Enhancement of Transfection Efficiency Through Rapid and Noncovalent Post-PEGylation of Poly(Dimethylaminoethyl Methacrylate)/DNA Complexes

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Purpose. The aim of this work was to develop a new strategy to introduce poly(ethylene glycol) (PEG) into methacrylate-based polymer/DNA complexes in order to produce hemocompatible particles able to transfect cells in the presence of serum.

Methods. Atom transfer radical polymerization was used to synthesize a well-defined poly(2-(dimethylamino)ethyl methacrylate) homopolymer (PDMAEMA) and a poly(2-(dimethylamino)ethyl methacrylate-b-poly(ethylene glycol) α-methyl ether, ω-methacrylate) palm-tree–like copolymer (P(DMAEMA-b-MAPEG)). The complexes obtained by self assembly of the pCMVβ plasmid and the polymers were used to transfect Cos-7 cells. Their physical properties—particle size and zeta potential—were characterized respectively by dynamic light scattering and electrophoretic mobility measurements. *Ex vivo* hemocompatibility was also determined.

Results. The PDMAEMA/pCMVβ complexes transfected Cos-7 cells exclusively in the absence of serum. Although the P(DMAEMA-b-MAPEG) copolymer had no transfection activity *per se,* the addition of the latter to pre-formed PDMAEMA/DNA complexes significantly enhanced the activity and allowed transfection even in the presence of serum. The presence of palm-tree–like copolymers also improved the hemocompatibility properties of the complexes. No effect on platelet counts was observed for P(DMAEMA-b-MAPEG)/pCMVβ complexes, whereas a decrease of platelets was clearly observed when blood cells were incubated with PDMAEMA/pCMVβ complexes.

Conclusions. Such a synergistic effect of noncovalent PEGylation of poly(amino methacrylate)/DNA complexes allows a new and versatile approach to tune up transfection efficiency.

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ABBREVIATIONS: β-gal, β-galactosidase; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; N/P, ratio between the polymer nitrogen atoms and the DNA phosphates; PEG, poly(ethylene glycol); PDMAEMA, poly(2-(dimethylamino)ethyl methacrylate) homopolymer; P(DMAEMA-b-MAPEG), poly(2-(dimethylamino)ethyl methacrylate-b-poly(ethylene glycol) α-methyl ether, ω-methacrylate); SD, standard deviation.

KEY WORDS: transfection; pegylation; atom transfer radical polymerization; gene delivery.

INTRODUCTION

Nonviral gene delivery systems are in full expansion. Indeed, these vectors present essential advantages over viral vectors, such as biosafety, low immunogenicity, higher capacity in terms of transgene size, and feasible scaling up of production. These systems involve cationic lipid formulations (*N*-[2,3-(dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP), Lipofectin) (1,2) and several cationic polymers such as poly-L-lysine (PLL) (3), polyethyleneimine (PEI) (4), derivatized chitosans (5), amidoamine dendrimers (6), and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) (7,8).

The major drawbacks of the nonviral vectors are their limited transfection efficiency *in vivo* compared to viruses, their cytotoxicity, and their clearance from the bloodstream when administrated intravenously, as a result of a nonspecific protein adsorption. One strategy developed to improve their biocompatibility and their lifetime *in vivo* is the modifications of the complexes with polymers containing hydrophilic segments such as poly(ethylene glycol) (PEG) (9), polymers of *N*-(2-hydroxypropyl)methacrylamide (poly(HPMA)) or *N*-vinyl-pyrrolidone (poly(NVP)) (10,11). PEG segments have also been shown to decrease the zeta potential of polymer/DNA complexes and to increase their solubility and their steric stability (as reflected by a reduced aggregation of the complexes in the presence of high salt concentrations) (9,10). The effect of PEGylation on transfection efficiency depends on the polymer structure and the strategy used to introduce the hydrophilic polyether segment into the polyplex. One of the first methods consists in the synthesis of two component vectors bearing a DNA condensing moiety and a hydrophilic part that are covalently linked in the form of either block or graft copolymers such as PEG-g-PLL (12,13), PEG-g-PEI (14,15), and PEGylated poly(amino methacrylate) (16). Although PEGylation has a negative effect on the transfection efficiency of methacrylate-based copolymers (16), it has been reported to increase that of PLL (13). Petersen *et al.* have reported that the effect of this kind of modification on the biological activity of PEG-g-PEI/DNA complexes is a function of the PEGylation level and of the molar mass of PEG chains that are used (15). Reduced transfection efficiency is thought to be mainly due to undesirable interactions between the PEG segment and the cationic counterpart of the copolymer, leading to a lower capacity to condense DNA. This problem can be overcome by the covalent attachment of PEG to the cationic polymers after the DNA condensation step (17,18). This method, called post-PEGylation, uses NHS-activated PEG which is thus added to pre-formed polymer/DNA complexes and left to react between 2 and 18 h (17,18). However, this procedure can request an additional step to neutralize remaining reactive groups (10). More recently, Kursa *et al.* have shown that complexes generated simply by mixing plasmid DNA with free PEI and PEGylated PEI ("flash" mixing) efficiently transfected K562 cells (19). However, this method does not avoid possible interactions between PEG and cationic elements that can impair transfection as mentioned above.

