Complex associates of plasmid DNA and a novel class of block copolymers with PEG and cationic segments as new vectors for gene delivery

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Abstract—Cationic block copolymers, consisting of a poly(ethylene glycol) block and a block deriving from the poly(dimethylamino)ethyl methacrylate were prepared via a two-step procedure, based on the use of macroinitiators. By appropriately changing the experimental conditions and reacting the poly(dimethylamino)ethyl methacrylate block with iodo- or bromo-alkyl derivatives, a variety of ionic block copolymers with tuned physicochemical properties were prepared. These block copolymers are able to spontaneously self-assemble with plasmid DNA to produce oriented and shielded vectors, with physicochemical properties appropriate for in vivo applications. In addition, the formation of a complex between the cationic block copolymer and the plasmid DNA results in a nuclease resistance increase due to the stable nature of the complex.

Key words: Gene deliver; plasmid DNA; polyion complex micelles; block copolymers; PEG.

INTRODUCTION

The use of DNA as a therapeutic agent in vaccine and gene therapy is frequently limited by the poor efficiency of gene delivery and expression in vivo [1]. Viruses are employed as vectors although immunogenicity and inflammatory effects may limit their usefulness. In addition, their inappropriate tropism further restrict their access to target tissues [2]. Liposomes have also been studied as the main synthetic
alternative with special attention to those containing cationic lipids. However, they often display poor pharmacokinetic characteristics and, in this case, their use is confined to local or intra-arterial applications [3–5].

An alternative class of nonviral gene delivery vectors is represented by cationic polymers specifically designed to self-assemble with DNA by electrostatic interactions [6, 7]. Simple mixing of DNA with poly-L-lysine (PLL) results in the formation of polyelectrolyte complexes whose size and transduction efficiency is influenced by the molar mass of the employed PLL [8–10]. However, these simple polyelectrolyte complexes exhibit certain practical limitations in their use. One major physicochemical limitation is their relatively low aqueous solubility, particularly when they are formed at charge neutrality, reflecting the formation of neutral, relatively hydrophobic complexes [6a]. A particular limitation to their use in vivo is a tendency to interact with serum proteins, resulting in a rapid clearance following i.v. administration [11]. It is well known that coating the liposome surface with flexible hydrophilic polymer chains, such as poly(ethylene glycol) (PEG), produces a steric barrier to the interaction with proteins and phagocytes resulting in a prolonged plasma circulation time of these liposomes [12], which are usually referred to as stealth liposomes. The same basic concept can be extended to the self-assembling approach by substituting the cationic polymer with a block or graft copolymer made of a cationic block, which is able to interact with DNA, and a hydrophilic nonionic block, capable to sterically stabilize the complex. So far, several polycations have been used in combination with hydrophilic nonionic polymers to produce polyelectrolyte complexes with improved biocompatibility. These include A–B type block copolymers in which the A block consists of a hydrophilic block of PEG, dextran or poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) and the B block of a polycation block of PLL, spermine, poly(dimethylamino ethyl methacrylate) (DMAEMA), or poly(trimethylammonio ethyl methacrylate chloride) (PTMAEMA-Cl); PEG-b-PLL [13–15], PEG-b-spermine [6], PHPMA-b-PTMAEMA [14, 16], or graft copolymers PLL-g-PEG, PLL-g-dextran and PLL-g-PHPMA, or PTMAEMA-g-PHPMA [17, 18]. These novel cationic block copolymers self-assemble with plasmid DNA to give micellar structure in aqueous media called block ionomer complexes or polion complex micelles. The supramolecular structure of these com-

