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Research paper

On the role of methacrylic acid copolymers in the intracellular delivery of antisense oligonucleotides

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Abstract

The delivery of active biomacromolecules to the cytoplasm is a major challenge as it is generally hindered by the endosomal/lysosomal barrier. Synthetic titratable polyanions can overcome this barrier by destabilizing membrane bilayers at pH values typically found in endosomes. This study investigates how anionic polyelectrolytes can enhance the cytoplasmic delivery of an antisense oligonucleotide (ODN). Novel methacrylic acid (MAA) copolymers were examined for their pH-sensitive properties and ability to destabilize cell membranes in a pH-dependent manner. Ternary complex formulations prepared with the ODN, a cationic lipid and a MAA copolymer were systematically characterized with respect to their size, zeta potential, antisense activity, cytotoxicity and cellular uptake using the A549 human lung carcinoma cell line. The MAA copolymer substantially increased the activity of the antisense ODN in inhibiting the expression of protein kinase C-alpha. Uptake, cytotoxicity and antisense activity were strongly dependent on copolymer concentration. Metabolic inhibitors demonstrated that endocytosis was the major internalization pathway of the complexes, and that endosomal acidification was essential for ODN activity. Confocal microscopy analysis of cells incubated with fluorescently-labeled complexes revealed selective delivery of the ODN, but not of the copolymer, to the cytoplasm/nucleus. This study provides new insight into the mechanisms of intracellular delivery of macromolecular drugs, using synthetic anionic polyelectrolytes.

Keywords: Methacrylic acid copolymers; Oligonucleotides; Intracellular delivery; Endosomal release; Drug delivery

1. Introduction

Antisense oligodeoxyribonucleotides (ODNs) are being investigated for the treatment of various diseases arising from genetic abnormalities, including cancer, viral infections and inflammatory disorders. These molecules can inhibit gene expression by several putative mechanisms, such as translational arrest and cleavage of target mRNA [1]. However, rapid degradation of ODNs by nucleases, poor cellular uptake and inability to reach their intracellular site of action (e.g. the cytoplasm and/or nucleus) in sufficient amount often compromise their biological activity. Naked ODNs are internalized by endocytosis and end up in acidic endosomal/lysosomal compartments [2,3]. These organelles represent a dead end for ODNs since from this location, they may either be released from the cell via exocytosis or be partially digested [4].

To circumvent problems related to ODN internalization, intracellular trafficking and premature degradation, a variety of positively-charged lipidic (lipoplexes) and polymeric (polyplexes) vehicles have been investigated (for recent reviews, see [5–7]). Cationic lipids, generally in combination with a fusogenic agent, can condense ODNs by electrostatic interactions and enhance their stability [8], cellular uptake [9] and antisense activity in vitro [10]. After internalization by endocytosis, ODN release is thought to be initiated by cationic lipids which induce the flip-flop of anionic lipids from the cytoplasmic facing monolayer [11]. Charge neutralization then leads to dissociation of the complex and ODN transfer into the cytosol, where it is rapidly transported into the nucleus [5,11–13]. Protection and facilitated transit of the ODN from the endosomes to the cytoplasm can also be achieved through the use of specific titratable polyamines (e.g. polyethylenimine), which have been shown to destabilize the endosomal membrane via a mechanism that is still a matter of debate. Indeed, the hypothesis initially put forward to explain the enhanced efficacy of some polyamines, the

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so-called 'proton sponge' effect [14], is now being seriously questioned [15,16].

In 1999, Hoffman and co-workers demonstrated that synthetic polyanions, such as poly(propylacrylic acid) (PPAA), exhibited membrane-lytic properties at pH values typically found in the endosomal compartment [17]. Upon protonation and charge neutralization in the acidic environment of endosomes, PPAA was shown to interact with the endosomal membrane and selectively disrupt it [18], thereby enhancing the cytoplasmic delivery of internalized macromolecules, such as plasmid DNA and polymer-antibody complex [19,20]. Moreover, it was demonstrated that PPAA enhanced the in vitro transfection of lipoplex formulations in the presence of 50% serum, and provided significant improvements in the efficacy of a plasmid DNA in an in vivo murine wound healing model [21]. Recently, our group reported that low molecular weight (MW ~ 20,000), pH-sensitive methacrylic acid (MAA) copolymers could also destabilize membrane bilayers at slightly acidic pH values [22]. As these copolymers are structurally related to PPAA, it was hypothesized that they could improve the intracellular delivery and biological activity of ODNs. This study investigates how MAA copolymers can enhance the cytoplasmic delivery of a 20-mer phosphorothioate anti-protein kinase C-alpha (PKC-α) ODN, which has been tested in phase III clinical trials [23]. The activity of the ODN incorporated into complexes composed of a cationic lipid and a MAA copolymer, is assessed in vitro for the first time. These results are put in perspective relative to cytotoxicity data and the activity of a conventional control lipoplex formulation. More importantly, the cellular internalization and trafficking of the ODN as well as of the copolymer are investigated by flow cytometry and confocal microscopy. Preliminary data reveal a drastic effect of the MAA copolymer on the uptake, intracellular distribution and antisense activity. Moreover, selective delivery of the ODN, but not of the copolymer, to the cytoplasm/nucleus is demonstrated.

2. Materials and methods

2.1. Purification and characterization of the copolymers

MAA copolymers were provided by Röhm GmbH (Darmstadt, Germany). They were prepared by free radical polymerization involving different chain transfer agents (Table 1). Prior to use, they were dissolved in ethanol and dialyzed against water for at least 5 days. Absolute number (M_n)- and weight (M_w)-average MWs were determined by size exclusion chromatography, with a Waters 1525 pump (Waters, Milford, MA) equipped with 4 Waters Ultrahydrogel (120, 250, 1000 and 2000) columns placed in series, a high-sensitivity differential refractive index detector (Waters 2410) and a PD 2000 light scattering detector (Precision Detectors, Franklin, MA). Samples were analyzed in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8, at 1 mL/min and 35 °C. Phase transition range of the different copolymers was evaluated by turbidimetry as previously reported [22].

2.2. Cell culture

A549 human lung carcinoma cells, obtained from the American Type Tissue Collection, were grown in Dulbecco's modified Eagle cell culture medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin (Invitrogen, Burlington, ON, Canada). The cells were routinely passed when they were 90–95% confluent.