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In this work, we describe the preparation of ternary complexes according to a rapid and versatile two-step mixing protocol (1 h) involving the condensation of the plasmid DNA by PDMAEMA homopolymers, followed by a noncovalent PEGylation of the complexes through the addition of P(DMAEMA-b-MAPEG) palm-tree copolymers. These ternary complexes transfect Cos-7 cells, even in the presence of serum. This strategy has already been described for instance to introduce PEGylated lipids into virosomes (20) or into "stabilized-plasmid lipid particles" (SPLP) (21), but is used for the first time for polyplexes. Moreover, in our study, PDMAEMA and PEGylated copolymers were synthesized by a well-controlled radical polymerization procedure leading to tailored composition and architecture, with a quite narrow molecular weight distribution (22). It is indeed of key interest to be able to finely control and tune up all the molecular parameters of such polycations in order to select the polymers that are not only efficient DNA vectors but are also hemocompatible and compatible with renal filtration.

MATERIALS AND METHODS

Materials

2-Ethylbromoisobutyrate (EB^{i}B), 1,1,4,7,10,10-hexamethyltriethylene tetramine (HMTETA), and CuBr were purchased from Aldrich (Bornem, Belgium) and used as received. Tetrahydrofuran (THF, 99+% from Chem-Lab), 2-(dimethylamino)ethyl methacrylate) (DMAEMA, from Aldrich), and poly(ethylene glycol) α-methoxy, ω-methacrylate (MAPEG from Aldrich, Mn MAPEG = 480 as determined by ^{1}H NMR spectroscopy, which corresponds to a PEO graft Mn of 395) were purified through a column of basic alumina in order to remove the stabilizing agents. The monomer and macromonomer were then stored under N_2 at –20°C and 5°C, respectively. The pCMVβ plasmid, with the β-galactosidase gene of *Escherichia coli* under control of the CMV promoter (Clontech, USA), was amplified and purified at large scale by Plasmid Factory (Germany). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen Corporation (UK). Penicillin and streptomycin were purchased from Biowhittaker (Verviers, Belgium). Mammalian Protein Extraction Reagent (M-PER) was obtained from Pierce (USA).

Synthesis of PDMAEMA Homopolymers

CuBr (0.238 mmol) and a magnetic bar were introduced in open air into a dry glass-tube, which was then closed by a three-way stopcock capped by a rubber septum and purged by three repeated vacuum/nitrogen cycles. In a 50-ml dry flask, DMAEMA (23.74 mmol) and HMTETA (0.473 mmol) were introduced and bubbled with nitrogen during 10 min before transferring the mixture into the glass-tube placed in a water bath maintained at 25°C. Degassed EB^{i}B (0.237 mmol) was added to the tube with a degassed syringe. After 16 h, the bulk polymerization was stopped by immersing the tube into a liquid nitrogen bath. The catalyst was removed by solubilizing the crude polymer in water at pH ~4 and then by increasing the pH at ca. 12 and the temperature at ca. 65°C for precipitating selectively the PDMAEMA chains. A global yield of 90% was determined while the average molar mass (Mn) and the polydispersity index (Mw/Mn) reached 37,500 and 1.18, respectively, as determined by size exclusion chromatography in reference to poly(methylmethacrylate) standards. The average mole number of tertiary amino groups per g of homopolymer (N) was determined by titration (N = 6.32 mmol/g).

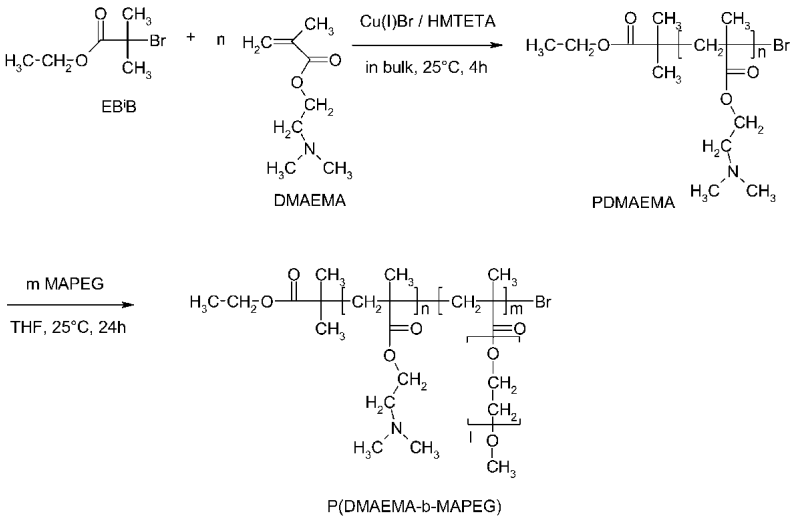
Synthesis of P(DMAEMA-b-MAPEG) Palm-Tree Copolymers