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**Table I.**

Physical properties of cationic block copolymers

<table>
<thead>
<tr>
<th>Block copolymer</th>
<th>R groups</th>
<th>m</th>
<th>p</th>
<th>$M_n$ (NMR)</th>
</tr>
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<tr>
<td>1</td>
<td></td>
<td>44</td>
<td>230</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>16</td>
<td>90</td>
<td>—</td>
</tr>
<tr>
<td>1a</td>
<td>CH$_3$</td>
<td>44</td>
<td>104</td>
<td>127</td>
</tr>
<tr>
<td>1b</td>
<td>CH$_3$</td>
<td>44</td>
<td>160</td>
<td>70</td>
</tr>
<tr>
<td>1c</td>
<td>(CH$_2$)$_3$CH$_3$</td>
<td>44</td>
<td>—</td>
<td>230</td>
</tr>
<tr>
<td>2a</td>
<td>CH$_3$</td>
<td>16</td>
<td>—</td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 1. Ideal formation scheme of polyion complex micelles from a pair of cationic block copolymers and plasmid DNA.

Complexes is a core-shell type micelle, in which the hydrophobic core is formed by DNA linked via electrostatic interactions to the charged block of the block copolymer and the outer shell is constituted by the non-charged hydrophilic block of the copolymer, as illustrated in Fig. 1 [19, 20]. The PEG-b-PLL diblock copolymers are highly attractive because of their biocompatibility. However, the modulation of the inherent complexing propensity of the polycation block toward DNA is still to be optimized.
In this paper, we introduce block copolymers 1, 2 whose general structure is as follows:

These block copolymers are constituted by a PEG block and a poly(DMAEMA) block and were prepared by free radical polymerization of the dimethylamino ethyl methacrylate using a high molar mass initiator containing a reactive azo group flanked by two PEG chains of variable length. Indeed, an alternative synthetic way to prepare similar block copolymers has been very recently described [21] by successive anionic polymerization of ethylene oxide and (N,N-dimethylamino)ethyl methacrylate. Narrow polydispersity indexes are observed.

In addition, copolymers 1 and 2 were partly or fully alkylated to give the corresponding cationic block copolymers 1a–c, 2a with the following structure:

The capability of block copolymers to spontaneously assemble with DNA and the stability of the resulting complexes as well as the nuclease resistance of the resulting PEG-b-PTMAEMA/DNA complex were tested on two DNA plasmids: pCV-Tat, an extensively characterized plasmid for in vitro HIV1-tat gene expression, and pCV0, the reference plasmid having the same sequence and lacking the HIV1-tat gene [22–25]. Plasmid pCV-Tat is being used for the development of a tat-based vaccine against HIV-1 infection [26, 27].

METHODS

Materials

Benzene, triethylamine, chloroform, and 2-DMAEMA were freshly distilled before use. 4,4′-Azobis(4-cyanopentanoyl chloride) (3) was prepared from 4,4′-azobis(4-
cyanopentanoic acid) (Aldrich) according to a procedure previously described [28]. Poly(ethylene glycol) mono methylether samples ($M_n = 750$ and $M_n = 2000$, Aldrich), 1-bromobutane, iodomethane and poly(vinyl sulfonic acid, sodium salts) (PVS) were purchased from Aldrich and employed without further purification.

**Physicochemical characterization**

Average molar masses were determined by SEC of water solutions with a 590 Waters chromatograph equipped with a TSK-GEL G3000PW column. A differential refractometer R401 Waters was used as a detector. PEG standard samples were used for the column calibration. $^1$H-NMR spectra were recorded on a Varian Gemini 200.

**Synthesis of macroinitiator 4a**

In a typical reaction, 2.0 g (6.3 mmol) of 3 were dissolved in 40 ml of benzene in a flask under constant nitrogen flow at 5°C, and a solution of 19.0 g (25 mmol) of poly(ethylene glycol) monomethylether ($M_n = 750$, $n = 16$), and 4.0 ml (28.6 mmol) of triethylamine in 10 ml of benzene were then added dropwise. After the addition was completed, the reaction mixture was heated to room temperature and stirred for 2 h. Then the reaction mixture was filtered, to remove the salt, and washed with a 10% sodium bicarbonate solution to remove the residues of PEG mono methylether and of 3. The organic phase was dried with sodium sulphate, filtered and the solvent was removed under vacuum at room temperature. Yield: 70%. $^1$H-NMR (CDC$_3$): 1.7 (d, CH$_3$), 2.5 (m, CH$_2$-CH$_2$), 3.4 (s, O-CH$_3$), 3.65 (s, O-CH$_2$-CH$_2$).

