °C were used. The instrument was calibrated with standard polystyrene latex particles of 200 nm in diameter. For the ζ-potential measurements the pCV-Tat plasmid was diluted in 10 mM sterile KH_2PO_4 buffer solution to a concentration of 20 μg/ml. The polymers were diluted in the same buffer to a concentration of 3.0 mg/ml. Then, the appropriate amount of the polymer solution was added to 1.5 ml of DNA solution, resulting in a N:P ratio ranging from 0.5 to 2.0. After 30 min incubation to allow complex formation, the ζ-potential was measured at a temperature of 25 °C with a Zetasizer 3000 HS and PCS software for Windows. The instrument was calibrated using latex particles with known ζ-potential.

2.4. DNAse protection assays

Block copolymers were resuspended at 10 mg/ml in PBS. Ten micrograms of the pCV-0 plasmid DNA were mixed with the appropriate amount of each copolymer solution, at N:P ratio 1.0 or 5.0, in 250 μl of 40 mM Tris–HCl (pH 8.0), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$. The control samples were the pCV-0 plasmid DNA (10 μg) without the copolymer and the block copolymer without the DNA, respectively. Optical densities were read with a spectrophotometer at 260 nm (time 0). RNAse-free *DNAse* I (1 u/μg; Stratagene, La Jolla, CA) was then added and optical densities were read following incubation for 15 and 60 min, respectively, at 37 °C.

2.5. Cell cultures and transfections

Monolayer cultures of human HL3T1 cells, containing an integrated copy of plasmid HIV-1-LTR-CAT, where expression of the chloramphenicol acetyl transferase (*CAT*) reporter gene is driven by the HIV-1 LTR promoter [39], were grown in DMEM (Gibco, Grand Island, NY) containing 10% FBS (Gibco). For DNA transfection, the calcium phosphate co-precipitation technique was used [38]. Alternatively, the lipofection procedure was used in some experiments, using the "Lipofectamin reagent" kit (Gibco), which is a 3:1 (w/w) liposome formulation of DOSPA:DOPE, according to the manufacturer's instructions.

2.6. Analysis of cytotoxicity in vitro

HL3T1 cells (1×10^4) were resuspended in 100 μl of DMEM containing 10% FBS, seeded in 96-well plates and cultured at 37 °C for 24 h. One-hundred microliters of medium containing pCV-0 (10 μg) plasmid DNA alone, or previously incubated with the appropriate volume of each copolymer to reach the N:P ratio 1.0 or 5.0, or containing the copolymers alone, were then added to the cells. Untreated cells were included as the control. Each sample was assayed in sestuplicate. Cells were incubated for 5 h at 37 °C, washed and incubated in fresh medium for 96 h. In some experiments, cells were cultured in the presence of the inoculum for 96 h. At the end of incubation, cell proliferation was measured using the colorimetric cell proliferation kit I (MTT-based) provided by Roche (Roche, Milan, Italy) [40].

2.7. Cellular uptake

HL3T1 cells (1×10^5) were seeded in 24-well plates containing 12 mm glass coverslips and cultured at 37 °C. Twenty-four hours later cells were replaced with 1 ml of fresh medium and incubated for 5 h at 37 °C in DMEM (without FBS) containing 100 ng of FITC-labeled pCV-0 DNA alone, or previously mixed with each copolymer at a 1.0 N:P charge ratio. Cells were then washed and cultured with fresh medium for 12 h. Controls were represented by cells transfected with the same amount of DNA using the lipofection procedure, untreated cells and cells incubated with the copolymers alone. Cells were washed and fixed with 4% cold paraformaldehyde. Samples were observed at a confocal laser scanning microscope LSM410 (Zeiss, Oberkochen, Germany). Image acquisition, recording and filtering were carried out using a Indy 4400 graphic workstation (Silicon Graphics, Mountain View, CA) as previously described [41]. Plasmid DNA was labeled with FITC-dUTP using the "Prime-It Fluor" kit (Stratagene), according to the manufacturer's instructions.

2.8. Evaluation of gene expression in vitro

HL3T1 cells (5×10^5) were seeded in 60 mm Petri dishes containing 12 mm glass coverslips and cultured at 37 °C. Twenty-four hours later cells were replaced with 10 ml of fresh medium and incubated for 5 h at 37 °C in DMEM (without FBS) containing pCV-Tat–EGFP DNA (10 μg) alone without the copolymers, or previously mixed with each copolymer at 1.0 or 5.0 N:P charge ratio. The same plasmid DNA (1–10 μg) was also transfected with the calcium phosphate co-precipitation technique or lipofected, as positive control of gene transfection/expression. Cells were washed and cultured with fresh medium for an additional 36 h. Gene expression was measured by both assaying the expression of CAT induced by Tat and by the detection of the chimeric Tat–EGFP protein with a fluorescence

microscope. CAT activity was measured in cell extracts after normalization to total protein content [37]. For detection of fluorescent Tat–EGFP, cells were fixed with cold 4% paraformaldehyde, colored with DAPI (0.5 μg/ml, Sigma, St. Louis, MI), and analyzed at a fluorescence microscope, as previously described [37].

2.9. Analysis of toxicity in vivo

Six-weeks-old Balb-c mice were injected with the copolymer K2 subcutaneously, at days 0, 15 and 30, in two sites in the back. Groups of two mice each were injected with 5, 10, 50, 100 and 250 μg of the copolymer in 100 μl of PBS, respectively, whereas one mouse was injected with 500 μg. Control animals were four untreated mice. In addition, groups of 5–10 mice each were injected by the intramuscular route with pCV-0 (30 μg) complexed to K1, K2 (N:P ratio 1.0) or K5 (N:P ratio 5.0), or with naked plasmid DNA as the control. Mice received six injections at days 0, 15, 30, 60, 90 and 120. Animals were observed daily at the site of injection and for their general conditions. Three weeks after the last inoculation animals were anaesthetized and sacrificed to collect and examine blood and organs.

2.10. Histological, histochemical and immunohistochemical procedures

Animals were sacrificed and subjected to autopsy. Sample of cutis, subcutis and skeletal muscle at the sites of injection and other organs (lungs, kidneys, intestine, lymph nodes, spleen and liver) were taken for histologic examination. Tissues were fixed in 10% formalin for 12–24 h and embedded in paraffin. Three 5 μm paraffin-embedded sections were then stained with hematoxylin and eosin, subjected to periodic acid Shiff (PAS) reaction, and treated with PAS–Diastase (Sigma). The avidin–biotin–peroxidase complex (ABC) technique was used for the immuno-histochemical studies performed on paraffin sections. The panel of antibodies included S-100 (DAKO, Denmark), HH-F 35 (DAKO) for detection of α-actin, CD68 and Mac387 (DAKO) for detection of macrophages. Briefly, after deparaffinization and rehydration, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol; samples were then incubated with the primary antibodies for 10–12 h at 4 °C. Biotinilated-anti-mouse and anti-rabbit immunoglobulins were utilized as secondary antibodies. Specific reactions were detected following incubation with avidin–biotin–peroxidase conjugated and development in diaminobenzidine (Sigma).