2.3. Hemolysis assays

This procedure was described previously by Murthy and coworkers [24]. Human red blood cells were collected from a healthy donor, centrifuged at 200*g* (5 min, 4 °C), and washed three times (centrifugation followed by redispersion) with saline 0.9% (w/v). The cells were counted with a hemacytometer and diluted in either isotonic phosphate (66 mM) or 2-(*N*-morpholino)ethanesulfonic acid (MES, 200 mM) buffer of the appropriate pH. Hemolysis assay was performed by adding the erythrocytes suspended in the appropriate medium (final cell concentration = 10^8 cells/mL) to a copolymer solution. To ensure that buffer pH did not influence the data, hemolysis was measured at each pH value in the absence of copolymer. Samples were incubated for 30 min under stirring at 37 °C, cooled on ice, and centrifuged at 5300*g* for 5 min at 4 °C. The extent of membrane disruption was established by measuring hemoglobin absorbance in the supernatant at 541 nm, with a PowerWave microplate reader (Biotek Instruments, Winooski, VT). To obtain 100% hemolysis, the cells (10^8/mL) were lysed by dispersion in water. Controls were prepared by mixing red blood cells with saline. The cells were used within 24 h after collection.

Table 1
Chemical composition and molecular weight of the different copolymers tested

Copolymer	Composition (molar ratio)	Chain transfer agent	Molecular weights			pH transition range
			M_n	M_w	PI	
1	EA/MAA (50:50)	2-ethylhexylthioglycolate	9200	12,400	1.35	4.2–5.2
2	EA/DMA/MAA (49:1:50)	2-ethylhexylthioglycolate	11,700	14,800	1.26	4.4–5.2
3	EA/DMA/MAA (49:1:50)	Dodecyl mercaptane	18,400	24,900	1.35	4.2–5.2
4	EA/BMA/MAA (40:10:50)	Dodecyl mercaptane	19,800	26,000	1.31	4.2–5.2

EA, ethyl acrylate; DMA, dodecyl methacrylate; BMA, butyl methacrylate; MAA, methacrylic acid.

2.4. Preparation of cationic liposomes and cationic lipid/ODN complexes

Dioleoyltrimethylammonium propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). DOTAP particles and DOTAP/DOPE liposomes were prepared by extrusion through 0.05-μm pore size membranes under sterile conditions [25]. Twenty-mer antisense phosphorothioate ODN (5′-GTTCTCGCTGGTGAGTTTCA-3′), designed to hybridize to the 3′ untranslated region of human PKC-α mRNA, its fluorescein-labeled derivative and a scrambled version (5′-GGTTTTACCATCGGTCTTGG-3′) were provided by Medicorp Inc. (Montreal, QC, Canada). Lipoplexes were prepared by incubating the ODN in HEPES (20 mM)—dextrose (5% w/v) (H/D, pH 7.4) with the cationic lipids for 30 min at room temperature under mild agitation. Formulations containing the endosomolytic polymer (i.e. ternary complexes) were obtained by further incubating the lipoplexes with copolymer 4 (Table 1) in H/D for 30 min. Complexes were formed at theoretical [+/−] ratios ranging from 2 to 8. Charge ratios were quantified as total positive-to-negative charges, and were calculated by assuming 40% copolymer ionization at pH 7.4, as determined from titration experiments (data not shown). Unless otherwise stated, the ODN concentration was fixed at 200 nM. Size and zeta potentials of the complexes were evaluated with a Malvern Autosizer 4800 and a Malvern ZetaSizer Nanoseries ZS (Malvern Instruments, Worcestershire, UK), respectively.

2.5. Gel electrophoresis

To ensure that ODN was not released from the complexes upon addition of the MAA copolymer, ternary complexes containing the fluorescent ODN were prepared as described above and loaded onto a 20% poly(acrylamide) gel. Following migration, ODN was visualized directly on the gel after exposition to UV light using a ChemiImager 5500 imaging system (Alpha Innotech Corp., San Leandro, CA).

2.6. Assessment of ODN antisense activity

A549 cells were plated in six-well tissue culture plates (1 mL medium containing 1×10^5 viable cells), and allowed to adhere at 37 °C in a humid atmosphere containing 5% CO_2. After 24 h, the cells were treated with 500 nM phorbol 12,13-dibutyrate (PDBu) (Sigma, St-Louis, MO) for 18 h to remove most of the immunoreactive PKC-α [26]. They were washed three times with DMEM, and 0.5 mL of Opti-Mem I reduced serum medium (Invitrogen, Burlington, ON, Canada) premixed with 0.5 mL of complexes in H/D was added to each well. One hundred percent PKC-α expression was obtained by adding Opti-Mem mixed with H/D to the cells. The cells were incubated for 4 h at 37 °C, washed once with complete DMEM and allowed to recover for 20 h. Then, they were washed twice with phosphate-buffered saline (PBS, 66 mM, NaCl 75 mM, pH 7.4), and extracted in 200 μL lysis buffer consisting of Tris–HCl, pH 8.0 (0.01 M), NaCl (0.14 M), sodium azide (0.0256 M), Triton X-100 (1% v/v), aprotinin (0.1 U/mL) and phenylmethylsulfonyl fluoride (0.5 mM). The cell lysates were incubated for 1 h at 4 °C, and centrifuged at 5000*g* for 5 min at 4 °C. Sample protein content was determined with the BCA protein assay kit using bovine serum albumin standards (Pierce, Rockford, IL). The remaining samples were electrophoresed through a 12% (w/v) poly(acrylamide) gel, and the resolved proteins were transferred to a poly(vinylidene fluoride) membrane by electrotransfer. PKC-α expression (MW 82,000 g/mol) was quantified by use of an anti-PKC-α monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). To confirm equal loading, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MW 36,000 g/mol) was measured simultaneously, using an anti-GAPDH monoclonal antibody (Advanced Immunochemicals, Long Beach, CA). All formulations were tested at least in triplicate.

2.7. Evaluation of the cellular toxicity of complexes by MTT assay

Inhibition of cell proliferation was assessed by tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [27]. A549 cells were plated in six-well tissue culture plates and treated with formulations prepared with scrambled ODN, as described above. After a 4-h incubation period, the medium was removed and replaced with 0.5 mL of complete DMEM. Twenty hours later, MTT dissolved in PBS (50 μL of a 5 mg/mL solution) was added to each well. After a further 3-h incubation, sodium dodecyl sulfate (500 μL of a 10% w/v solution containing 0.01 N HCl) was added to dissolve the reduced MTT. Absorbance was measured 24 h later at 570 nm. Each experiment was performed in quadruplicate.