**Synthesis of macroinitiator 4b**

The macroinitiator 4b was synthesized in the same way as 4a using 50.0 g (25 mmol) of poly(ethylene glycol) mono methylether ($M_n = 2000$, $n = 44$). Yield: 70%. $^1$H-NMR (CDC$_3$): 1.7 (d, CH$_3$), 2.5 (m, CH$_2$-CH$_2$), 3.4 (s, O-CH$_3$), 3.65 (s, O-CH$_2$-CH$_2$).

**Synthesis of block copolymers 1, 2**

Copolymer 1 was obtained by solution polymerization of macroinitiator 4b with DMAEMA. In a typical reaction, 8.5 g (2.0 mmol) of macroinitiator 4b and 6.0 ml (35.0 mmol) of DMAEMA were dissolved in 30 ml of benzene in a Pyrex glass ampoule. The reaction mixture was degassed by three freeze–thaw cycles. The ampoule was then charged with nitrogen and placed in a thermostatic bath at 60 ± 0.1°C for 24 h. The reaction mixture was then poured into 300 ml of petroleum ether and the product collected and dried under vacuum at room temperature. The solid was then dissolved in water, purified by dialysis against water (dialysis membrane Medicell International, MWCO 12 000–14 000 Da) and freeze-dried. Yield: 66%.
\(^1\)HNMR (CDCl\(_3\)): 1.0 (d, CH\(_3\)), 1.9 (m, CH\(_2\)), 2.3 (s, N(CH\(_3\))\(_2\)), 2.6 (s, CH\(_2\)-N), 3.4 (s, O-CH\(_3\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 4.1 (s, COOCH\(_2\)).

Copolymer 2 was obtained by bulk polymerization of macroinitiator 4a with DMAEMA. In a typical reaction, 3.0 g (1.7 mmol) of macroinitiator 4a were dissolved in 12.0 ml (71 mmol) of DMAEMA and poured in a Pyrex glass ampoule. The reaction mixture was degassed by three freeze–thaw cycles and then charged with nitrogen and placed in a thermostatic bath at 60 ± 0.1°C for 24 h. The polymerization reaction and purification were performed as described for copolymer 1. Yield: 65%. \(^1\)HNMR (CDCl\(_3\)) = 1.0 (d, CH\(_3\)), 1.9 (m, CH\(_2\)), 2.3 (s, N(CH\(_3\))\(_2\)), 2.6 (s, CH\(_2\)-N), 3.4 (s, 2 O-CH\(_3\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 4.1 (s, COOCH\(_2\)).

**Synthesis of cationic block copolymers 1a–c, 2a**

Cationic block copolymer 1a was obtained by reacting block copolymer 1 with iodomethane in stoichiometric defect. In a typical reaction, 2.0 g of 1 were dissolved in 50 ml of ethyl acetate at room temperature in a three-neck flask under constant stirring. 0.2 ml (3.2 mmol) of methyl iodide was then added and the reaction mixture was placed at 40°C. After 2 h, the reaction mixture was dried under vacuum and the solid product was washed with ether and subsequently with methyl alcohol. Yield: 70%. \(^1\)HNMR (D\(_2\)O) = 1.0 (m, CH\(_3\)), 2.0 (m, CH\(_2\)), 2.3 (s, N(CH\(_3\))\(_2\)), 2.6 (s, CH\(_2\)-N), 3.3 (s, N\(^+\)(CH\(_3\))\(_3\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 3.9 (s, CH\(_2\)-N), 4.5 (s, COOCH\(_2\)).