2.11. Serology

Serological responses against copolymers were measured by an enzyme-linked immunosorbent assay (ELISA) that was accurately standardized in preliminary experiments. Ninety-six wells immunoplates (Maxisorp, Nunc, Naperville, IL) were coated with 100 μl/well of copolymers (5–100 μg/ml in 0.05 M carbonate buffer, pH 9.6), sealed and incubated in the dark for 2 h at 37 °C. Each sample was tested on two coated wells (specific reactivity) and on one uncoated well (unspecific reactivity). Wells were washed five times with 0.05% Tween 20 in PBS (PBS–Tween) in an automated washer (Immunowash 1575, Bio-Rad Laboratories, Hercules, CA) and blocked with 150 μl/well of PBS containing 3% BSA for 90 min at 37 °C. Sera were diluted in PBS containing 3% BSA. The minimal serum dilution was 1:100. After extensive washing, 100 μl aliquots were added and incubated for 90 min at 37 °C. Plates were washed five times with PBS–Tween, and 100 μl of horse-radish peroxidase-conjugated sheep anti-mouse IgG (Amersham Life Science, Buckinghamshire, UK), diluted 1:1000 in PBS–Tween containing 1% BSA, were added to each well. Plates were incubated for 90 min at room temperature, washed five times and incubated with 100 μl/well of peroxidase substrate (ABTS) (Roche, Milan, Italy) for 40 min at room temperature. The reaction was blocked with 100 μl of 0.1 M citric acid and the optical density (OD) was measured at 405 nm in an automated plate reader (ELX-800, Bio-Tek Instruments, Winooski, UT). In each assay, the blank corresponded to copolymer-coated wells incubated with the labeled secondary antibody and ABTS (control of the labeled antibody); two uncoated wells were also incubated with ABTS alone (ABTS control). The reaction value was then calculated as the difference between the OD of coated and uncoated wells (ΔOD_{405}), previously subtracted of the blanks' OD. The cut-off corresponds to the mean ΔOD_{405} (+3 S.D.) of sera of untreated mice ($n = 4$), or mice injected with pCV-0 naked DNA ($n = 6$), tested in three independent assays. Samples ΔOD_{405} higher than the cut-off were considered positive.

2.12. Evaluation of gene expression in vivo

Six-weeks-old Balb-c mice were injected by the intramuscular route with pCV-Tat (1 μg) complexed to K2 (N:P ratio 1.0) or with naked pCV-Tat (10 μg). Control mice were injected with naked pCV-0 plasmid DNA (10 μg). Mice received six injections at days 0, 15, 30, 60, 90 and 120. Mice were observed daily and sacrificed 3 weeks after the last inoculation. Muscle at the site of injection was collected, immediately frozen in liquid nitrogen and stored at −80 °C. Expression of the *tat* gene was evaluated by RT–PCR analysis. RNA was extracted using the "Tripure" reagent provided by Roche, according to manufacturer's instructions. RNA (5 μg) was incubated with RNAse-free *DNAse* (1 u/μg; Promega, Madison, WI) for 30 min at 37° (*DNAse* treatment was repeated three times), and purified by phenol–chloroform extraction. Before retrotranscription (RT), the absence of contaminating DNA was controlled on RNA samples by PCR using actin-specific primers [5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (forward), 5′-AGTCATAGTCCGCCTAGAAGCATTTG-

CGGT-3′ (reverse) at 95 °C for 90 s, 63 °C for 30 s, 72 °C for 90 s (35 cycles)]. RNA (2 μg) was reverse transcribed into cDNA using an oligo dT as the primer and the "RT–PCR system" provided by Promega. One-fifteenth of the RT-reactions was amplified by PCR using primers specific for the cellular actin gene, as described above. One-sixth (one-third for naked pCV-Tat) of the RT-reactions was amplified by PCR using primers specific for the *HIV-1 tat* gene [5′-GGGCTGCAGTCTCTGTCTCTCTCTC-3′ (forward), 5′-GGGCTGCAGGGCGACTGAATTGGT-3′ (reverse), at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min (35 cycles)]. PCR reactions were carried out in a DNA thermal cycler (Perkin-Elmer Cetus), and PCR products were analyzed on 1–1.5% agarose–gel electrophoresis.

2.13. Statistical analysis

Student's *t*-test was performed as described [42].

3. Results

3.1. Block copolymers

The various block copolymers, named K1–5, are characterized by a PEG block of variable length and a poly(dimethylamino)ethyl methacrylate block, either partly or fully alkylated. Their specific structural characteristics are reported in Table 1. Block copolymer–plasmid complexes (Fig. 1) were prepared by mixing in buffer DNA with the given block copolymer at various concentrations in buffer solutions, as described in Section 2.

3.2. Block copolymer–plasmid complex assembly: particle size and ζ-potential

The surface charge characteristics of the complexes were determined by measuring the ζ-potential value as a function of the N:P ratio (Fig. 2). The results indicate that the ζ-potential trend has a typical sigmoid shape and is very similar for all complexes. The ζ-potential turned from negative at low N:P ratio, corresponding to an excess of phosphate groups in the complexes, to positive at very high N:P ratio, corresponding to an excess of quaternary ammonium positive groups in the complexes. The *Z*-average particle size of the complexes, determined by dynamic light scattering

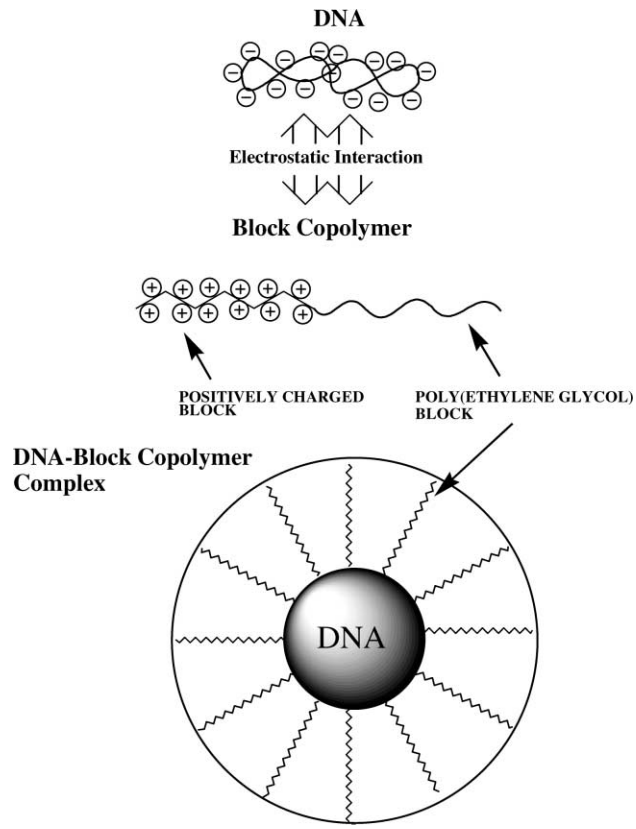
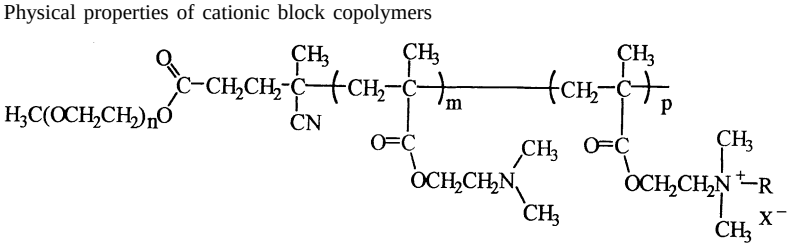


Fig. 1. Schematic representation of DNA/block copolymer micellar-type complexes.

Table 1
Physical properties of cationic block copolymers



Block copolymer	R groups	*n*	*m*	*p*	M_n (NMR)
K1	$-CH_3$	16	–	90	28000
K2	$-CH_3$	44	–	300	91000
K3	$-CH_3$	44	104	127	56400
K4	$-(CH_2)_3CH_3$	44	–	230	80600
K5	$-CH_3$	44	160	70	48200

Physical properties of cationic block copolymers: alkyl groups (R), number of oxyethylene units in the PEG chains (*n*), number of amino groups (*m*) and of quaternary ammonium groups (*p*) in the positive charged block, and molecular weight (M_n) (evaluated by HNMR).