PDMAEMA chains were synthesized by solvent-free atom transfer radical polymerization as described in the preceding paragraph, except that the polymerization time was limited to 4 h instead of 16 h (Fig. 1). Then, a previously degassed THF solution of MAPEG (4.5 mmol in 7 ml of THF) was transferred into the glass-tube and the copolymerization was carried out for 24 h under a nitrogen atmosphere. The reaction was stopped by immersing the tube into a liquid nitrogen bath. The organic solution diluted with fresh THF was passed through a column of basic alumina and poured into a large volume of heptane. The precipitate was recovered by filtration and dried up to constant weight (yield = 76%). It was then dissolved in Millipore water, and the aqueous solution was dialyzed against water for 48 h by using a regenerated cellulose tubular membrane with a molecular weight cut-off of 3500. Finally, the purified copolymer was recovered by lyophilization. The average molar mass (Mn) and polydispersity index (Mw/Mn) reached 24,100 and 1.45, respectively, as determined by size-exclusion chromatography with reference to poly(methylmethacrylate) standards. The molar fraction in MAPEG was determined by ^{1}H NMR spectroscopy (300 MHz, $CDCl_3$) from the relative intensity of PEG methylene protons at 3.65 ppm (I_{PEG}) and α-amino methylene and methyl protons of DMAEMA repetitive units at 2.1–2.7 ppm (I_{MA}) ($F_{MAPEG} = [(I_{PEG} \times MW_{EO})/4 \times (Mn_{MAPEG} - MW_{MMA})]/[[(I_{PEG} \times MW_{EO})/4 \times (Mn_{MAPEG} - MW_{MMA})] + [I_{MA}/8]] = 0.11$, where MW_{EO}, MW_{MMA}, and Mn_{MAPEG} are the molecular weights of ethylene oxide repetitive units, methyl methacrylate, and MAPEG, respectively). The average mole number of tertiary amino groups per g of copolymer (N) was determined by titration (N = 4.22 mmol/g).

Polymer/DNA Complex Formation

For transfection experiments, polymer stock solutions were prepared at a concentration of 1–2 mg/ml in HEPES-buffered solution (HBS: 20 mM HEPES, 155 mM NaCl, pH 7.4). Complexes were formed with different molar ratios of methacrylate polymer nitrogen atoms to DNA phosphates (N/P). For the preparation of ternary complexes, a small volume of concentrated PDMAEMA was mixed with the DNA and left to condense for 30 min at room temperature. After the addition of the copolymer and a second incubation of 30 min, complexes were diluted in DMEM with or without FBS and added to the cells (final concentration of DNA was 2 μg/ml). When PDMAEMA was tested alone, HBS was added after the first 30 min incubation instead of the PEG-based copolymer.

For granulometric characterization and hemocompatibility tests, complexes were respectively prepared in water and PBS (phosphate buffer 10 mM, pH 7.4 with 0.9% NaCl) to



(Co)polymer	Mn_{SEC}[1]	Mw/Mn[1]	F_{MAPEG}[2]	N[3](mmol/g)
PDMAEMA	37500	1.18	0.00	6.32
P(DMAEMA-b-MAPEG)	24100	1.45	0.11	4.22

[1] Determined by size exclusion chromatography with reference to poly(methylmethacrylate) standards
[2] Molar fraction in MAPEG was determined by ^{1}H NMR spectroscopy
[3] Average mole number of nitrogen atoms per g of (co)polymer was determined by titration

Fig. 1. Synthesis and characterization of PDMAEMA and P(DMAEMA-b-MAPEG).

ensure both hemocompatibility and isoosmotic pressure of the buffer.

Particle Size Characterization

Particle size was determined by dynamic light scattering (Brookhaven Inst. BI 2030 AT Digital Correlator). Complexes were formed in water and prepared at a DNA concentration of 13.5 μg/ml, with a N/P ratio of 8.4/1 for PDMAEMA/pCMVβ (i.e., a ratio of 4/1, w/w) and 11.4/1 for P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ ternary complexes (i.e., a ratio of 5/2/1, w/w/w). The influence of the incubation time was tested on the size and the stability of the PDMAEMA/pCMVβ and P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ complexes.

Zeta potential measurements were performed using a Coulter DELSA 440 SX, and complexes were prepared in water in order to avoid any artefactual signals, as described by Ogris *et al.* (9).

Transfection

Cos-7 cells were cultured in DMEM medium supplemented with HEPES (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and FBS (10%). The day before transfection, cells were seeded in 12-well plates (2×10^4 cells/well) in complete culture medium. The cells were incubated with the complexes, in the absence or in the presence of 5% FBS, at 37°C and under a humidified 5% CO_2 atmosphere. After 3 h, the excess of complexes was removed, the cells were washed with DMEM and were further cultured for 24 h in culture medium with 5% FBS. In order to evaluate β-galactosidase expression, cells were washed twice with PBS and lysed with the Mammalian Protein Extraction Reagent (M-PER). One hundred microliters of β-galactosidase substrate (*o*-nitrophenyl β-D-galactopyranoside, or ONPG, 4.4 mM) were mixed with 100 μl of cell lysates and incubated at 37°C. After 30 min, A_{405} was measured. For each sample, protein concentration was determined according to the BIO-RAD Protein Assay protocol (with BSA as standard). Results were expressed in arbitrary units or a.u. (absorbance units corrected for the dilution of the cell lysate) per 100 μg of proteins, as the mean ± standard deviation of triplicate determinations. The viability of the cells was determined using the colorimetric MTT assay (23) and expressed as percentages of the MTT values obtained for non transfected cells.

Hemocompatibility

Hemocompatibility was evaluated using the automated Cell-Dyn 3500 hematology analyser (Abbott), for blood cell counting. Fresh blood was punctured from 300 g male rats and collected on EDTA. Complexes were then incubated with blood for 30 min at 37°C, and the influence of the complex concentration on white blood cells, red blood cells and platelets was determined by cell counting. Complexes were prepared in PBS, with a pCMVβ concentration varying from 0.13 to 1.3 μg/ml. The animal welfare committee of the University of Liège has approved all animal experiments (reference: no. LA1610001).