2.8. Effect of metabolic inhibitors on ODN delivery

Prior to incubation with the complexes, A549 cells were treated for 15 min with cytochalasin B (25 μg/mL) or for 30 min with bafilomycin A (200 or 500 nM) in Opti-Mem I medium. Inhibitors were present concomitantly with the complexes during the 4-h incubation period.

2.9. Labeling of copolymer with rhodamine

The carboxylic acid group activator 1,3-diisopropylcarbodiimide (0.543 mmol) was added to a solution of copolymer 4 in anhydrous dimethylformamide (DMF) (50 mg, 1.0 mL). After 30 min, Lissamine® rhodamine B ethylenediamine (Molecular Probes, Eugene, OR) (2.05×10^{-3} mmol) dissolved in DMF and catalytic amounts of triethylamine and 4-(dimethylamino)pyridine were added. The reaction was carried out for 3 days in an inert atmosphere, in the dark at room temperature. The crude product was dialyzed against methanol (MW cut-off 3500) for 3 days to remove unreacted rhodamine. The methanol was then replaced progressively by water, and the purified product was freeze-dried. The absence of free

rhodamine was confirmed by thin layer chromatography, using a chloroform/methanol (80/20 v/v) mobile phase. Rhodamine content of the copolymer was assayed by spectrofluorimetry in methanol ($\lambda_{ex}=560$ nm, $\lambda_{em}=581$ nm) and found to be 0.28 mol% (yield: 70%).

2.10. Flow cytometry

Complexes containing fluorescein-labeled ODN (20% of total ODN) ± rhodamine-labeled copolymer 4 (15% of total polymer) were prepared as described above, and incubated with cells for 4 h. The cells were then washed twice with cold PBS and resuspended in 0.5 mL PBS. Mean green and red fluorescence intensities for 10,000 cells were recorded at 530/30 (FL1-height) and 585/42 (FL2-height) nm, respectively, with a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA) at a laser excitation wavelength of 488 nm.

2.11. Intracellular distribution of ODN and copolymer

A549 cells were grown on coverslips and incubated for varying lengths of time with complexes containing fluorescein-labeled ODN and rhodamine-labeled copolymer 4. The cells were then washed twice in cold PBS and fixed for 15 min in PBS containing 3% (w/v) paraformaldehyde. The fixed cells were mounted in Mowiol® (EMD Biosciences Inc., Darmstadt, Germany) and examined by LSCM under a Leica DMIRBE inverted microscope coupled with a Leica TCS SP confocal system (Leica Microsystems, Heidelberg, Germany). The cells were excited at 488 or 568 nm, and fluorescence was collected by emission windows set at 505–555 and 585–635 nm, respectively. Images collected by LCS Lite software (Leica Microsystems) were exported as TIFF files and prepared for publication by Adobe Photoshop v7.0 software (Adobe Systems, San Jose, CA).

2.12. Statistical analysis

Significant differences between two groups were evaluated by Student's *t*-test. Multiple comparisons for more than two groups were evaluated by one-way analysis of variance followed by Scheffé's post hoc test to determine the significance of paired combinations. *P*-values lower than 0.05 were considered to be significant.

3. Results

3.1. Characterization of the different MAA copolymers

In a previous study, we showed that among a series of novel MAA copolymers, those containing ethyl acrylate (copolymer 1, Table 1) demonstrated the best membrane-destabilizing properties at acidic pH [22]. To further improve membranolytic activity, hydrophobized derivatives of copolymer 1 were synthesized. These derivatives bear a small proportion of alkyl chains (butyl or dodecyl) that was incorporated either randomly and/or at one extremity of the polyanion. The chemical composition and respective MWs of the MAA copolymers are presented in Table 1. As these copolymers are a priori not biodegradable, their MW was kept below 40,000 to ensure elimination by renal glomerular filtration [28]. Number-average (M_n) MWs ranged from 9200 to 19,800 with polydispersity indices (PI) between 1.2 and 1.4. Table 1 also shows the pH transition ranges of the different copolymers. Despite differences in their chemical composition, all copolymers precipitated at similar pH values (4.2–5.2), and exhibited sharp transitions encompassing 0.8–1.0 pH units. It is noteworthy that the turbidimetry method used to monitor phase transition generally underestimates the pH value at which the onset of coil-to-globule transition takes place [29].

A useful endosomolytic agent should have membrane-destabilizing properties at the mildly acidic pH (5.0–6.5) found in endosomes and should be inert at physiological pH. The pH-dependent membrane-lytic activity of MAA copolymers was assessed at 100 μg/mL with red blood cells serving as an endosomal membrane model (Fig. 1a). At pH 7.4, slight hemolytic activity was noticed for copolymers 3 and 4 only. However, this destabilizing effect disappeared at concentrations below 25 μg/mL (data not shown). All copolymers showed enhanced membrane-disruptive activity at decreased pH values and were highly hemolytic at pH 5.0–5.5. Copolymer 4 was the most hemolytic, as it caused 50 and 100% hemolysis at pH 6.5 and 6.0, respectively. It was followed by copolymers 3, 2, and 1. To determine the lowest concentration at which hemolysis occurred under acidic

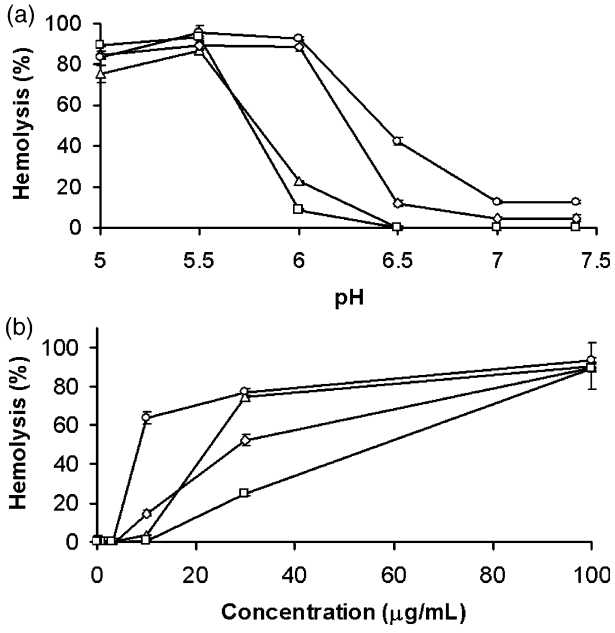

Fig. 1. pH-dependent red blood cell hemolysis induced by MAA copolymers. The copolymers were incubated with human erythrocytes for 30 min at 37 °C. The samples were centrifuged, and the extent of hemolysis was measured by reading the absorbance of the supernatant at 541 nm. (a) Red blood cells suspended in isotonic phosphate or MES buffers of the appropriate pH were added to copolymers 1 (squares), 2 (triangles), 3 (diamonds), or 4 (circles) dissolved in mildly alkaline saline at 100 μg/mL (final concentration). (b) Red blood cell hemolysis caused by the copolymers at pH 5.5 at various concentrations (0.1–100 μg/mL). Symbols are the same as in (a). Values are mean ± SD, $n=3$. No hemolysis was observed in the absence of copolymer.