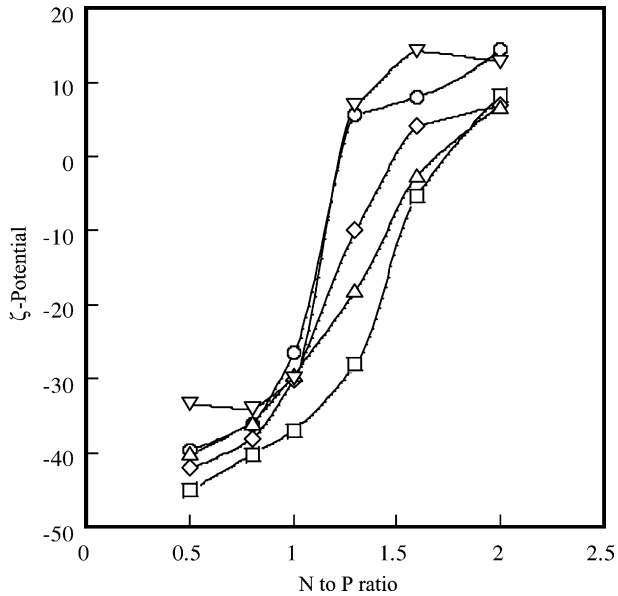


Fig. 2. ζ-Potential of copolymers/plasmid complexes as a function of the molar ratio of amino or quaternary ammonium groups to DNA phosphate groups (N:P ratio). DNA/K1 complex (▽), DNA/K2 complex (○), DNA/K3 complex (□), DNA/K4 complex (◇), DNA/K5 complex (△).

at 25 °C, ranged from 100 to 300 nm with a PI comprised between 0.2 and 0.5 (Table 2). The results also indicate that the *Z*-average particle size of the complexes decreases as the N:P ratio increases. This effect is in agreement with other reports [43–45], and is usually explained in terms of DNA condensation, deriving from the increase in the counter charge density, as a consequence of the increase of block copolymer concentration.

3.3. Polymers protect DNA from DNAse I degradation

One of the most important characteristics required for DNA delivery systems is the nuclease resistance. This feature was examined using the plasmid pCV-0. To this purpose, 10 μg of free DNA or DNA complexed with each copolymer at 1.0 or 5.0 N:P charge ratios were incubated with *DNAse* I for different time periods at 37 °C. As shown in Table 3, the addition of *DNAse* I to naked DNA produced an immediate increase in the absorbency of the solution that is due to DNA fragmentation. In contrast, the DNA–copolymer complex solutions showed small changes, at both charge ratios, in the absorbency values following the addition of *DNAse* I. Thus, each block copolymer was able to effectively protect DNA from *DNAse* I digestion.

Table 3
Block copolymers protect DNA from *DNAse* I digestion[a]

Complex	N:P ratio	$\Delta OD_{260\,nm}$	
		15 min	60 min
pCV-0/K1	1:1	0	0
	1:5	0	0
pCV-0/K2	1:1	0	0
	1:5	0	0
pCV-0/K3	1:1	0	0
	1:5	0	0
pCV-0/K4	1:1	0	0
	1:5	0	0
pCV-0/K5	1:1	0	0
	1:5	0	0
pCV-0 naked		0.96	0.92

[a] Ten micrograms of pCV-0 plasmid DNA alone or associated to K1–5 copolymers, at a 1:1 and 1:5 charge ratios, were incubated for 30 min to allow complex formation. Then, optical density at 260 nm was read before (OD_{260} range 0.203–0.275) and after addition of *DNAse* I. ΔOD_{260} were measured after 15 and 60 min incubation at 37 °C.

3.4. Measurement of in vitro cytotoxicity of free and DNA-complexed copolymers

The cytotoxicity of copolymers K1–5 was assayed in HL3T1 cells following exposure to pCV-0 DNA–copolymer complexes, at the N:P charge ratio of 1.0 or 5.0, or to the corresponding amount of free copolymers. Cells incubated with free DNA, in the absence of the copolymers, or untreated cells, represented the control samples.

Block copolymers K1–5 associated to DNA at N:P charge ratio of 1.0 were not toxic for the cells after both 5 and 96 h incubation (Fig. 3a and c, black bars), with the only exception of the pCV-0/K5 complex that caused a significant reduction ($P < 0.05$) of cell viability after 96 h incubation

Table 2
Z-average particle size and polydispersity index of the complexes[a]

N:P ratio	*Z*-average ± PI (nm)				
	K1	K2	K3	K4	K5
0.5	218 ± 0.328	200 ± 0.500	278 ± 0.310	230 ± 0.342	135 ± 0.391
0.8	145 ± 0.480	171 ± 0.313	140 ± 0.374	185 ± 0.275	138 ± 0.230
1.0	118 ± 0.239	118 ± 0.359	106 ± 0.255	130 ± 0.245	123 ± 0.280
1.6	107 ± 0.250	105 ± 0.201	117 ± 0.300	110 ± 0.278	106 ± 0.220

[a] The complexes were prepared in water at defined molar ratio of amino or quaternary ammonium groups to DNA phosphate groups (N:P ratio). After 30 min incubation to allow complex formation, *Z*-average particle size and polydispersity index (PI) of the complexes were determined by dynamic light scattering (DLS) at 25 °C.