Erythrocyte Hemolysis and Aggregation

The hemolysis and aggregation assays were performed as described by Petersen *et al.* (15). Briefly, fresh human blood was collected on EDTA, centrifuged for 10 min, and washed several times with PBS until the supernatant was colorless. For the hemolysis detection, complexes prepared as described above (100 μl) were added to 900 μl of erythrocyte suspension (2.5%, v/v). After an incubation of 30 min at 37°C, red blood cells were removed by centrifugation and the A_{540}

read for the release of hemoglobin as described by Petersen *et al.* (15).

To study the erythrocyte aggregation, 150 μl of complexes and 150 μl of red blood cells (0.25%, v/v) were mixed and incubated at 37°C for 2 h in 24-well plates (Nunc) and the aggregates were visualized with a Nikon phase contrast microscope (20× magnification).

RESULTS

Cationic Polymers

As previously published by some of us (22), a well-defined PDMAEMA homopolymer has been synthesized at room temperature by solvent-free atom transfer radical polymerization (ATRP) using copper bromide complexed by HMTETA ligand as the catalyst and 2-ethylbromoisobutyrate (EB^{i}B) as initiator, (Fig. 1). Pure and colorless PDMAEMA was isolated from residual monomers, ligand, and transition metal salt by taking advantage of the pH-dependence and thermo-responsive behavior of PDMAEMA in aqueous media. Practically, acidic water (pH ~4) was added to solubilize the crude polymerization product at room temperature while PDMAEMA chains selectively precipitated by increasing both pH at ca. 12 and temperature at ca. 65°C. A P(DMAEMA-b-MAPEG) palm-tree copolymer was synthesized according to a two-step strategy involving the aforementioned solvent-free ATRP of DMAEMA for 4 h, followed by the controlled radical polymerization of poly(ethylene glycol) α-methyl ether, ω-methacrylate (MAPEG) in THF for 24 h (Fig. 1). After the removal of the catalyst residues by passing the THF solution through a column of basic alumina, extensive purification of the P(DMAEMA-b-MAPEG) copolymer was carried out by dialysis against water. These protocols yielded (co)polymers with monomodal molecular weight distributions and rather low polydispersity indexes: 1.18 for the PDMAEMA and 1.45 for the block polymer (Fig. 1). The PEG grafts have a Mn of 395.

Transfection of Cos-7 Cells with Ternary Complexes in the Absence of Serum

The transfection efficiency of these (co)polymers was tested on Cos-7 cells using the pCMVβ gene as a reporter construct. In a first step, complexes were prepared with either PDMAEMA or P(DMAEMA-b-MAPEG) (co)polymers. As shown in Fig. 2a, significant β-gal activity was detected in cells transfected with PDMAEMA, this activity slightly increased with the N/P molar ratio (where N and P represent respectively the polymer nitrogen atoms and DNA phosphates). However, at high concentration, the PDMAEMA homopolymer seems to be toxic as reflected by a decrease in cell viability (Fig. 2b). Cell viability was first determined by the well known MTT assay. A major disadvantage of this method is that separate samples have to be prepared for the MTT assay and for the measurement of the β-gal activity. So we checked if this test could not been replaced by the quantification of the residual protein content (Bradford test) in the cell lysate used to measure the β–gal activity. As shown in Fig. 2b, both methods give similar results.

The P(DMAEMA-b-MAPEG) copolymer alone, in the absence of serum, had no transfection activity at all (Fig. 3a).

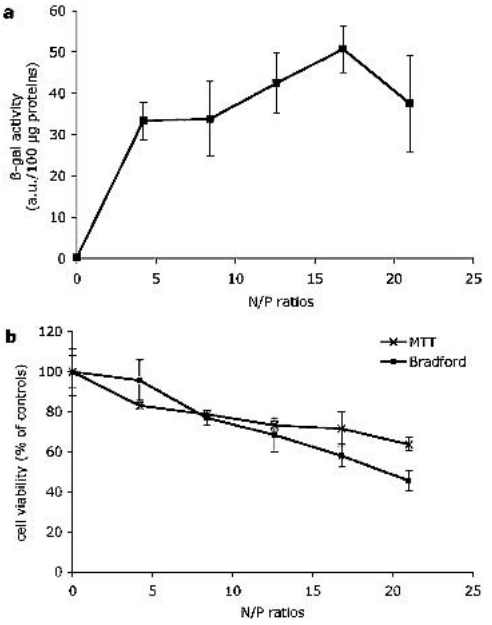


Fig. 2. Transfection of Cos-7 with PDMAEMA/pCMVβ complexes in the absence of serum. The cells were transfected with 1 μg of DNA and different amounts of PDMAEMA. (a) β-gal activity was measured 24 h after transfection. (b) Cell viability was estimated by the MTT test or by measuring the residual protein content in the cell lysates used for the β-gal activity determination (Bradford). Results are expressed as mean ± SD of triplicate determinations in one representative experiment.