Cationic block copolymer 1b was obtained by reacting block copolymer 1 with iodomethane in stoichiometric defect. In a typical reaction, 2.0 g of 1 were dissolved in 50 ml of ethyl acetate at room temperature in a three-neck flask under constant stirring. 0.1 ml (1.6 mmol) of iodomethane was then added and the reaction mixture was placed at 40°C. After 2 h, the reaction mixture was dried under vacuum and the solid product was washed with ether and subsequently with cyclohexane. Yield: 65%. \(^1\)HNMR (D\(_2\)O) = 1.0 (m, CH\(_3\)), 2.0 (m, CH\(_2\)), 2.3 (s, N(CH\(_3\))\(_2\)), 2.6 (s, CH\(_2\)-N), 3.3 (s, N\(^+\)(CH\(_3\))\(_3\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 3.9 (s, CH\(_2\)-N), 4.5 (s, COOCH\(_2\)).

Cationic block copolymer 1c was obtained by reacting copolymer 1 with an excess of 1-bromobutane. In a typical reaction, 2.0 g of 1 were dissolved in 40.0 ml (0.37 mol) of 1-bromobutane and the reaction mixture was placed at 50°C for 24 h. The reaction mixture was filtered and the solid product was washed with methyl alcohol and dried under vacuum at room temperature. Yield: 70%. \(^1\)HNMR (D\(_2\)O) = 1.0 (m, CH\(_3\)), 1.4 (m, CH\(_3\)CH\(_2\)CH\(_2\)), 1.4 (m, CH\(_3\)CH\(_2\)CH\(_2\)), 2.0 (m, CH\(_2\)), 2.3 (s, N(CH\(_3\))\(_2\)), 2.6 (s, CH\(_2\)-N), 3.3 (s, N\(^+\)(CH\(_3\))\(_2\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 3.9 (s, CH\(_2\)-N), 4.5 (s, COOCH\(_2\)).

Cationic block copolymer 2a was obtained by reacting copolymer 2 with an excess of iodomethane. In a typical reaction, 7.0 g (80 mmol) of copolymer 2 were dissolved in 200 ml of chloroform at room temperature in a three-neck flask under constant stirring. 5.0 ml of methyl iodide was then added and the reaction...
was performed at room temperature for 2 h. The reaction mixture was filtered and the solid product was washed with methyl alcohol and dried under vacuum at room temperature. Yield: 90%. \(^1\)HNMR (D\(_2\)O): = 1.0 (m, CH\(_3\)), 2.0 (m, CH\(_2\)), 3.3 (s, N\(^+\)(CH\(_3\))\(_3\)), 3.65 (s, O-CH\(_2\)-CH\(_2\)), 3.9 (s, CH\(_2\)-N), 4.5 (s, COOCH\(_2\)).

**Plasmid DNA**

Plasmid pCV-Tat containing the cDNA of the HIV-1 tat gene under the transcription control of the adenovirus major late promoter was previously described [22]. Plasmid pCV0 is identical to pCV-Tat, but it lacks the tat gene sequence. Plasmid DNA was purified on CsCl gradient, according to a standard procedure [29], and resuspended in sterile phosphate-buffered saline solution (PBS) with calcium and magnesium. The concentration of plasmid preparation used in the experiments was 3.1 mg ml\(^{-1}\), as determined by UV absorbance reading at 260 nm at room temperature.

**Assembly of complexes at different charge ratios**

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 \(^1\)HNMR spectra. Complexes were prepared in buffer solution by adding a polycation solution to DNA at various concentrations, as described below. To compare the complexing behavior of the various block copolymers, the complexes were prepared at defined molar ratio of amino or quaternary ammonium groups to DNA phosphate groups (N-to-P ratio).