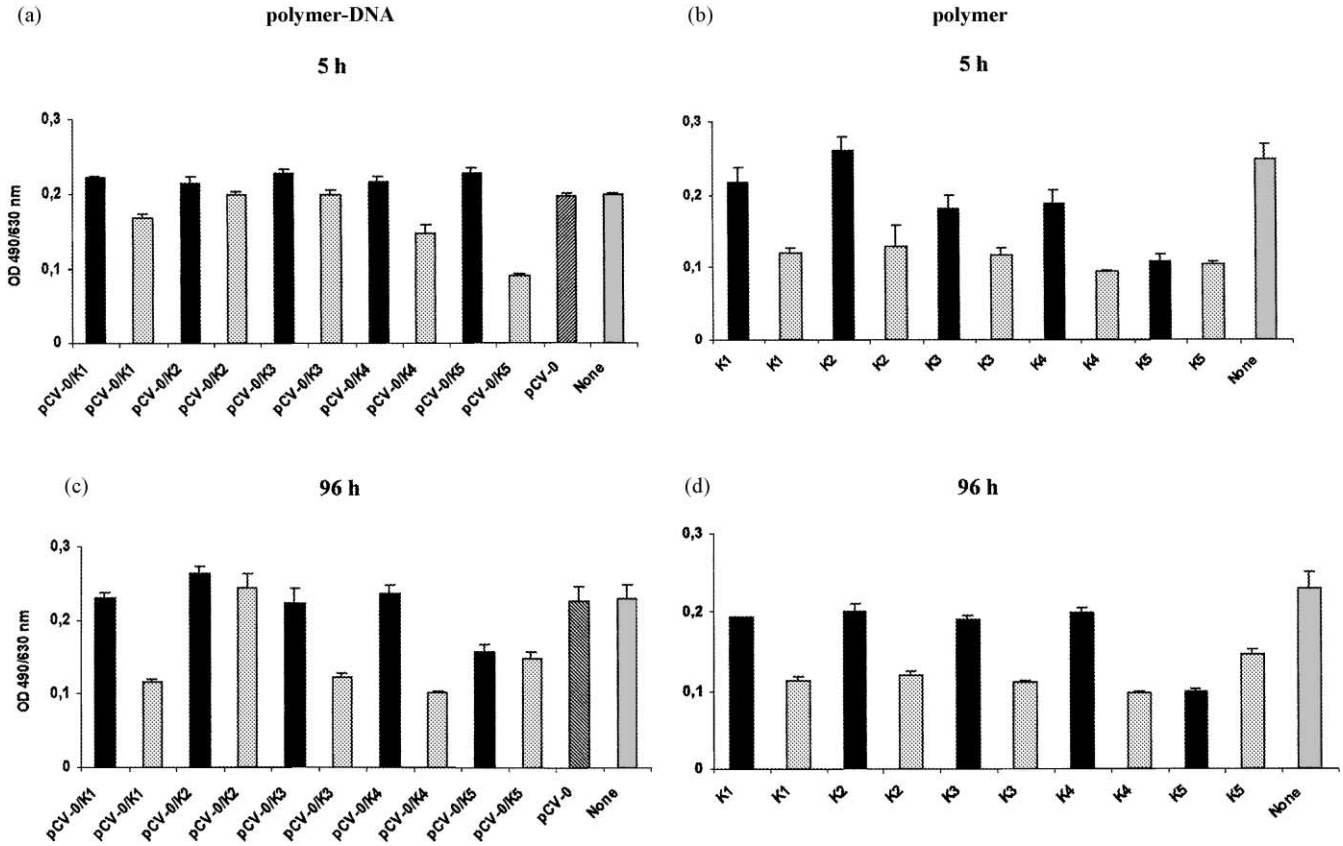


Fig. 3. Evaluation of cell proliferation in the presence of block copolymer–DNA complexes (a, c) or free block copolymers without DNA (b, d) after 5 or 96 h incubation. HL3T1 cells were cultured with pCV-0 plasmid DNA (10 μg) associated with different concentrations of K1–5, at 1.0 (black bars) or 5.0 (dotted bars) N:P charge ratios, with pCV-0 without copolymer (stripped bars), or with medium alone (gray bars). Results are expressed as the mean (±S.D.) of sestuplicates.

(Fig. 3c, black bar). Interestingly, early after the addition to the cells, all DNA–copolymer complexes induced a statistically significant ($P < 0.05$) increase in cell proliferation (Fig. 3a, black bars), which persisted after 96 h only in cells treated with pCV-0/K2 (Fig. 3c, black bar). In contrast, all DNA–copolymer complexes assembled at N:P charge ratio of 5.0 were slightly toxic and reduced cell proliferation to various extent, with the exception of pCV-0/K2 (Fig. 3a and c, dotted bars). Early after incubation, this effect was statistically significant ($P < 0.05$) for pCV-0/K1, pCV-0/K4 and pCV-0/K5 (Fig. 3a, dotted bars), whereas for pCV-0/K3 it was observed only after 96 h (Fig. 3c, dotted bar).

When free block copolymers (without DNA) were added to the cells, at the same doses used for the DNA–copolymer complexes at 1.0 and 5.0 N:P charge ratios, a significant decrease ($P < 0.05$) in cell viability and proliferation was generally observed, both with the lower (Fig. 3b and d, black bars) or the higher doses (Fig. 3b and d, dotted bars), with the exception of K1 and K2 at the lower dose after 5 h (Fig. 3b, black bars).

These data agree with the observed cytotoxicity of the charged poly(dimethylamino) ethyl methacrylate, which is drastically reduced upon electrostatic interaction with DNA and subsequent complex formation [46]. In the case of the free copolymers, and of the aggregates at N:P ratio of 5.0, the free positively charged groups may be responsible for non-specific interactions with the cell surface and/or negatively charged biopolymers inside the cells (proteins, RNA and even DNA), thus, interfering with normal cellular functions.

3.5. Cellular uptake of DNA–copolymer complexes

The capability of block copolymers K1–5 to deliver the DNA into the cells was evaluated using fluoresceinated pCV-0 plasmid DNA, complexed with each copolymer at N:P charge ratio of 1.0. Confocal microscopic analysis showed that the cells internalized the DNA–copolymer complexes. Indeed, intracellular fluorescence was detected in all samples incubated with the pCV-0/copolymer complexes (Fig. 4a–e), in a fashion similar to that observed in the positive controls, where fluorescinated-pCV-0 DNA was introduced into the cells by means of lipofection (Fig. 4g). The extent of cellular uptake of the DNA–copolymer complexes was comparable to that of naked DNA (Fig. 4h), and lower as compared to lipofection.