Table 2
Size and zeta potential of the lipid particles

	H/D buffer		H/D:opti-mem media (50:50 v/v)	
	Size (PI) (nm)	Zeta potential ±SD (mV)	Size (PI) (nm)	Zeta potential ±SD (mV)
DOTAP	118 (0.131)	48.3±3.0	111 (0.073)	49.9±1.7
DOTAP/ODN (21/1)*	117 (0.101)	44.9±3.5	125 (0.122)	47.2±0.6
DOTAP/ODN/ copolymer 4 (4/1)*	195 (0.200)	32.0±1.9	329 (0.259)	31.4±2.1
DOTAP/ODN/ copolymer 4 (3/1)*	255 (0.280)	31.1±1.5	440 (0.332)	23.7±2.7
DPTAP/ODN/ copolymer 4 (2/1)*	345 (0.289)	26.8±1.2	599 (0.324)	6.7±2.7

*The [+/−] ratio is indicated in parentheses.

conditions, red blood cells were incubated with the copolymers at pH 5.5 at concentrations ranging from 0.1 to 100 μg/mL (Fig. 1b). Again, copolymer 4 was the most hemolytic agent, with 60% lysis at 10 μg/mL. As this copolymer demonstrated high membrane-lytic properties under mildly acidic conditions, it was selected for incorporation into the lipoplexes. Ternary complexes prepared at three different [+/−] charge ratios were characterized with respect to their size and zeta potential. As shown in Table 2, the addition of ODN only had a moderate effect on the zeta potential of DOTAP particles, which remained highly positive. Further charge neutralization was achieved upon complexing the copolymer, resulting in a net drop of zeta potential values. The ternary complexes also exhibited larger sizes compared to DOTAP aggregates and DOTAP/ODN complexes. In H/D, the 4/1, 3/1 and 2/1 ternary complexes consisted of monodisperse populations with mean hydrodynamic diameters of about 200, 250 and 350 nm, respectively (Table 2). As more copolymer was incorporated into the complexes, zeta potential values decreased and slight particle aggregation occurred. The size of the ternary complexes increased further in cell culture medium, whereas zeta potential values decreased. Both phenomena can be attributed to charge screening and complex aggregation induced by the presence of salts in cell culture medium [30,31]. Gel electrophoresis of the 4/1, 3/1 and 2/1 complexes revealed that ODN was not released from the complexes upon the addition of the MAA copolymer (data not shown).

3.2. Inhibition of PKC-α expression and cytotoxicity experiments

Fig. 2a shows the effect of treating A549 cells with ternary complexes (DOTAP/ODN/copolymer 4) at different [+/−] ratios on the expression level of PKC-α and cell survival. These complexes were compared to a positive control composed of DOTAP/DOPE/ODN (Fig. 2b). In both formulations, the charge ratio was adjusted by varying the amount of cationic lipids. For the ternary complexes, maximal antisense effect (60% PKC-α inhibition) was achieved at [+/−]=4/1. A further increase in cationic charge density did not result in enhanced antisense activity as the cytotoxic level of the complexes was reached. On

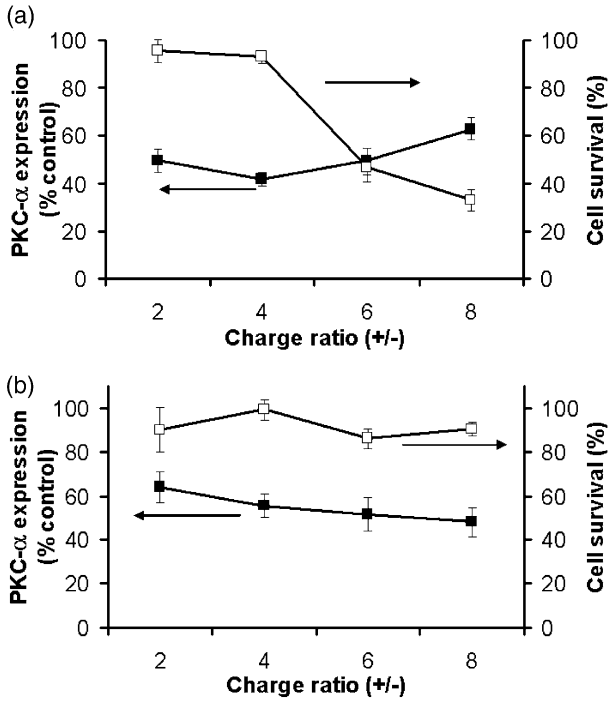

Fig. 2. Effect of charge ratio on the expression of PKC-α (closed squares) and cytotoxicity (open squares). A549 cells in serum-free medium were treated with 500 nM PDBu for 18 h and washed to remove it. Then, the cells were incubated for 4 h with either DOTAP/ODN/copolymer 4 (a) or DOTAP/DOPE/ODN (b) complexes. The ODN and copolymer concentrations were 200 nM and 40.8 μM, respectively. The [+/−] ratio was adjusted by varying the amount of cationic lipids. After the washing step, the cells were allowed to recover for an additional 20 h. Proteins were extracted, and immunoreactive PKC-α was determined by immunoblotting. Protein level is expressed as PKC-α % control (buffer treatment). Cell survival was determined using colorimetric MTT assay. Data are expressed relative to 100% cell survival (buffer treatment). Values are mean ± SD, n = 3–6 experiments.

the other hand, when the ODN was complexed to DOTAP/DOPE, a slight rise in antisense activity was observed at increasing [+/−] ratios, with 50% PKC-α inhibition achieved at [+/−]= 8/1. No cytotoxicity was apparent since the amount of cationic lipids was inferior to that of ternary complexes at an equivalent charge ratio (e.g. 15.2 vs. 80 μM at 4/1 [+/−], respectively).