**Scanning force microscopy (SFM) analysis**

Complexes at a known N-to-P ratio were assembled in low ionic strength buffer (4 mM HEPES pH 7.4) containing DNA molecules in nanomolar concentration and a millimolar concentration of Mg\(^{2+}\), and then incubated at room temperature for 10 min. 10–15 µl of solution containing the complexes were deposited on a 1.5 cm\(^2\) disc of freshly cleaved ruby mica (Mica, New York, NY, USA). Deposition of the complexes was allowed to take place for 1 min. Then the discs were rinsed with approximately 2 ml of milliQ deionized water (Millipore, USA) and dried with a gentle flow of nitrogen gas. The specimens were analyzed with tapping-mode scanning force microscopy (TM-SFM) in a NanoScope IIIa Scanning Force Microscope equipped with a Multimode head (Digital Instruments, Santa Barbara, CA, USA).

**Inhibition of ethidium bromide-DNA fluorescence by cationic block copolymers**

The ability of cationic block copolymers to form complexes with DNA was determined by loss of ethidium bromide-DNA fluorescence using a LS-50B Perkin-Elmer spectrofluorimeter [15]. 2.0 ml of a solution of DNA (20 µg ml\(^{-1}\)) and
ethidium bromide (0.40 μg ml⁻¹) in water, 20 mM buffer sodium phosphate buffer (pH 7.4) and 20 mM buffer sodium acetate buffer (pH 5.0) was prepared. Fluorescence was recorded at λ_ex = 366 nm, λ_em = 590 nm and set to 100%. Then, a polycation solution was added stepwise (4.0 μl each addition, to account for a 0.2 increase of N-to-P ratio), sample was mixed and fluorescence was read after 5 min. The concentration of polymer causing 50% fall in measured fluorescence (IF₅₀), and the lowest fluorescence level achieved (minF) were determined for each sample.

**Ethidium bromide-DNA fluorescence restoration by PVS**

2.0 ml of a solution of DNA (20 μg ml⁻¹) and ethidium bromide (0.40 μg ml⁻¹) in water were prepared and fluorescence was set to 100%. Then, block copolymers were added to form complexes adjusting the N-to-P ratio equal to 1.4. Finally, portions of 5.0 μl of 6.06 mM poly(vinyl sulfonic acid, sodium salts) (PVS) solution were added with gentle mixing. After each addition, the fluorescence was detected. The concentration of each anion required to restore 50% original ethidium bromide fluorescence (RF₅₀), and the maximum value of ethidium bromide fluorescence restoration (maxF) were determined.

**DNAse protection assays**

Solutions containing 1.0, 10, 50, and 100 μg of PCV0 or PCV-Tat plasmid in 1.0 ml of Tris-HCl 40 mM, CaCl₂ 10 mM, MgCl₂ 6 mM (pH 7.9) were mixed with the appropriate amounts of a block copolymer solution to obtain complexes at N-to-P ratio of 1 and 5. The solutions were incubated for 30 min at room temperature. Control samples represented by pCV0 plasmid DNA at the same concentrations but without the block copolymer were included. Optical densities were read at 260 nm (time 0). RNase-free DNase (10 μg, Promega, Madison, WI, USA) was added and the solutions were incubated at 37°C. Optical densities were read after 15 and 60 min at room temperature.