The two (co)polymers were then combined in the following way: DNA was first condensed with PDMAEMA (N/P = 4.2) and the pre-formed complexes were then PEGylated by adding the P(DMAEMA-b-MAPEG) palm-tree copolymer. Interestingly, when tested in transfection ternary complexes showed a noticeable increase of the transfection efficiency compared to the PDMAEMA homopolymer alone (Fig. 3b). The time of incubation used to prepare the ternary complexes had clearly an effect on the efficiency of the complexes. When both condensation and PEGylation were performed for 15 min, β-gal activity was biphasic with a maximum observed at a N/P value of 5.4–5.6 (values of N/P take into account both polymers). When DNA condensation and PEGylation were performed for 30 min, the increase of β–gal activity was observed for a broader range of N/P molar ratios (from 5.6 to 11.4). This difference cannot be explained by a high toxicity of the free copolymer at increasing N/P ratios. Indeed, at the N/P ratio of 7.2, where the shorter incubation period severely affects the transfection efficiency, there is no significant differences in the residual protein content of cells transfected with polyplexes prepared in 2 × 15 min or in 2 × 30 min: the amounts of protein represent respectively 77 ± 17% and 77 ± 19% of the protein content of untransfected control cells. In the following experiments, complexes were formed in 60 min.

Transfection of Cos-7 Cells with Ternary Complexes in the Presence of Serum

As already reported for other polycations (24), transfection of Cos-7 cells with the PDMAEMA polymer was drastically reduced in the presence of fetal bovine serum (FBS). The very low activity of the complexes detected with the co-

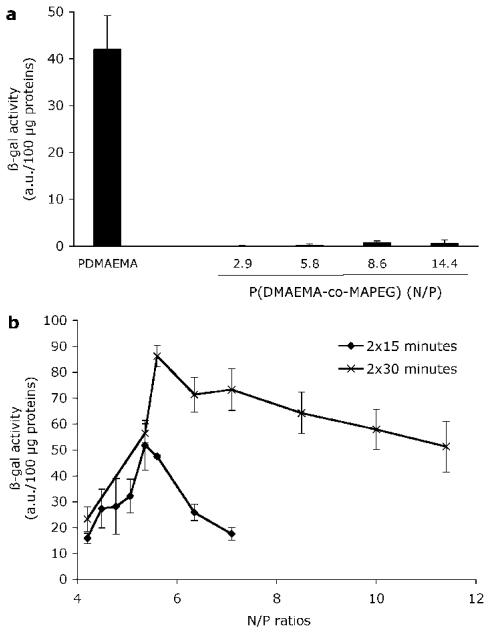


Fig. 3. Transfection of Cos-7 cells with P(DMAEMA-b-MAPEG)/DNA or ternary complexes in the absence of serum. (a) The cells were transfected with different amounts of P(DMAEMA-b-MAPEG). As a control, PDMAEMA was used at the N/P ratio of 4.2. (b) DNA was complexed with PDMAEMA (N/P = 4.2) for 15 or 30 min. Then, increasing amounts of the P(DMAEMA-b-MAPEG) copolymer were added to the pre-formed complexes. After another incubation of 15 or 30 min, complexes were added to the cells for 3 h, in the absence of serum and β-galactosidase activity was measured 24 h later. Results are expressed as mean ± SD of triplicate determinations in one representative experiment. In the N/P ratio, N takes into account both polymers.

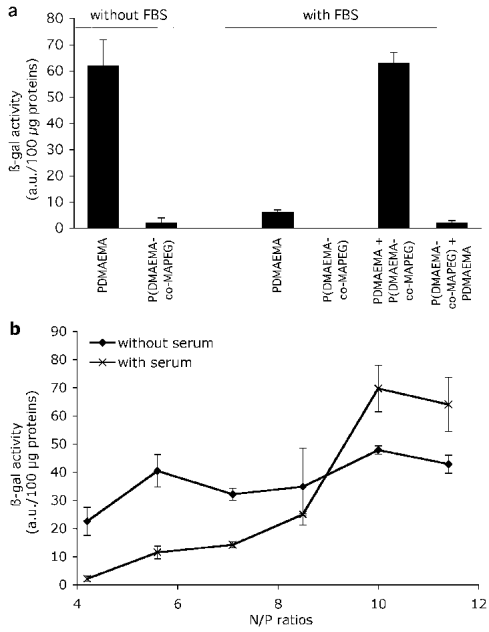


Fig. 4. Transfection of Cos-7 cells with ternary complexes. (a) Importance of the sequence of polymer addition in the preparation of ternary complexes. Cos-7 cells were transfected with PDMAEMA/pCMVβ complexes (N/P = 4.2) or P(DMAEMA-b-MAPEG)/pCMVβ complexes (N/P = 7.2), in the absence or in the presence of 5% serum. Ternary complexes were prepared by adding P(DMAEMA-b-MAPEG) to pre-formed PDMAEMA/pCMVβ complexes (PDMAEMA + P(DMAEMA-b-MAPEG) condition) or by adding PDMAEMA to pre-formed P(DMAEMA-b-MAPEG)/pCMVβ complexes (P(DMAEMA-b-MAPEG) + PDMAEMA condition). Polymers were used at the N/P ratio of 11.4 in both cases. Ternary complexes were used in the presence of 5% of serum. (b) Effect of the relative amount of P(DMAEMA-b-MAPEG) on the transfection efficiency of the ternary complexes. Cos-7 cells were transfected with PDMAEMA/pCMVβ complexes (N/P = 4.2) or ternary complexes prepared with increasing quantity of P(DMAEMA-b-MAPEG), as described above, in the absence or in the presence of 5% serum. In the N/P ratio, N takes into account both polymers. β-galactosidase activity was measured 24 h after transfection. Results are expressed as mean ± SD of triplicate determinations in one representative experiment.