To assess whether ODN activity was influenced by polymer concentration, A549 cells were treated with complexes containing increasing amounts of copolymer 4 (Fig. 3a). The 4/1 formulation was titrated with the copolymer to reach [+/−] ratios of 3 and 2. As shown in Fig. 3a-I, complexes with the lowest amount of polymer (i.e. 4/1) were as effective as those with the highest polymer content (i.e. 2/1). Surprisingly, a lower antisense effect was noted with complexes prepared with intermediate amounts of polymer (i.e. 3/1) ($P<0.05$). These results suggest that for ternary complexes, antisense activity is not directly correlated to polymer concentration. As discussed in the next section, other factors such as cationic charge density influence cellular internalization of the complexes and, consequently, delivery of the ODN to its site of action. In addition to cellular uptake, the surface charge also modifies the cytotoxicity of the complexes; increasing complex concentration by 2.5-fold relative to that used in Fig. 3 did not affect

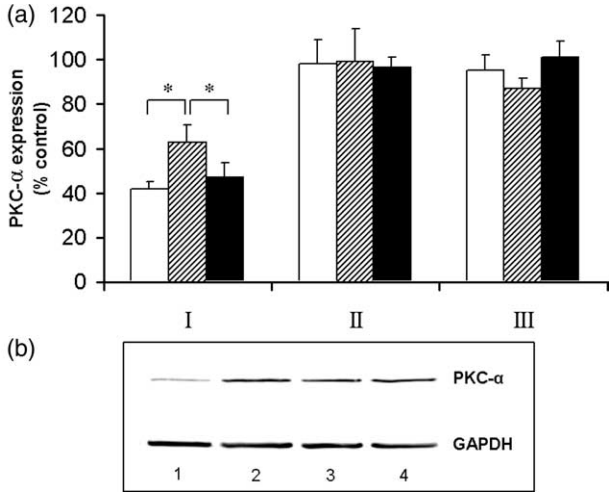

Fig. 3. Effect of copolymer concentration on PKC-α expression. (a) A549 cells in serum-free medium were treated with 500 nM PDBu for 18 h and washed to remove it. Then, the cells were incubated for 4 h with either ternary complexes prepared with antisense ODN (I), ternary complexes prepared with scrambled ODN (II) or complexes without copolymer (III). In all complexes, the ODN concentration was 200 nM. The [+/−] ratio was adjusted to 4/1 (open bars), 3/1 (grey bars) or 2/1 (solid bars) by varying the concentration of copolymer 4 in the ternary complexes, and the amount of cationic lipids in the DOTAP/ODN complexes. After the washing step, the cells were allowed to recover for an additional 20 h. Proteins were extracted, and immunoreactive PKC-α was determined by immunoblotting. Protein level is expressed as PKC-α % control (buffer treatment). Values are mean ± SD, $n = 3$–4 experiments. *$P < 0.05$. (b) Representative blot of cells treated with different complexes at a 4/1 charge ratio. Top band, immunoreactive PKC-α isozyme. Bottom band, immunoreactive GAPDH, demonstrating equal loading in each lane. Cells were incubated with: lane 1, ternary complexes prepared with the antisense ODN; lane 2, ternary complexes prepared with the scrambled ODN; lane 3, complexes without copolymer prepared with the antisense ODN; lane 4, H/D buffer.

A549 cell survival in the case of 2/1 complexes, whereas it induced cytotoxicity in the case of 4/1 complexes (data not shown). It is worth mentioning that control formulations that lacked polymer did not inhibit PKC-α expression at any charge ratio (Fig. 3a-III and b). Similarly, no antisense activity was detected with complexes formulated with scrambled ODN (Fig. 3a-II and b), and with complexes containing no ODN (data not shown). Indeed, this particular antisense ODN was previously shown to be sequence-specific [26,32].

3.3. Interaction of ternary complexes with cancer cells

Lipoplex uptake by A549 cells was first examined by flow cytometry. The complexes were prepared using fluorescein-labeled ODN and rhodamine-labeled copolymer 4. Control cells incubated with free ODN showed practically no uptake as the cell-associated fluorescence approximated that of untreated cells (Fig. 4b). In the absence of the copolymer, DOTAP/ODN lipoplexes interacted avidly with the cell membrane, as demonstrated by the strong cell-associated fluorescence (Fig. 4a). Interestingly, the addition of copolymer 4 to the lipoplexes produced a drastic drop in cellular uptake, despite the fact that the [+/−] ratios were identical to those of control lipoplexes (Fig. 4a). Cells incubated with 4/1 ternary complexes presented higher levels of both green (ODN) and