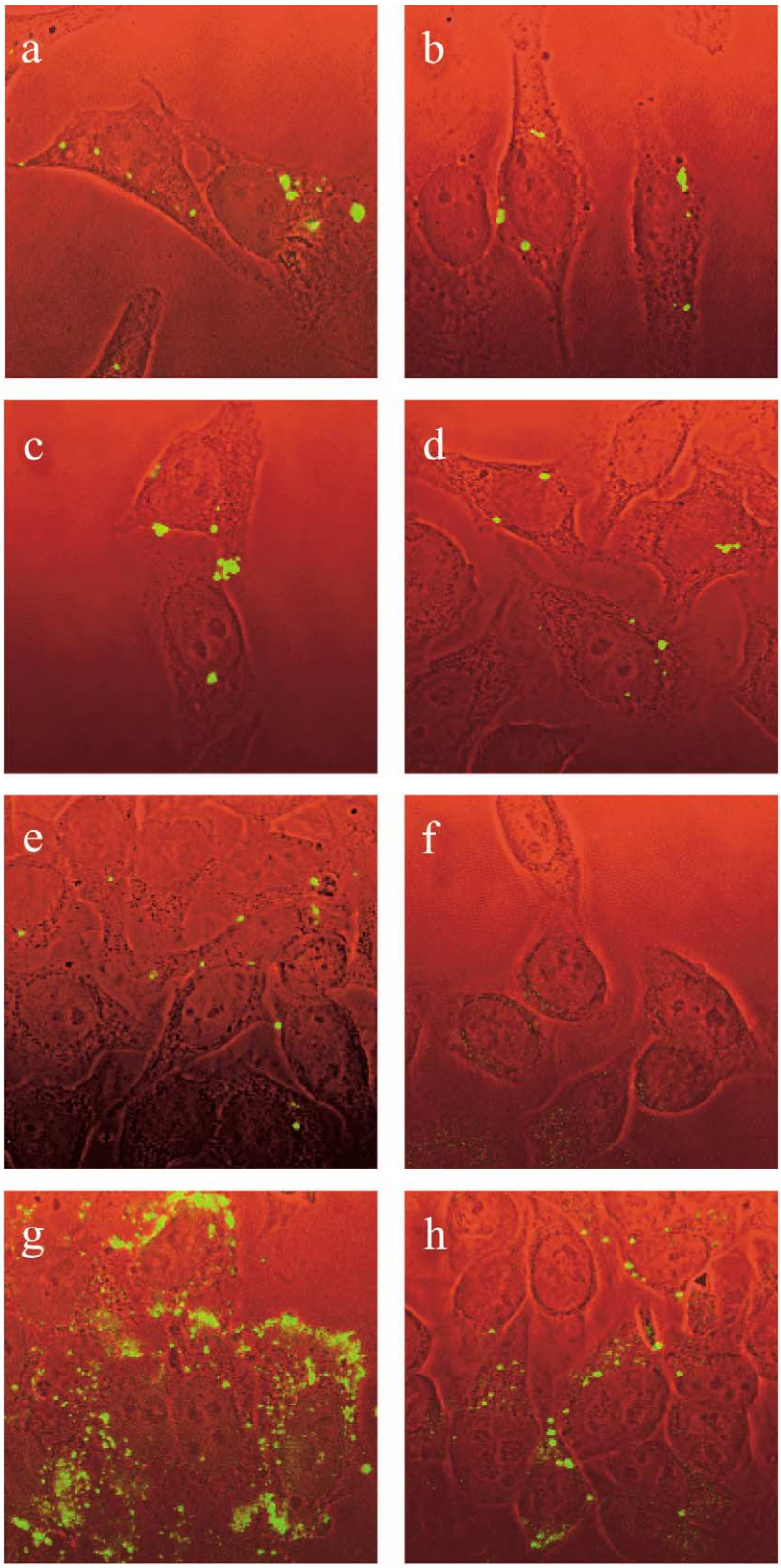


Fig. 4. Block copolymers–DNA complexes are internalized by the cells. Cells were incubated with 100 ng of FITC-labeled pCV-0 plasmid DNA associated (N:P charge ratio of 1.0) to K1 (a), K2 (b), K3 (c), K4 (d), K5 (e) or with FITC-labeled DNA without the copolymers (h). In addition, cells were treated with each copolymer without DNA (f) or lipofected with FITC-labeled DNA (g). In part (f), the results of one representative block copolymer without DNA are shown.

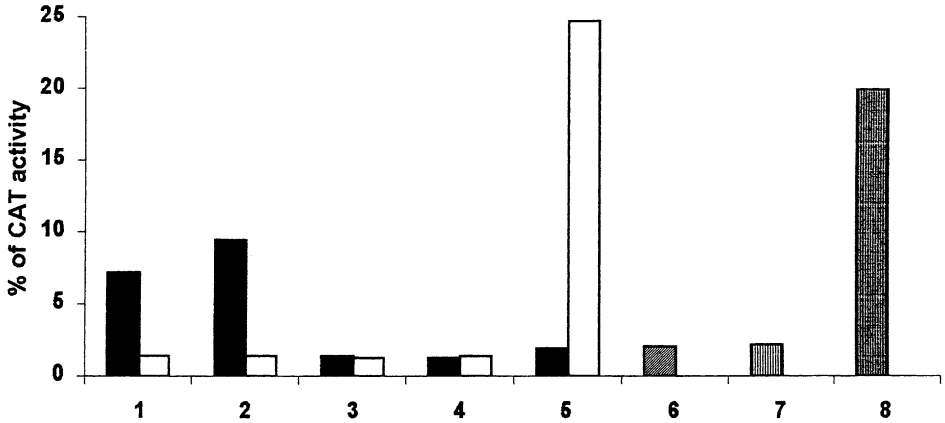


Fig. 5. Analysis of expression of pCV-Tat–EGPF (10 μg) complexed to block copolymers. (1) pCV-Tat–EGFP/K1; (2) pCV-Tat–EGFP/K2; (3) pCV-Tat–EGFP/K3; (4) pCV-Tat–EGFP/K4; (5) pCV-Tat–EGFP/K5; (6) pCV-Tat–EGFP without copolymers; (7) HL3T1 without DNA (cell background); (8) pCV-Tat–EGFP lipofected. HL3T1 cells, containing an integrated copy of the reporter vector HIV-1 LTR-CAT, were incubated with pCV-Tat–EGFP/block copolymers complexes, at 1.0 (black bars) or 5.0 (white bars) N:P charge ratios. As a positive control of gene transfection/expression, pCV-Tat–EGFP (1–10 μg) was introduced into the cells by lipofection. The percentage of CAT activity was calculated by the formula [cpm of the acetylated ^{14}C-chloramphenicol/total cpm of acetylated and unacetylated ^{14}C-chloramphenicol] × 100, as described previously [37]. Results are the mean of two independent experiments. Results of lipofection, reported in lane 8, are referred to 1 μg of DNA. After lipofection of 5 and 10 μg of DNA, the percentage of CAT activity was >98 (not shown).

3.6. Evaluation of gene expression in vitro

The capability of intracellular DNA–copolymer complexes to release DNA and to allow its expression was evaluated in HL3T1 cells, containing the CAT reporter gene under the transcriptional control of the HIV-1 LTR promoter. Cells were incubated with pCV-Tat–EGFP complexed to each copolymer, both at 1.0 or 5.0 N:P charge ratio, and with pCV-Tat–EGFP alone (in the absence of the copolymers). Cells transfected with pCV-Tat–EGFP using lipofection or the calcium phosphate precipitation technique represented the positive control of gene transfection/expression. After 48 h incubation, expression of Tat–EGFP was simultaneously analyzed by a fluorescence microscope and CAT assays. As shown in Fig. 5, CAT activity was detected only in cells treated with pCV-Tat–EGFP/K1 and pCV-Tat–EGFP/K2 complexes at 1.0 N:P charge ratio, and with pCV-Tat–EGFP/K5 complex at 5.0 N:P charge ratio, as well as in the positive controls. Observation of the cells at the fluorescence microscope confirmed these results (data not shown). Since activation of the HIV-1 LTR promoter and expression of the *CAT* gene is induced by Tat, this result indicates that K1, K2 and K5 allow the delivery, the release and the expression of the pCV-Tat–EGFP plasmid DNA. Therefore, these three copolymers were tested in vivo.

3.7. Evaluation of toxicity of free block copolymers in vivo

The toxicity of free copolymers was evaluated in mice. Since copolymers have the same basic molecular structure, except for the number of positive charged groups in the poly(dimethylamino)ethyl methacrylate block, we reasoned that any in vivo toxic effect of free copolymer should be related to the number of positively charged groups. Therefore, copolymer K2 that possesses the highest number of positive charged groups was chosen for a detailed investigation of in vivo toxic effects. Five groups of two mice each were inoculated three times subcute with free K2. Each group received the same dose of copolymer (5, 10, 50, 100 or 250 μg). In addition, one mouse was injected with 500 μg. No general or local signs of toxicity were observed during the duration of the experiment as compared to control mice. Three weeks after the last injection, all animals were sacrificed, and skin at the sites of injection, and other organs were examined histologically. All animals constantly showed normal appearance of the skin (epidermis and skin appendages) and of the connective tissue (dermis), except for occasional and scattered lymphocytes infiltrated around hair follicles or in the epidermal–dermal interface. However, at the site of injection, inflammatory infiltrates were always observed with variable distribution and intensity between the dermis and the subcutaneous connective tissue (Fig. 6a). Inflammatory cell infiltrates were composed almost exclusively of macrophages (Fig. 6b) with immunohistochemical reactivity to CD68 and Mac387 (Fig. 6e); few lymphocytes and neutrophil granulocytes were also evident. Inflammatory infiltrates were more intense in mice which received higher doses of K2. Indeed, a dose-related number of macrophages in subcutis was evident, with the highest number found in mice injected with 250 or 500 μg of K2. The inflammatory reaction was low or absent at the 5 μg-dose of K2. Macrophages cytoplasm appeared stuffed