polymer alone without serum was completely abolished in the presence of serum (Fig. 4a). Interestingly enough, in the presence of serum, ternary complexes prepared as described above, restored the activity of PDMAEMA complexes at a very high level. The sequence of addition of both PDMAEMA and the copolymer seemed to be crucial, as ternary complexes prepared in the other way (the palm-tree copolymer added before the PDMAEMA) were inefficient (Fig. 4a). The ability of these ternary complexes to transfect Cos-7 cells in the presence of serum was also dependent on the relative amount of P(DMAEMA-b-MAPEG) added to the initial binary complexes (N/P = 4.2). Figure 4b illustrates the results obtained for increasing amounts of added PEG-based copolymer. The level of β-gal activity in cells transfected in the presence of serum increased progressively up to a N/P molar ratio of 10, where it reached a plateau.

In Fig. 4b, the plateau value was higher for cells transfected in the presence of serum than for cells transfected without serum. However, over all the experiments performed, the β-gal activity detected in cells transfected with ternary complexes (N/P = 11.4), in the presence of serum, was very close to the activity detected in cells transfected with PDMAEMA/DNA polyplexes in the absence of serum: indeed we observed respectively 30 ± 13 a.u./100 µg proteins (22 independent experiments with serum) and 32 ± 15 a.u./100 µg proteins (25 independent experiments without serum).

Both types of complexes are also comparable in terms of toxicity. When Cos-7 cells are transfected with PDMAEMA/DNA polyplexes (N/P = 4.2) or with ternary complexes (N/P = 11.4), we recovered respectively 94 ± 14% (39 independent experiments) and 92 ± 10% (31 independent experiments) of the residual protein content detected in non transfected cells.

Particle Size and Zeta Potential Characterization

The physicochemical characteristics of both PDMAEMA/DNA (N/P = 8.4) and P(DMAEMA-b-MAPEG)/PDMAEMA/DNA complexes (N/P = 11.4) were studied in water, with a DNA concentration of 13.5 µg/ml. The size of the complexes was studied by photon correlation spectroscopy. In that case, the size of both types of particles was relatively small (Table I), and stability was reached after 30 min of PDMAEMA or P(DMAEMA-b-MAPEG)/PDMAEMA incubation (Fig. 5).

As described in the literature (9,15), transfections are more efficient when complexes are neutral or slightly positive (zeta potential between 5 to 30 mV). Moreover, an excess of positive charges can deeply activate the alternative comple-

Table I. Physicochemical Characteristics of the Binary and Ternary Complexes

Complex	N/P	Zeta potential*,† (mV)	Mean diameter*,† (nm)
PDMAEMA/pCMVβ	8.4/1	18 ± 3	76 ± 4
P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ	11.4/1	14 ± 3	110 ± 15

* The complexes were prepared in de-ionized water at a DNA concentration of 13.5 μg/ml.
† Mean ± SD (n = 4).

ment pathway or can lead to strong erythrocyte aggregation and hemolysis. Zeta potential, measured at N/P ratios leading to high transfection efficiencies (i.e., 8.4/1 and 11.4/1 for respectively PDMAEMA/pCMVβ and P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ complexes), was positive for both complexes and in the range of 5 to 30 mV (Table I). The ternary complexes had a slightly lower zeta potential than the PDMAEMA/DNA complexes, as shown for other types of PEG-modified vectors (10,15,18).

Hemocompatibility *ex Vivo*

As described in "Materials and Methods," blood compatibility tests were performed *ex vivo* with fresh rat blood. Blood cells were counted after a 30 min incubation of the complexes with blood at 37°C. Figure 6 shows the influence of both complexes formed in PBS on white blood cells, red blood cells, and platelets. No significant effect was observed on white (Fig. 6a) and red (Fig. 6b) blood cell counts. This suggests that no hemolysis occurred after 30 min of incubation of blood cells in the presence of binary or ternary complexes. The absence of hemolysis was confirmed by using the erythrocyte hemolysis assay of Petersen *et al.* (15) (data not shown). The erythrocyte aggregation was also studied with isolated cells following the method described by the same authors (15). As shown on Fig. 7, some small aggregates were detected in samples incubated with polyplexes, especially with ternary complexes at the DNA conentration of 1.3 μg/ml. Finally, PDMAEMA/pCMVβ complexes caused a slight decrease of platelets (15%), after 30 min of incubation at a DNA concentration of 1.3 μg/ml (Fig. 6c). At the lower concentration of 0.13 μg/ml, no significant decrease of platelets was detected. On the contrary, ternary complexes had no noticeable effect on platelets even at the highest DNA concentration.

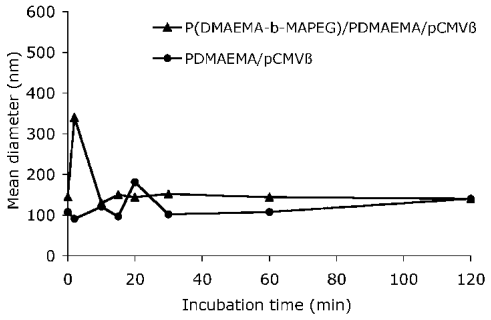


Fig. 5. Influence of time incubation on the size of PDMAEMA/pCMVβ and P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ complexes. Complexes were prepared in water as described in "Materials and Methods," with an N/P ratio of 8.4 and 11.4, respectively, for binary and ternary complexes. Mean diameter was determined by dynamic light scattering at 25°C.