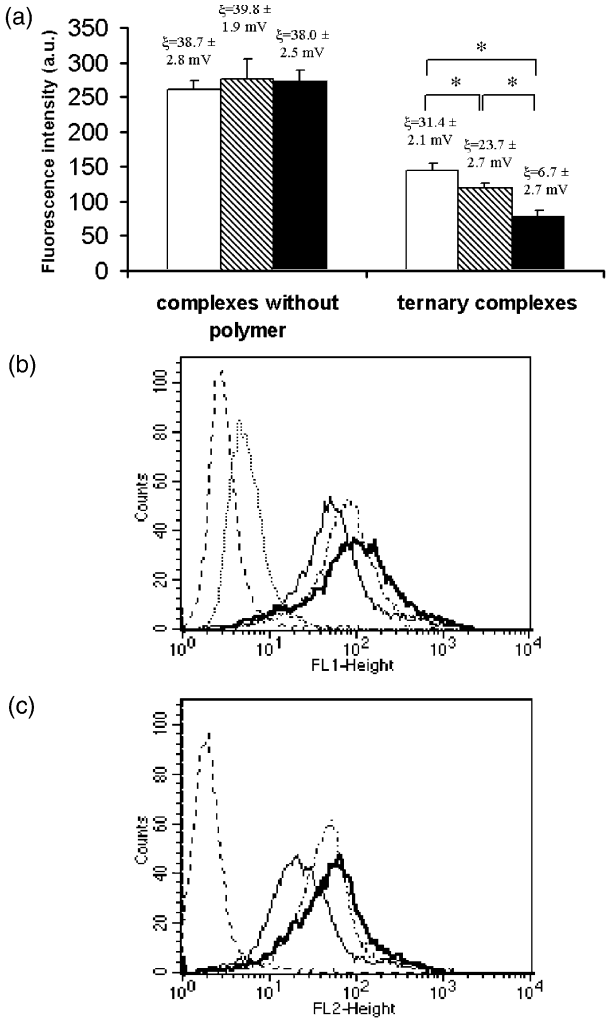

Fig. 4. Cellular uptake of lipoplexes. A549 cells were incubated with DOTAP/ODN complexes ± copolymer 4 using a fluorescein-labeled ODN and rhodamine-labeled copolymer. After a 4-h incubation period, the cells were rinsed and resuspended in PBS. The mean fluorescence intensity of 10,000 cells was measured by flow cytometry (see Section 2). Values are mean ± SD ($n = 3$ experiments). (a) Green fluorescence intensity of cells incubated with DOTAP/ODN complexes or ternary complexes. The ternary complexes were composed of DOTAP (80 μM), antisense ODN (200 nM), and copolymer 4 at 40.8 μM, 4/1 [+/−] (open bars), 57.0 μM, 3/1 [+/−] (grey bars) or 90.5 μM, 2/1 [+/−] (solid bars). For DOTAP/ODN complexes, the DOTAP concentration was kept constant (80 μM), and the charge ratio was adjusted by varying ODN concentration. Zeta potentials (± SD) measured in cell culture medium are indicated for each formulation. *$P < 0.05$. Green (b) and red (c) fluorescence of cells incubated with free ODN (dotted line), 4/1 (thick line), 3/1 (dashed-dotted line) or 2/1 (thin line) ternary complexes. Dashed lines represent unstained control cells. The results shown in (b) and (c) are representative of several independent experiments.

red (polymer) fluorescence than those exposed to the 3/1 and 2/1 formulations (Fig. 4b and c) ($P < 0.05$). In contrast, there were no marked differences between binding of the 4/1, 3/1 and 2/1 complexes with no polymer (Fig. 4a). These results may be explained by the surface charge of the complexes. As shown in Fig. 4a, the zeta potential values of the three DOTAP/ODN complexes were not noticeably distinct, as reported previously [33]. In contrast, for ternary complexes, they were markedly different between the three charge ratios.

To confirm that ternary complexes were internalized via an endocytic pathway, A549 cells were incubated with 4/1 or 2/1 complexes in the presence of cytochalasin B. This fungal metabolite depolymerizes the microfilaments of actin and blocks uncoated pit-mediated endocytosis [34]. Fig. 5 shows that treatment with cytochalasin B completely blocked the antisense activity of the lipoplex formulations ($P<0.05$). These data confirm that endocytosis is the major internalization pathway for ternary complexes. The mechanism of ODN delivery was examined further in the presence of bafilomycin A, an inhibitor of endosomal/lysosomal acidification that blocks the H^+-ATPase pump located in the vesicle membrane [35]. In principle, a higher endosomal pH should decrease the extent of protonation of MAA, which would, in turn, prevent polymer-triggered endosomal membrane disruption and cytoplasmic release of ODN. At a concentration of 200 nM, bafilomycin A strongly inhibited the antisense activity of the 2/1 complexes ($P<0.05$), whereas no effect was observed for the 4/1 system (Fig. 5). However, when the bafilomycin concentration was increased further to 500 nM, the 4/1 complexes exhibited a significant decrease in antisense activity. Thus, the acidification of endosomes is required for polymer-mediated ODN activity.

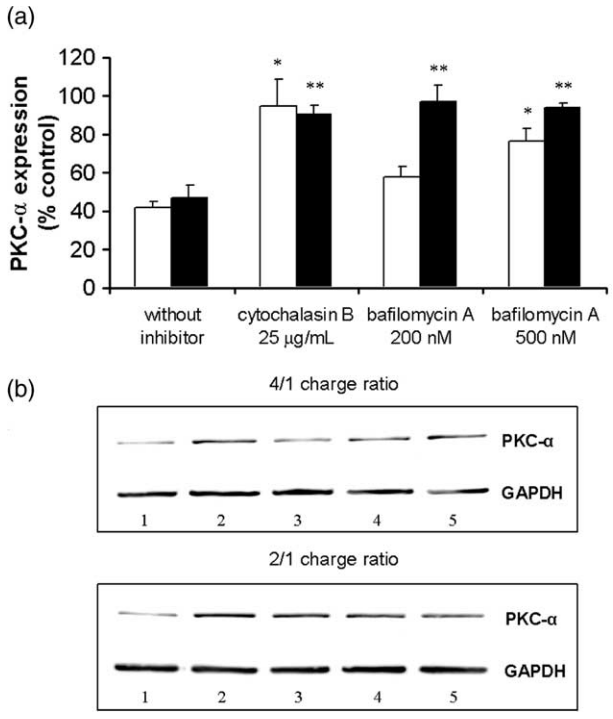

Fig. 5. Effect of metabolic inhibitors on PKC-α expression. (a) A549 cells in serum-free medium were treated with 500 nM PDBu for 18 h and washed to remove it. Before complex addition, the cells were preincubated for 15 min with cytochalasin B (25 μg/mL), or for 30 min with bafilomycin A (200 or 500 nM). Ternary complexes prepared with DOTAP (80 μM), antisense ODN (200 nM) and copolymer 4 at 40.8 μM, 4/1 [+/−] (open bars) or 90.5 μM, 2/1 [+/−] (solid bars) were then added. The inhibitors were present during the 4-h incubation period. After the washing step, the cells were allowed to recover for an additional 20 h. Proteins were extracted, and immunoreactive PKC-α was determined by immunoblotting. Protein level is expressed as PKC-α % control (buffer treatment). Values are mean ± SD ($n=3$). $P<0.05$ relative to cells incubated in the absence of inhibitor with 4/1 (*) or 2/1 (**) complexes, respectively. (b) Representative blots of cells treated with ternary complexes at a 4/1 or 2/1 [+/−] ratio in the presence or absence of metabolic inhibitors. Top band, immunoreactive PKC-α isozyme. Bottom band, immunoreactive GAPDH, demonstrating equal loading in each lane. Lane 1, no inhibitor; lane 2, cytochalasin B 25 μg/mL; lane 3, bafilomycin A 200 nM; lane 4, bafilomycin A 500 nM; lane 5, H/D buffer.