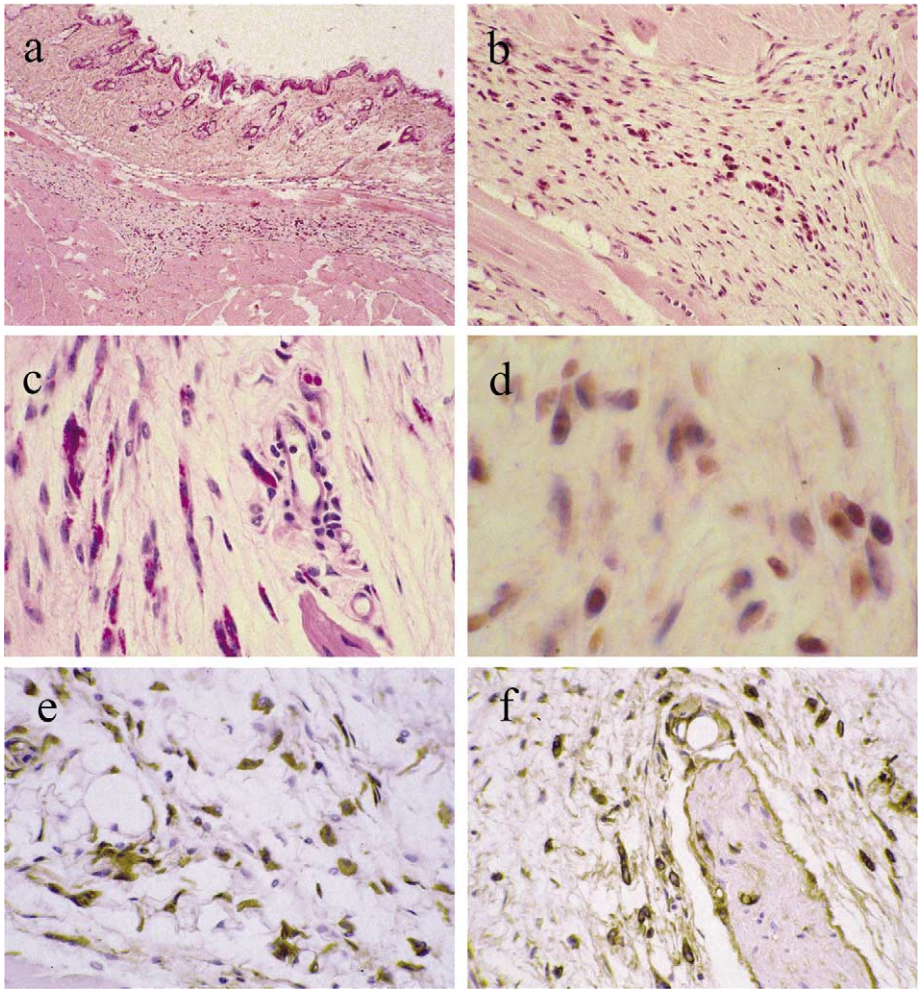


Fig. 6. Histologic examination of cutis and subcutis of mice after subcutaneous injection of free block copolymer K2. A representative mice injected with 250 μg of K2 is shown. An inflammatory cell infiltrate in the subcutis just beneath the "panniculus carnosus" (a), consisting almost exclusively of macrophages (b), with PAS reactive cytoplasm next to capillaries (c) is evident. Macrophages show cytoplasmic inclusions (d), and strong immunohistochemical reactivity with the anti-CD68 antibody (e, f). Hematoxylin–eosin staining: (a) 63×, (b) 160× and (d) 1000× magnifications; PAS reaction: (c) 250× magnification; anti-CD68 avidin–biotin–peroxidase reaction: (e) and (f) 160× magnifications.

and contained microspherular eosinophilic inclusions that showed strong PAS–Diastase reactivity (Fig. 6c, d), which may be related to the K2 block copolymer. Indeed, the principle of the reaction [47] is that periodic acid (oxidant) will cause oxidative cleavage of the carbon-to-carbon bond of glycols or their amino or alkyl-amino derivatives to form di-aldehydes which react with fucsin-sulphorous acid, which in turn combines with the basic pararosaniline to form a magenta colored compound (alkyl sulphonate type). To this respect, free block copolymer K2 shows PAS-reactivity in cell-free assays (not shown). Infiltrates were found in the subcutis next to capillaries or throw the muscle cells or irregularly and diffusely dispersed; eosinophilic inclusions laden cells were also observed around and in the perineural spaces (Fig. 6f). No inflammatory infiltrates with K2-related spherular inclusions were observed in the other organs (lungs, kidneys, intestine, lymph nodes, spleen and liver) that were examined histologically, or in untreated control mice.

3.8. Evaluation of toxicity of DNA–copolymer complexes in vivo

The toxicity of K1, K2 and K5 copolymers complexed with pCV-0 was evaluated in mice injected by the intramuscular route with the DNA–copolymer complexes. Controls were represented by mice injected with naked pCV-0 DNA. Mice received six injections at days 0, 15, 30, 60, 90 and 120. Small foci of necrosis involving one or two muscle fibers with a poor cellular inflammatory reaction were observed at the site of injection of mice receiving pCV-0/K2 complex, but not in control mice. Macrophages were constantly present in the muscular fascia and in the surrounding adipose tissue (Fig. 7a–d). The cytoplasm of

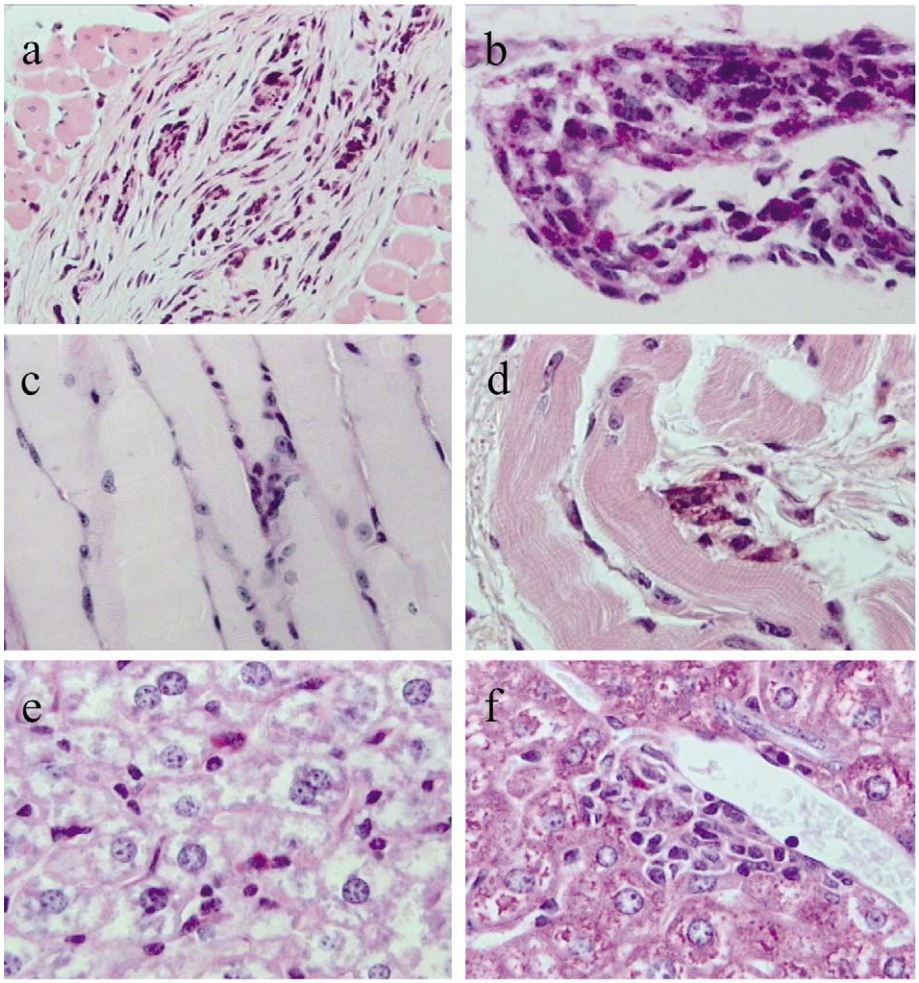


Fig. 7. Histological examination of mice tissues after injection of pCV-0/K2 complex by the intramuscular route. Macrophages infiltration is conspicuous near muscular fibers at the injection site (a) and show large cytoplasm containing PAS reactive granules (b). Inflammatory infiltration is also evident in the interstitium (c) and around the muscle fibers (d). PAS positive Kupffer cells (e) and macrophages (f) are found in the liver parenchyma. Hematoxylin–eosin staining: (a) 63×, (d) 250× magnifications; PAS reaction: (b) 250×, (c) 160×, (e) and (f) 250× magnifications.