**RESULTS AND DISCUSSION**

The various block copolymers were synthesized via the procedure illustrated in Fig. 2. The first step consists in the free radical polymerization of the dimethylamino ethyl methacrylate using the high molar mass initiators 4a and 4b, containing a reactive azo group flanked by two PEG chains of variable length leading to block copolymers 1 and 2. In the second step, 1 and 2 were reacted with iodomethane or 1-bromobutane, leading to the corresponding cationic block copolymers 1α–c and 2a. In detail, macroinitiators 4 were synthesized by reacting 4,4’-azobis(4-cyanopentanoyl chloride) with PEG mono methyl ethers containing either sixteen or forty four oxyethylene units. These macroinitiators were used to start up the free-radical polymerization of the methacrylate monomer DMAEMA through the
thermal decomposition of the azo groups at 60°C. It is well established that free-radical polymerization of methacrylate monomers terminates via a disproportionation process, which, in the present polymerization system, results in the formation of AB diblock copolymers consisting of one PEG block and one poly(DMAEMA) block. However, any preference for a combination termination mechanism would eventually yield minor amount of ABA block copolymers. Copolymers 1 and 2 were precipitated several times from chloroform solution into methanol, to eliminate un-
reacted monomers, and purified by dialysis against water to eliminate the unreacted PEG. The block copolymers 1 and 2 were reacted with methyl iodide to obtain the positively charged block copolymers 1a, 1b and 2a. In particular, a large excess of methyl iodide with respect to the monomeric units deriving from DMAEMA was employed, to obtain block copolymer 2a. In contrast, for copolymers 1a and 1b, the methyl iodide was adjusted to obtain respectively a 50 and 30% of quaternary ammonium groups with respect to the total DMAEMA units. Block copolymer 1 was also reacted with a large excess of 1-bromobutane to give block copolymer 1c. As a typical example, Fig. 3 reports the SEC traces of poly(ethylene glycol) mono methylether \((n = 44)\) (a), macroinitiator 4b (b) as well as copolymers 1 (c) and 1a (d). The SEC trace of the macroinitiator is monomodal and translated toward lower values along the elution volume scale with respect to the starting PEG. In the SEC chromatogram of copolymer 1a, no trace of PEG and DMAEMA was observed after the above purification procedure whereas a slight amount of residual PEG (less than 5%) is observed in the SEC trace of copolymer 1. In addition, SEC analysis of block copolymers 1a–c and 2a indicated that the alkylation reactions did not degrade the polymeric chains. Under the assumption of an AB structure, not only the composition, but also the molar mass characteristics of the copolymers 1 and 2 were evaluated by $^1$HNMR as the usual PEG standard samples are not reliable standards for SEC column calibration in the present polyelectrolytic polymeric system. Figure 4 reports representative examples of the $^1$HNMR spectra of the macroinitiator 4a (a), of the block copolymer 2 (b) and of the cationic block copolymer 2a (c).
Figure 4. $^1$HNMR spectra of macroinitiator 4a (a), block copolymer 2 (b) and block copolymer 2a (c).

DNA-block copolymer complexes for gene delivery

Block copolymer–plasmid complexes were prepared by adding in buffer solution a block copolymer to DNA at various concentrations, as described in the experimental part. The morphological analysis of the complexes was performed using scanning force microscopy [30]. A typical TM-SFM image of the deposition of together with the relevant assignments. The composition of the block copolymers 1 and 2 (Table 1) was determined from the intensity of the $^1$HNMR signals due to the oxyethylene groups (3.65 ppm) and to the methyl groups linked to the nitrogen as well as the methylene groups comprised between the nitrogen and the oxygen in the poly(dimethylamino)ethyl methacrylate unit (2.3, 2.6, and 4.1 ppm, respectively).
pCV-Tat plasmid DNA is presented in Fig. 5. SFM images represent topographies of the sample surface, where the height of the features on the substrate is coded in shades of color according to the attached look-up table. The shapes of the plasmid DNA in Fig. 5 was in agreement with those previously reported from this and other imaging techniques [31]. The contour length of the molecules is that expected for the DNA in its hydrated form (B-DNA). The apparent width of the DNA double strands is significantly exaggerated due to the broadening effect inherent in SFM imaging. Figure 6 shows a TM-SFM image of deposited DNA–polymer complex at N-to-P ratio of 5 in a low ionic strength buffer and 1.0 mM MgCl₂. Magnesium ions were added to ensure a reproducible deposition of DNA molecules on the negatively charged mica surface [32, 33]. The topography shows complexed DNA plasmid appearing as globular structures often displaying segments of DNA protruding from their body. Non-complexed cationic copolymer molecules are also present on the mica substrate and appear as small dot-shaped objects as also observed in SFM images of the polymer alone in the same conditions (data not shown). A preliminary evaluation of the volume of the complexes shown seems to confirm that only one DNA molecule is contained in each globular object.