To gain insight into the intracellular trafficking of the ternary complexes, the cellular distribution of the ODN and copolymer was examined by laser scanning confocal microscopy (LSCM). After a 3-h incubation period, cells treated with free ODN showed multiple fluorescent intracellular vesicles, probably corresponding to endocytic compartments (Fig. 6a). Similar subcellular distribution was observed at an earlier time point (0.5 h), although the fluorescence intensity was lower (data not shown). In all cases, fluorescence remained weak and was never detected in the nucleus of the cells. The hydrophilicity and negative charge of ODN molecules are responsible for their limited internalization and poor ability to cross intracellular barriers. Association of the ODN with DOTAP (2/1 [+/−]) resulted in increased cellular fluorescence (Fig. 6b). Although the punctuate fluorescent pattern was still present, the vesicles were less abundant and larger in size. Some cells showed more diffuse fluorescence, indicating partial release of the ODN into the cytosol. Staining of nuclei was weak and, most of the time, undetectable. As for ODN/DOTAP/copolymer 4 (2/1 [+/−]) complexes, intense green fluorescence was detected in the cytoplasm after 0.5 h, suggesting rapid escape of ODN from endocytic organelles (Fig. 6c). At that stage, some ODN was already present in the nucleus. Conversely, the copolymer (red color) remained

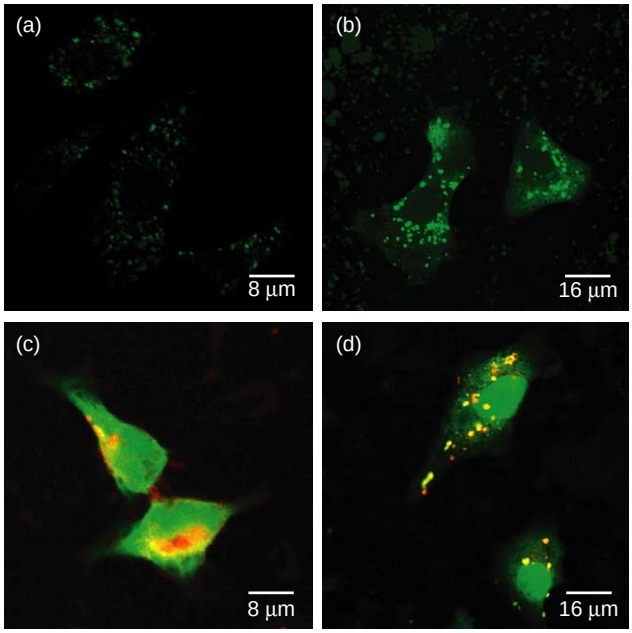

Fig. 6. Intracellular distribution of the ODN and copolymer. A549 cells grown on coverslips were incubated with free ODN for 3 h (a), DOTAP/ODN complexes for 3 h (7.6 μM/200 nM, 2/1 [+/−]) (b), or DOTAP/ODN/copolymer 4 complexes (80 μM/200 nM/90.5 μM, 2/1 [+/−]) for 0.5 h (c) or 3 h (d). Complexes were prepared using a fluorescein-labeled ODN and rhodamine-labeled copolymer. After the incubation period, the cells were rinsed with PBS and fixed in a 3% (w/v) paraformaldehyde solution. Coverslips were mounted on slides and examined by LSCM (see Section 2).

mainly localized in the globular compartments. Colocalization of ODN and the copolymer was also detected in most cells (yellow color), corresponding to complexes retained in intracellular organelles. After 3 h of incubation, cytoplasmic fluorescence was less pronounced, while bright green nuclear fluorescence could be seen, indicating almost complete diffusion of the ODN into the nucleus (Fig. 6d). In a few cells, weak and diffuse red fluorescence was detected in the cytoplasm (data not shown), which may correspond to low MW copolymer chains released from the endosomes (vide infra). Collectively, these findings demonstrate that cytoplasmic delivery of the ODN was facilitated by the copolymer, the latter being mostly retained in the endosomal/lysosomal compartments.

4. Discussion

Polyelectrolytes bearing pendant carboxylic acid group units undergo coil-to-globule conformational transition upon a decrease in pH [36]. Previous work showed that the sharpness of phase transition was related to carboxylate content [22]. Polymers with a high proportion of MAA units (typically more than 30%) exhibited abrupt phase transition and increased membrane destabilizing properties at acidic pH. Moreover, it was demonstrated that copolymerization of MAA with a non-ionizable hydrophobic monomer, such as ethyl acrylate, potentiated the membrane-lytic activity of the copolymers. The hemolysis data presented in this work revealed that further enhancing copolymer hydrophobicity by incorporating monomers bearing long alkyl chains had a dramatic impact on the destabilization of cell membranes. It was previously demonstrated by Murthy et al. [24,37] that increasing the alkyl chain length of monomeric units from 2 to 4 carbons enhanced the membrane-lytic activity of poly(alkylacrylic acid) homopolymers. Longer alkyl chains facilitate polymer insertion into the phospholipid bilayers, and destabilize them more readily. As shown in Fig. 1, the presence of a docecyl (C_{12}) group at the extremity of the polymeric chain (compare copolymers 3 and 4 vs. 1 and 2) improved its lytic properties substantially. By comparing copolymers 3 and 4, it can be seen that the addition of 10 mol% butyl methacrylate had a greater effect on hemolytic activity than 1 mol% dodecyl methacrylate, reflecting the higher overall hydrophobicity of copolymer 4.

The ability of copolymer 4 to efficiently destabilize cell membranes at pH values corresponding to those found in endosomes (Fig. 1) led us to hypothesize that it might represent an ideal candidate for enhancing the cytosolic delivery of fragile biomacromolecules, such as ODNs. Thus, copolymer 4 was complexed to an antisense ODN by means of electrostatic interactions with a positively-charged lipidic carrier (DOTAP). Ternary complexes prepared with either endosomolytic polymers or peptides have indeed been investigated as transfection aids for cationic lipoplexes loaded with plasmid DNA [20,38,39]. In the present work, a synthetic pH-sensitive polyanion was shown to increase the activity of an antisense ODN directed against PKC-α in A549 human lung carcinoma cells. PKC-α is an isozyme that functions as an intracellular receptor in the phosphoinositide signal transduction pathway. This pathway is often constitutively activated in tumor cells, and altered PKC-α regulation has been implicated in tumor promotion and carcinogenesis [23]. Therefore, inhibiting PKC-α expression might help to halt or slow tumor cell proliferation. A549 cells are known to strongly express PKC-α [32] and were thus an interesting model for the present study. DOTAP/ODN/copolymer 4 complexes having a 4/1 or 2/1 [+/−] ratio were highly active toward target protein (Figs. 2a and 3). Their efficiency was, in fact, comparable to that of a reference system in which the ODN was complexed to DOTAP/DOPE liposomes (Fig. 2b). DOPE is an inverted cone-shaped lipid with non-bilayer-forming properties that is thought to trigger cytosolic release by fusing to and/or disrupting the endosomal membrane [40]. Complexes lacking an endosomolytic agent (i.e. composed solely of DOTAP and ODN) were unable to inhibit protein expression under the conditions tested (Fig. 3a-III), reflecting their poor efficiency in mediating ODN escape from the endosome.