the macrophages was stuffed with PAS and PAS–Diastase positive microspherules, that may be related to DNA/K2 complexes. However, there were also hemosiderin granules deposits, a likely consequence of the injection procedure. Macrophages showed always good reactivity to CDE68 and Mac 387 monoclonal antibodies. T and B lymphocytes were not found in the inflammatory reaction. In mice injected with pCV-Tat/K2, lymph nodes showed dilated sinuses containing isolated or aggregated macrophages with microspherules in the cytoplasm, and liver presented normal lobular architecture with increased number of the Kupffer cells. Significantly, some of these cells and macrophages in the portal tracts contained microspherules in their cytoplasm (Fig. 7e and f); no specific alterations were evident in the hepatocytes. All other organs were similar to control mice injected with the naked pCV-0 DNA. Future biodegradation and biodistribution studies with these copolymers will address their degradation/elimination rate and route.

Table 4
Analysis of the antibody response after injection of free K2

Group/no. of mice	K2 injected dose[a] (μg)	Anti-K2 IgG titers[b]
1/1	5	500
1/2	5	500
2/1	10	500
2/2	10	500
3/1	50	500
3/2	50	500
4/1	100	500
4/2	100	500
5/1	250	500
5/2	250	500
6/1	500	500

[a] Mice were injected three times by the subcutaneous route with different amounts of K2. Sera were collected 3 weeks after the last injection when animals were sacrificed, and were assayed by ELISA using K2 as the antigen.

[b] Results are expressed as end point ELISA IgG titers.

Table 5
Humoral response to cationic block copolymers complexed to DNA

Complex injected (dose)	No. of mice	Copolymer (dose)	Anti-copolymer IgG titers[a]			
			Bleeding I	Bleeding II	Bleeding III	Bleeding IV
pCV-0/K1 (30 μg/30 μg)	3	K1 (30 μg)	0	0	0	0
	5		0	0	ND	0
	6		0	0	1000	0
	7		0	0	1000	0
pCV-0/K2 (30 μg/30 μg)	1	K2 (30 μg)	500	1000	1000	1000
	4		ND	0	1000	ND
	5		0	500	500	1000
	6		500	1000	1000	1000
	7		ND	ND	0	1000
pCV-0/K5 (30 μg/600 μg)	2	K5 (600 μg)	0	1000	500	500
	3		0	0	0	500
	4		0	0	0	500
	6		0	0	500	1000
	7		0	1000	1000	1000

[a] Mice sera were assayed by ELISA, using K1, K2 or K5 block copolymers as the antigen, after the 3rd, 4th, 5th and 6th injection (bleedings I–IV). Results are expressed as end point ELISA IgG titers.

3.9. Evaluation of immunogenicity of block copolymers

Since one of the most important characteristics required for DNA delivery systems is the lack of immunogenicity, we determined whether copolymers were capable of eliciting an antibody response. The presence of anti-copolymer IgG was analyzed by ELISA in sera of mice injected with high doses of free or DNA–complexed copolymers. As shown in Table 4, a weak reactivity was observed up to 1:500 serum dilution in all animals injected three times with free K2 by the subcutaneous route. The antibody response did not correlate with the dose of the copolymer injected.

The presence of antibody to K1, K2 and K5 in mice injected, six times by the intramuscular route, with pCV-0–copolymer complexes was analyzed both during the course of immunization and at sacrifice (bleedings I–IV). The results, reported in Table 5, indicate that an antibody response to K1, K2 and K5 was developed in a few mice with titers ranging from 1:500 to 1:1000. These responses generally correlated with the number of boosts. Indeed, the number of responders and the antibody titers were generally absent or lower after three or four administrations (bleedings I and II), whereas the number of responders and the IgG titer slightly increased after five and six injections (bleedings III and IV). These results compulsively suggest that the cationic block copolymers are scarcely immunogenic.

3.10. Analysis of gene expression in vivo

To assess whether DNA is released and expressed from the DNA/copolymer complexes also in vivo, mice were injected in the quadriceps muscles with 1 μg of pCV-Tat complexed to K2 block copolymer, or with 10 μg of naked pCV-Tat DNA. Control mice were injected with 10 μg of the pCV-0 empty plasmid. Expression of the *tat* gene was searched in the muscle at the site of injection by RT–PCR analysis. As shown in Fig. 8, expression of Tat was easily detectable only in mice receiving the pCV-Tat/K2 complex, whereas in mice injected with a 10-fold higher dose of naked *tat* DNA expression of Tat was barely detectable. This indicates that in vivo the DNA/copolymer complexes are taken up by the cells, where DNA is protected from enzymatic degradation, released from the complexes and expressed at detectable levels.

4. Discussion

Nucleic acids show much promise for use as vaccines [1,5,17,22]. However, immunization with naked DNA is relatively inefficient requiring high doses of DNA and multiple injections, whereas viral vectors, although induce far greater immune responses than DNA vaccines, have several disadvantages including unwanted immunogenicity. In addition, they are usually expensive, difficult to prepare in

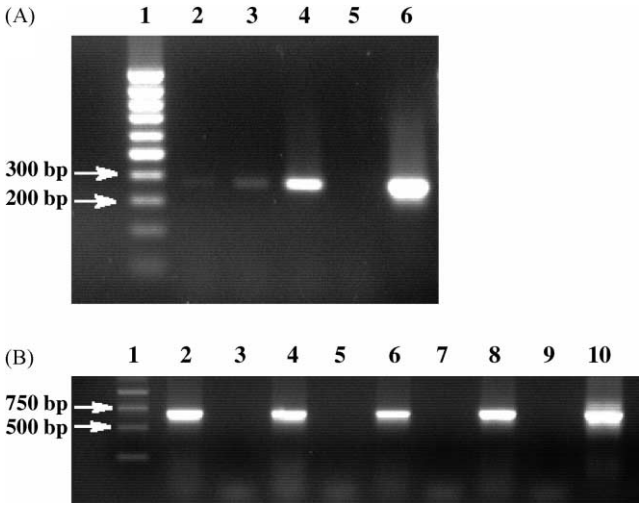


Fig. 8. RT–PCR analysis of gene expression in vivo. Mice were injected six times in the posterior quadriceps muscle with 1 μg of pCV-Tat/K2 complexes, or with 10 μg of naked pCV-Tat DNA. Control mice were injected with 10 μg of the pCV-0 empty plasmid. Four representative mice are shown. At the site of injection, expression of Tat (250 bp) (A) and actin (600 bp) (B), as an house-keeping gene, was evaluated. The absence of contaminating DNA was controlled by PCR on each RNA sample before reverse transcription (odd lanes of panel B). In part (A): lane 1, molecular weight marker 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania); lane 2, one representative mouse injected with 10 μg of naked pCV-Tat; lanes 3 and 4, two representative mice injected with 1 μg of pCV−Tat/K2; lane 5, one mouse injected with pCV-0 empty plasmid; lane 6, positive control of PCR reaction represented by pCV-Tat plasmid DNA. PCR reaction in lane 2 was performed on one-third of the RT-reaction volume; PCR reactions of lanes 3 and 4 were performed on one sixth of the RT-reaction volume. In (B): lane 1, molecular weight marker 1-Kb DNA ladder (MBI Fermentas); lane 2, one representative mouse injected with 10 μg of naked pCV-Tat; lanes 4 and 6, two representative mice injected with 1 μg of pCV-Tat/K2; lane 8, one mouse injected with pCV-0 empty plasmid; lane 10, positive control of PCR reaction represented by cellular DNA. All PCR reactions were performed on one-fifteenth of the RT-reaction volume. In lanes 3, 5, 7 and 9, PCR on RNA samples of lanes 2, 4, 6 and 8 before reverse transcription.