Some of the imaged complexes display incomplete condensation of DNA and appear to be structurally similar to others reported in literature [34]. The DNA
DNA-block copolymer complexes for gene delivery

Figure 6. TM-SFM image of the complexes of cationic block copolymer 2a with pCV-Tat plasmids. The complexes were assembled in solution at N-to-P ratio = 5, then deposited on the surface of freshly cleaved mica, rinsed with milliQ ultrapure water, and dried. The image has been recorded with the microscope operating in air. (This figure is published in colour on http://www.vsppub.com/jconts/JBS)

chains seem to form loops entering and exiting a central globular core, plausibly the expanding center of nucleation for the polycation-driven DNA condensation.

The efficiency of block copolymers to complex plasmids was determined following the fluorescence decrease of ethidium bromide with DNA after addition of the various block copolymers. The experiment was performed in water, in 20 mM sodium phosphate buffer (pH 7.4) and in 20 mM sodium acetate buffer (pH 5.0). Table 2 reports the relevant data whereas Fig. 7 illustrates the trend of ethidium bromide fluorescence upon addition of the block copolymers at pH 7.4 (a) and 5.0 (b). The trends of ethidium bromide fluorescence in pure water and in buffer solution at pH 7.4 are very similar. The percentage of quaternary ammonium groups with respect to the total DMAEMA units in the copolymer chain seems to influence the loss of ethidium bromide fluorescence, with the copolymer containing the lower percentage of quaternary ammonium groups requiring the higher concentration to achieve effective complexation. As reported in Table 2, copolymers 1 and 2, with no quaternary ammonium groups, showed IF$_{50}$ values of 1.06 and 1.11, and minF values of 19.82 and 21.40%. Copolymers 1c and 2a, both having 100% of quaternary ammonium groups, showed IF$_{50}$ values of 0.89 and 0.72, and minF values of 16.30 and 12.10%, respectively. Copolymers 1a and 1b, with 50 and 30% of quaternary ammonium groups respectively, showed an intermediate behavior with IF$_{50}$
Figure 7. Formation of copolymers/DNA complexes as measured by loss in ethidium bromide fluorescence. Experiments performed in 20 mM sodium phosphate buffer (pH 7.4) (a) and 20 mM sodium acetate buffer (pH 5.0) (b). Fluorescence of DNA-ethidium bromide solution was measured and set to 100%. Fluorescence was measured after step-wise addition of copolymer solution in water. Complexes of DNA with block copolymers 1 (◇), 2 (×), 1a (○), 1b (▽), 1c (△), and 2a (□).
Table 2.
Biological properties of complexes formed by self-assembly of DNA with cationic block copolymers

<table>
<thead>
<tr>
<th>Block copolymer</th>
<th>Water F&lt;sub&gt;50&lt;/sub&gt; (N-to-P ratio)</th>
<th>Buffer pH 7.4 minF (%) original</th>
<th>Buffer pH 5.0 minF (%) original</th>
<th>Water RFI&lt;sub&gt;50&lt;/sub&gt; (PVS/cation molar ratio)</th>
<th>maxF (%) original</th>
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<tr>
<td>1</td>
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<td>1.06</td>
<td>19.82</td>
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<tr>
<td>2</td>
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<td>28.14</td>
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<tr>
<td>1a</td>
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<tr>
<td>1b</td>
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<td>2a</td>
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<td>10.00</td>
<td>0.72</td>
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</table>
Figure 8. Stability of complexes to disruption by PVS as measured by restoration of ethidium bromide fluorescence. Fluorescence of DNA-ethidium bromide solution was measured and set to 100%. Complexes were formed with an N-to-P ratio of 1.4. PVS solution in water was added in sequential aliquot portion (5 μl). Complexes of DNA with block copolymers 1 (◇), 2 (×), 1a (○), 1b (△), 1c (△), and 2a (□).

value of 0.94 and 1.19, and minF value of 17.32 and 25.50%. This probably reflects the poor protonation of DMAEMA free base units. In contrast, at pH 5.0, the IF\textsubscript{50} values of block copolymers 1, 1a, and 1b as well as the relevant minF values are substantially lower than those observed at pH 7.4 in agreement with a high degree of protonation of the DMAEMA units (pK\textsubscript{a} ≈ 7).