Although the use of pH-sensitive endosomolytic polymers for enhancing ODN delivery has been previously proposed by the group of Murthy et al. [24], studies investigating the actual mechanisms by which these agents act are lacking. In a recent work, Jones et al. [18] examined the pathway by which poly(alkylacrylic acid)s, such as poly(ethylacrylic acid) (PEAA) and PPAA, enhanced gene transfection. They suggested that the copolymers were mainly destabilizing late endosomes, and that sufficient polymer hydrophobicity was essential to mediate the intracellular delivery of DNA. However, the ternary complexes used in this study were not fully characterized. Besides, although the ability of the polymers to disrupt endosomes was examined in cultured cells, interaction of the polymeric lipoplexes with cells was not investigated.

In the present work, three ternary complex formulations that differed only in their respective copolymer content were systematically compared in terms of size, zeta potential, activity and cellular uptake. The MAA copolymer was shown to neutralize DOTAP particles, and substantially decrease the surface charge of the complexes (Table 2). It is fairly well-established that a residual positive charge is required for effective delivery, since lipoplexes bind to the cell surface by means of electrostatic interactions [9,41]. Flow cytometry experiments demonstrated that a more positive surface charge does increase the cellular uptake of complexes (Fig. 4). Indeed, the cellular association of the 3/1 and especially the 2/1 ternary complexes was decreased vs. their 4/1 counterparts. Although 2/1 ternary complexes were less charged than their 4/1 analogs, they were equally effective in inhibiting PKC-α expression (Fig. 3a-I). These results were surprising and imply that zeta potentials and, thus, extent of uptake are not directly predictive of complex efficiency. The equipotency of the 2/1 and 4/1 formulations may be ascribed to the greater efficiency of 2/1 ternary complexes in triggering ODN escape from the endosomes. Although these complexes are less taken up than the 4/1 lipoplexes, their higher polymer content may destabilize the endosomal membrane more efficiently. This hypothesis may also account for the lower efficiency of the intermediately-charged 3/1 complexes (Fig. 3a-I). Indeed, the

combined effects of decreased uptake (vs. 4/1 complexes, see Fig. 4) and insufficient copolymer concentration (vs. 2/1 complexes) may reduce the antisense activity of the 3/1 lipoplexes.

The activity of the ternary complexes was substantially decreased when the endosomal/lysosomal compartments were alkalinized by inhibiting the H^+ATPase pump (Fig. 5). Accordingly, acidification of endosomes is necessary for the efficient release of ODN into the cytoplasm. The lack of effect of bafilomycin toward 4/1 ternary complexes at low concentration (200 nM) vs. the 2/1 system indirectly corroborates the fact that the 4/1 complexes were taken up to a greater extent. Indeed, at 200 nM, there might not be enough bafilomycin to inhibit acidification of a larger number of endocytic vesicles. This hypothesis is supported by the almost complete inhibition of complex activity obtained when bafilomycin concentration was increased.

The mechanism by which titratable polyanions destabilize membranes at acidic pH is not fully elucidated. Confocal microscopy experiments have revealed that after only 30 min of incubation with ternary lipoplexes, the ODN largely diffuses in the cytoplasm. Since, the endocytosed material generally reaches the lysosomes after $T_{1/2} \approx 35$ min [42], it can be speculated that ODN was mostly released from the endosomes. A previous study [18], demonstrated that poly(alkylacrylic acid)s allowed the cytoplasmic delivery of both a small molecule (i.e. calcein, 622 g/mol) and a macromolecule (i.e. cathepsin B-GFP fusion protein, MW 64,000 g/mol) through the specific destabilization of late endosomes. In addition, it was hypothesized that these polyanions could sufficiently disrupt endosomes to effect the indiscriminant release of their content into the cytosol. However, since the copolymers were not directly tracked, it was not known whether they also escaped the endosomes. In the present work, copolymer 4 was probed in the cell after labeling with rhodamine. Although low amounts of polymer were detected in the cytoplasm of a few cells, the polyanion remained mostly sequestered in vesicular structures, probably corresponding to endosomal/lysosomal compartments (Fig. 6). Thus, it appears that polymer pH-dependent conformational change induces transient and small membrane defects to the endosomal membrane, allowing escape of the ODN (MW = 6988 g/mol). Chung et al. [43] have reported the formation of cation-selective channels through artificial membranes induced by PEAA in a pH-dependent manner. Since MAA copolymers are structurally related to PEAA, a similar mechanism of membrane destabilization may be hypothesized, although the exact nature of the defects remains to be determined. One could be tempted to attribute the weak and sporadic red cytoplasmic fluorescence seen in some cells (data not shown) to the complete rupture of the endosomal membrane. However, since the polymers used had a PI > 1.2, this phenomenon could also be ascribed to the partial endosomal escape of copolymer chains with low MW via pores formed in the endosomes. Obviously, more work is required to answer this question. Experiments conducted with fractionated copolymer samples with narrower PI could help clarify this issue.

This study revealed, for the first time, that the incorporation of a MAA copolymer into cationic lipid particles markedly increased the activity of an antisense ODN. Following internalization via endocytosis, endosomal acidification triggers coil-to-globule conformational change of the copolymer, endosomal membrane destabilization and release of the ODN into the cytosol. Important insights into the mode of interaction of polymeric lipoplexes were highlighted in this work. First, the polymer concentration of the complexes greatly modifies the surface charge which, in turn, was found to influence the extent of uptake by A549 cells. Second, decreased cellular uptake does not necessarily lead to a decline in complex activity, as long as a sufficient polymer concentration can be achieved in the endosomes. Third, within cells, the MAA copolymer remains mostly sequestered in endosomal/lysosomal compartments, which suggests that ODN release was mediated through the induction of transient membrane defects in the endosomal membrane. Future work will aim to determine the effect of the presence of serum in the cell culture medium on complex activity, cellular uptake and intracellular trafficking. Previously, it was found by Cheung et al. that the incorporation of the anionic polyanion PPAA into complexes made of plasmid DNA and DOTAP markedly enhanced the serum stability of the formulations in vitro [20] and in vivo [21]. In contrast, cationic liposomes containing DOPE failed to deliver efficiently ODN into cells in the presence of 10% serum [44,45] and their application to animals has been disappointing [46]. Mechanistic studies under physiological conditions are thus essential in order to contribute to the successful implementation of these copolymers in vivo.

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