bulk, and raise safety concerns for their use in humans, because of the potential pathogenicity of viral nucleic acids [21,22]. For a versatile gene-targeted delivery the ideal vector should be safe, biocompatible, efficient and simple to produce and store. Hence, synthetic delivery systems are being widely sought as attractive alternatives and are particularly appealing because of their simplicity of use, ease of large-scale production and lack of specific immune response [23].

In a previous study, we described the synthetic procedure and the physico-chemical characterization of a novel class of cationic block copolymers, consisting of a neutral hydrophilic PEG block and a positively charged poly(dimethylamino)ethyl methacrylate block, that was partly or fully alkylated to generate several cationic block copolymers [35]. These novel block copolymers self-assemble with DNA to give stable dispersed complexes in aqueous media with micellar structures called polyion complex micelles. Accordingly, the supramolecular structure of these ordered aggregates is a core–shell-type, with an average diameter ranging from 100 to 300 nm and a PI comprised between 0.2 and 0.5, in which the inner core is constituted by DNA linked via electrostatic interactions to the charged block of the block copolymer, and the outer shell is formed by the neutral hydrophilic PEG (Table 2 and [35]). The *Z*-average particle size of the complexes decreases as the N:P ratio increases, steeply at first and then more gradually until a limiting value of about 100 nm is reached. The surface charge characteristics, determined by ζ-potential measurements, are very similar for all the complexes. In particular, the ζ-potential turns from negative at low N:P ratio, corresponding to an excess of phosphate groups in the complex, to positive at very high N:P ratio, corresponding to an excess of quaternary ammonium positive groups in the complex (Fig. 2). In addition, cell-free experiments showed that the DNA is released from the polyion complex micelles with an exchange reaction with anionic polymers [35], suggesting that these novel block copolymers possess physico-chemical characteristics well suited for their in vivo application. To this respect, it is worth mentioning that the exchange capacity of DNA in the polyion complex micelles is crucial for biological properties, such as nuclease resistance and gene release and expression, and therefore, for in vivo application [48,49]. This type of exchange reaction seems to take place for DNA complexed with cationic block copolymers in biological environments [48–50], where various types of polyanions (e.g. anionic proteins, sulfated sugars and mRNA) are present.

Although K1–5 copolymers share the same overall structure, they are quite different in terms of subtle molecular features of both blocks. In particular, they present short or relatively long PEG chains in the neutral block and different length as well as quaternary group linear density in the positively charged block (Table 1 and [35]). Thus, in the present study we have examined the behavior of these new block copolymers, and their safety in vitro and in vivo, in order to assess their usefulness as possible vehicles for gene delivery for vaccination.

The present results indicate that, irrespective of the specific structural details, all K1–5 copolymers protect very efficiently the DNA from enzymatic degradation at both 1.0 and 5.0 N:P charge ratios (Table 3). All DNA–copolymer complexes were not cytotoxic at N:P ratio of 1.0 and, immediately after their addition to the cultures, they induced a slight, but significant ($P < 0.05$) increase of cell proliferation, which was still observed after a longer incubation time in the case of the DNA–K2 complex. A slight cytotoxicity was observed at the N:P charge ratio of 5.0. All DNA–copolymer complexes, except K2, displayed similar cytotoxicity, causing 40–50% reduction of cell viability and proliferation as compared to controls (Fig. 3). The toxic effect at the high N:P charge ratio could depend on non-specific interactions between free positive charges of the copolymers with cell surface molecules, or with

negative charged biopolymers inside the cells, causing interference with normal cellular functions. Thus, cytotoxicity of DNA–copolymer complexes in vitro appears to correlate with variation in the charges of the complex rather than with the presence of a specific alkyl group, or with the number of positive charged groups in the poly(dimethylamino)ethyl methacrylate block.

The extent of cellular uptake and internalization of the five DNA–copolymer complexes was comparable with naked DNA (1–2%), and it was lower as compared to a well established liposome-based system, such as Lipofectamine (5–10%) (Fig. 4). This difference may depend on different internalization pathways of DNA–copolymer complexes and liposomes (e.g. endocytosis versus fusion) that may have different efficiency. In addition, the binding and the internalization of the DNA–copolymer complexes into the cells may be more difficult because the PEG chains increase the hydrophilicity of the complexes, as suggested by others [45]. This may explain the similar level of cellular internalization of DNA–copolymer complexes and naked DNA. However, expression of the *HIV-1 tat* gene was detected only when DNA was delivered by the cationic block copolymers (K1, K2 and K5), but not in the lane of naked DNA (Fig. 5). In accordance with the internalization studies, the expression of the *tat* gene delivered by K1, K2 or K5 was lower (10–30-fold) as compared to *tat* expression after lipofection (20% of CAT activity with 1 μg of DNA). These observations are in agreement with findings by others [34,45], and are not surprising since DNA uptake and expression are generally lower with synthetic polymers as compared to liposome-based vectors [30,45].

The reason why *tat–EGFP* gene expression was not detectable at N:P charge ratio of 5.0 with K1 and K2 and at N:P charge ratio of 1.0 with K5, and why the gene is not expressed when associated with K3 and K4 copolymers, it is not presently clear. Since the results indicate that the extent of cellular internalization of the complexes is similar, probably the dissociation of the DNA–copolymer complexes and/or the preservation of the DNA topology in a biological environment is inadequate under these conditions. In addition, copolymers have different subtle molecular features of both blocks, in that each copolymer (at each N:P charge ratio) may generate complexes with different configurations and supramolecular structures, with characteristics and activity that are similar in a cell-free system, but may differ in a biological environment. These results indicate that the efficiency of gene release and expression is not simply related to specific structural parameters of these novel block copolymers, including molecular weight, length of the PEG chain or number of positively charged groups in the poly(dimethylamino)ethyl methacrylate block, at least in the compositional range investigated (Table 1 and [35]). Moreover, the results indicate that, in addition to deliver and release active DNA intracellularly, K1, K2 and K5 copolymers protect it from *DNAse* attack also intracellularly. This is further supported by the in vivo experiments showing detectable expression of *tat* only when DNA is complexed with the copolymer, whereas when a 10-fold higher dose of naked DNA is injected, expression of *tat* was more difficult to detect.

Thus, the block copolymer system shown here allows DNA to be protected from *DNAse* attack extending DNA half-life, and reducing the DNA dose required for detectable expression, both in vitro and in vivo. This indicates that DNA is not only protected, but also released in a controlled fashion from the polyion complexes. In addition to these properties, block copolymers displayed very little cytotoxicity in vitro, were safe in vivo and showed scarce immunogenicity. The above characteristics, together with the ease of production and the possibility to tailor these molecules according to a specific target, render the present block copolymer system very attractive as a delivery vehicle for DNA vaccination [51].

Acknowledgements

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