DISCUSSION

For *in vivo* applications, gene delivery systems need to be protected at least from nonspecific interactions with blood components in order to avoid coagulation or reactions with some components of the immune system. This problem can be overcome by modifying the complexes with shielding agents such as PEG, poly(NVP) or poly(HPMA). Classical methods for the PEGylation of the DNA containing complexes include the synthesis of suitable PEG-based copolymers or the covalent post-PEGylation of the complexes using NHS-activated PEG. However, these methods are time consuming and they often produce polymers with a weak transfection efficiency.

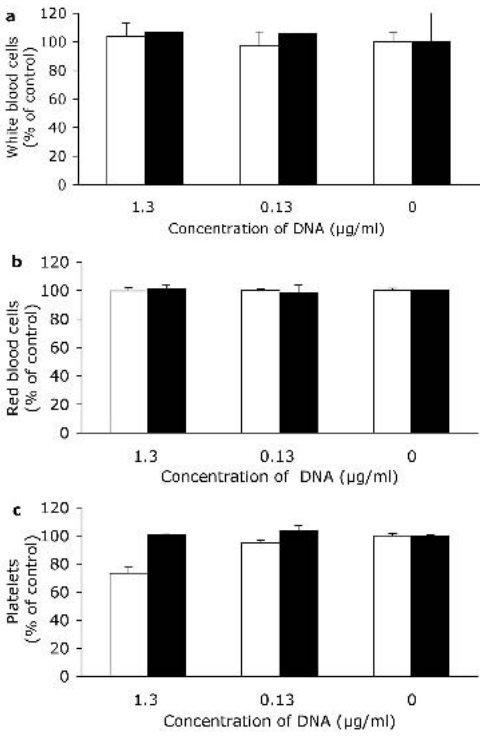


Fig. 6. Effect of the DNA concentration on blood cells incubated *ex vivo* with PDMAEMA/pCMVβ complexes (open bars) or with P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ complexes (closed bars). After collecting fresh rat blood on EDTA, white blood cells (a), red blood cells (b), and platelets (c) were incubated 30 min at 37°C with the complexes at two DNA concentrations (0.13 and 1.3 μg/ml). The N/P ratio was 8.4 and 11.4, respectively, for binary and ternary complexes. Data are given as percentages of cells counted in the sample incubated in the absence of the complexes, and results are expressed as mean ± SD of duplicate determinations.

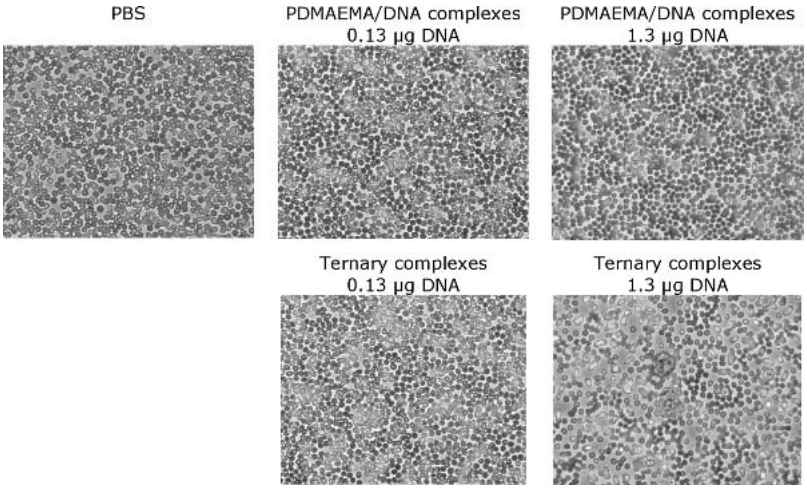


Fig. 7. Polyplexes-induced erythrocyte aggregation. Erythrocytes were incubated in the presence of PBS (control) or in the presence of the different polyplexes for 2 h, and pictures were taken by phase contrast light microscopy (magnification ×20).

Here, we report the rapid formation of polymer/DNA complexes according to a two-step protocol with well-defined polymers. The (co)polymers of methacrylate described herein are chains of 24,000–37,000 Da. This range of molecular weight (Mn) has been chosen on purpose for further *in vivo* experiments, as the polymers used in this study are compatible with renal filtration. Moreover, we used the method of atom transfer radical polymerization which allows the production of polymers with a very narrow molecular weight distribution: the polydispersity is 1.18 and 1.45 for the homopolymer and copolymer, respectively. Some PDMAEMA polymers described in the literature have a polydispersity index of 28 (25). It means that the molecular weight distribution is very broad. In this case it is difficult to determine which subpopulation of polymers is really responsible for the transfection activity and which one is responsible of the side effects (erythrocyte aggregation, for example). Even if the chemical composition is the same, the polymers described in this paper and those used by Verbaan *et al.* are different and their behavior is different (25).