Restoration of ethidium bromide fluorescence by addition of PVS solution to preformed copolymer-DNA complexes can be taken as an indicator of the complex stability. Figure 8 reports the trends of ethidium bromide fluorescence after sequential additions of PVS to the copolymers/DNA complexes in water with N-to-P ratio of 1.4, whereas Table 2 reports the relevant data.

Copolymer 1c, with butyl groups attached to quaternary ammonium groups, shown an RFI\textsubscript{50} value of 0.16, whereas all the other copolymers, with methyl groups attached to quaternary ammonium groups, shown RFI\textsubscript{50} values ranging from 0.23 to 1.48. Moreover the maximum value of ethidium bromide fluorescence restoration is found for copolymer 1c. In fact, the maxF value of 1c is 86.70%, whereas those of the other copolymers range from 72.76 to 63.60%.

It is interesting to note that DNA complexes with copolymers 1, 2 and 1b have maxF values of 64.10, 62.20, and 63.60%, respectively. This suggests that complexes formed from copolymers with the lower percentage of quaternary
ammonium groups with respect to the total DMAEMA units are the most stable thus indicating an active participation of the uncharged DMAEMA units to the copolymer–DNA complexing mechanism.

The efficiency of the cationic block copolymers to protect the DNA against nuclease digestion through the formation of copolymer–DNA complexes was tested by measuring the absorbance of a DNA solution and a solution of copolymer–DNA complex at the same concentration after addition of nuclease as a function of time. Figure 9 reports as a typical example the variation of absorbance at 260 nm of naked DNA 10 μg ml⁻¹ and copolymer 1a–DNA and 2a–DNA complexes (DNA 10 μg ml⁻¹ and N-to-P ratio = 1.5) following the addition of DNase. The addition of nuclease to the solution of naked DNA results in an absorbance increase determined by a rapid DNA fragmentation. In contrast, the copolymer–DNA complexes are practically unaffected by the nuclease activity as clearly indicated by the absorbance constancy after addition of nuclease. High nuclease resistance was also observed for the other block copolymers thus indicating the stable nature of the copolymer–DNA complex.

**CONCLUSIONS**

Neutral and cationic diblock copolymers were prepared via a two-step procedure, based on the use of macroinitiators. The length of each block and the
number of charged units in the polycation block can be predetermined and adjusted to specific purposes. This novel block copolymer system is able to spontaneously self-assemble with plasmid DNA and to produce oriented and shielded complexes, with physicochemical properties appropriate for in vivo applications. The copolymer/DNA complexes appear as globular structures displaying segments of DNA protruding from their body.

Formation of copolymers/DNA complexes was monitored by the loss in ethidium bromide fluorescence. The percentage of quaternary ammonium groups, with respect to the total DMAEMA units in the copolymer chain, has a definite influence on the complex formation propensity with the copolymer containing the lower percentage of quaternary ammonium groups requiring the higher concentration to achieve complexation. In addition, the uncharged DMAEMA units appear to be able to further participate in the DNA–copolymer complexes, especially at pH lower than 7, at which the residual DMAEMA units are protonated.

The formation of a complex between the cationic block copolymer and the plasmid DNA results in a resistance increase to nuclease digestion, due to the stable nature of the complex. This improved resistance of plasmid DNA against enzymatic breakdown is probably due to a decreased accessibility of the DNA to the enzymes.

These results indicate that the strategy utilized is suitable for the preparation of DNA to be delivered for gene therapy or vaccine studies. From work in progress [35], we can anticipate that the neutral as well as the cationic block copolymers display little cytotoxicity in the case of HL3T1 cells. Immunogenicity and delivery efficiency experiments of pCV-Tat complexed with the above PEG-based cationic diblock copolymer vectors are in progress both in vitro and in animal models, including mice and monkeys.

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DNA-block copolymer complexes for gene delivery


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