In this study, the PEGylated complexes were formed as followed: DNA was condensed with PDMAEMA homopolymers and then the preformed complexes were PEGylated by the addition of P(DMAEMA-b-MAPEG) palm-tree copolymers. The ternary complexes were thus prepared within 1 h, whereas conventional covalent post-PEGylation required incubations of 2–18 h at room temperature (17,18). Our results showed that, although P(DMAEMA-b-MAPEG) had no transfection activity *per se*, it did not reduce the transfection activity of PDMAEMA. On the contrary, the presence of the PEG-based copolymer improved gene transfer with PDMAEMA in the absence of serum and even more drastically in the presence of serum. The sequence of addition of the different polymers to prepare the complexes was crucial: indeed, as expected from their respective hypothesized contributions, it is mandatory to add the palm-tree copolymer after PDMAEMA to produce efficient complexes. First, DNA is condensed by the PDMAEMA homopolymer. The P(DMAEMA-b-MAPEG) copolymer is then added to avoid undesirable effects of the PEG segments on DNA complexation and moreover to force these chains to be exposed at the surface of the complexes. The results show that the duration of the 2 steps (condensation and PEGylation) is another critical parameter: 2 incubations of 30 min leading to a much higher transfection efficiency than 2 incubations of 15 min. The differences observed are not due differences in toxicity of the free copolymer. However, free copolymers probably negatively affect transfection efficiency, by interfering with the assembled ternary complexes.

PEGylation was thought to occur by noncovalent interactions between the methacrylate backbones of both types of (co)polymers as illustrated in Fig. 8. These interactions seemed to be strong enough to resist in the presence of serum proteins, as in these conditions transfection with the ternary complexes is still efficient (Fig. 4b). Ogris *et al.* (9) reported that noncovalently bound PEG chains did not prevent protein binding to transferrin-modified PEI/DNA complexes. The difference between both systems is that we did not use free

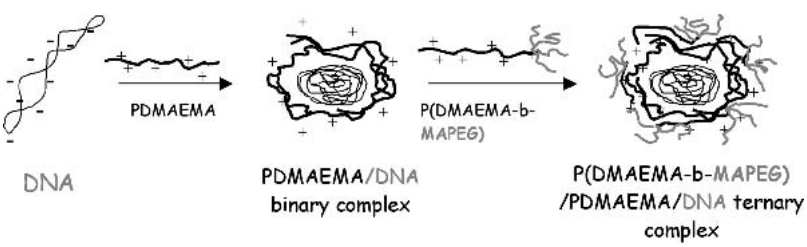


Fig. 8. Hypothetic structure of the P(DMAEMA-b-MAPEG)/PDMAEMA/pCMVβ ternary complexes.

PEG chains, but PEG-modified methacrylate polymers. Another argument in favor of interactions between the two types of polymer is that the size of the ternary complexes is slightly greater than the size of the binary structures (the sizes are respectively around 110 and 76 nm).

Physicochemical characteristics seem to be of major importance to choose the appropriate complexes for gene delivery. However, as discussed by Petersen *et al.* (13), stressing how complex the gene transfer process is, it is far too simplistic to try to correlate transfection data with a single physicochemical characteristic (zeta potential, particle size, and so forth) or biological parameter (cytotoxicity). Actually, these parameters are extremely sensitive to modifications regarding the composition of the medium used for complex formation (salt or phosphate concentration, N/P ratio, pH, and so forth). The size of both binary and ternary complexes we studied was around 100 nm and their electrokinetic potential was slightly positive. This is in agreement with the criteria reported in the literature to favor transfection and blood compatibility (9).

The influence of the P(DMAEMA-b-MAPEG) palm-tree copolymer on the behavior of the complexes in blood was also studied. When fresh rat blood was incubated with polyplexes, we did not observed any modification of red blood cell counts, suggesting that no hemolysis or hemoagglutination occurred. The absence of hemolysis was confirmed by a specific assay (data not shown). On the contrary, the erythrocyte aggregation assay, performed with isolated cells, revealed the formation of some small aggregates in the presence of both PDMAEMA/DNA complexes and ternary complexes. This discrepancy could could be explained by the differences in the experimental conditions. Verbaan *et al.* recently reported severe aggregation of erythrocytes in the presence of PDMAEMA/DNA complexes (25), however it is difficult to compare their results with ours, as they did not show the extent of the aggregation. The hemagglutination we have observed is however clearly less important than the hemagglutination reported for PEI/DNA complexes (15).

Finally, a decrease in platelets counts could be detected after a 30 min incubation of blood cells with PDMAEMA/DNA complexes, which was probably caused by complex aggregation leading to platelet adsorption, and thus activation. Actually, the hematological analyzer used in this study differentiates platelets from the other cells according to their size. Therefore, we hypothesize that the decrease in platelet counts could be due to a change of shape, caused by activation. Such a negative effect on platelet number was not observed with P(DMAEMA-b-MAPEG)/PDMAEMA/DNA complexes. The ternary complexes had no influences on platelets during 30 min of incubation with DNA, at concentrations up to 1.3 μg/ml. Complexes seem to be protected by PEG chains grafted on the palm-tree copolymer: hydrophilicity and mobility of the side chains could prevent platelets from adsorption onto the complexes and from activation.

Taken together, that noncovalent PEGylation of poly(amino-methacrylate)/DNA complexes, using PEG-modified methacrylate polymers, represents a new and versatile approach for gene transfer: the ternary polyplexes described in this study are as efficient as the non-PEGylated polymers in transfecting the cells *in vitro*, they allow transfection in the presence of serum, and they are good candidates for *in vivo* experiments. Effects of (co)polymer molecular parameters, for example, molecular weight, compositions, as well as topology (diblock, grafted, comb-like, brush like copolymers), on the transfection efficiency and hemocompatibility are currently under investigation and will be the topic of a forthcoming paper.

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