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Research paper

Enhanced antisense efficacy of oligonucleotides adsorbed to monomethylaminoethylmethacrylate methylmethacrylate copolymer nanoparticles

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Abstract

The purpose of this study was the investigation of cationic nanoparticles as drug delivery systems for antisense oligonucleotides. Cationic monomethylaminoethylmethacrylate (MMAEMA) copolymer nanoparticles were prepared from *N*-monomethylaminoethylmethacrylate hydrochloride and methylmethacrylate. Oligonucleotides were adsorbed onto MMAEMA nanoparticles. Cell penetration was investigated in vitro with fluorescently labeled oligonucleotides and nanoparticles. Antisense effects of oligonucleotides adsorbed to MMAEMA nanoparticles were evaluated by sequence specific inhibition of ecto-5′-nucleotidase expression. The amount of enzyme expressed in PC12 cells was detected and quantified by immunocytochemistry using fluorescein isothiocyanate-labeled antibodies. Oligonucleotides were adsorbed to MMAEMA nanoparticles by the formation of ion-pairs between the positively charged secondary amino groups located on the particle surface and the anionic phosphodiester or phosphorothioate backbones of the oligonucleotides. Adsorption to nanoparticles led to an increased cellular uptake of oligonucleotides and to a significantly enhanced antisense efficacy of unmodified phosphodiester oligonucleotides as well as phosphorothioates. The results of the cell penetration and the antisense assay demonstrated that MMAEMA nanoparticles are promising carriers for oligonucleotide administration.

Keywords: Nanoparticle; Aminoalkylmethacrylate; Antisense oligonucleotide; Cell penetration; PC12 cell; Ecto-5′-nucleotidase

1. Introduction

Oligodeoxynucleotides (ODNs) are short segments of single-stranded DNA. They act as highly sequence-specific modulators of genetic expression [1] and therefore may be used for the treatment of viral diseases [2,3], cancer [4], or other diseases based on an uncontrolled overexpression and overproduction of proteins. However, oligodeoxynucleotides exhibit some drawbacks due to their low penetration through lipophilic cell membranes and their short half-life in biological fluids. At higher concentrations non-sequence-specific effects are often observed. The naturally occurring phosphodiester linkages in ODNs are very susceptible to degradation by serum and by endogenously occurring intracellular nucleases [5]. Therefore, most frequently phosphorothioates were used in antisense therapy. Phosphorothioates are structural modifications of oligonucleotides in which one of the non-bridging O-atoms of the phosphodiester backbone is replaced by a sulfur atom. These derivatives are more stable towards degradation caused by nucleases [6] but as polyanions they also penetrate across lipid bilayers poorly. An alternative approach to enhance penetration is the binding of natural ODNs to carrier systems [7–11]. In previous studies by our workgroup [12–14], cationic aminoalkylmethacrylate copolymer nanoparticles were developed as potential carrier systems for anionic drugs. Among different aminoalkylmethacrylate derivatives, monomethylaminoethylmethacrylate methylmethacrylate

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MMA, methylmethacrylate; MMAEMA, *N*-monomethylaminoethylmethacrylate; NP, nanoparticles; ODN, oligonucleotide; FITC, fluorescein isothiocyanate; FODN, 5′-rhodamine-labeled oligonucleotide; CLSM, confocal laser scanning microscopy.

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copolymer nanoparticles (MMAEMA) were found to be the most suitable carrier system for oligonucleotides [14]. These particles possessed a sufficient binding efficacy even at physiological pH under isotonic conditions and neither induced cytotoxic effects nor significantly influenced the integrity of biological membranes. The objective of the present study was to examine whether the extent of cell penetration and the subsequent antisense effects of oligonucleotides could be enhanced by adsorption to these nanoparticles. Cellular uptake of the oligonucleotide-nanoparticle formulations was investigated in cell cultures using rhodamine-labeled oligonucleotides. Subsequently, the cellular distribution of the entrapped oligonucleotides was determined by confocal laser scanning microscopy. The enhanced sequence-specific effect of oligonucleotides adsorbed to MMAEMA nanoparticles was assessed by the inhibition of the enzyme ecto-5′-nucleotidase at the protein expression level.

2. Materials and methods

2.1. Materials

Methylmethacrylate (MMA) as well as all other chemical compounds used for the preparation of nanoparticles and the subsequent loading studies were purchased from Merck (Darmstadt, Germany). The basic monomer monomethylaminoethylmethacrylate (MMAEMA) was not commercially available and, therefore, was synthesized as described previously [13]. Ammonium persulfate (APS) was a gift from Hüls AG (Marl, Germany). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and heat-inactivated fetal bovine serum were purchased from GIBCO (Eggenstein, Germany). FITC-concanavalin A, poly-D-lysine hydrobromide ($M_r > 30\,000$), as well as NGF-β were obtained from Sigma (Deisenhofen, Germany).

2.2. Oligonucleotides

The oligonucleotides used in this study were synthesized by MWG Biotech (Ebersberg, Germany). Antisense oligonucleotide unmodified and phosphorothioate-modified sequence: 5′-GTGAGCTCCCAGGATTTGGC-3′ (M_r 6149). Control oligonucleotide unmodified and phosphorothioate-modified sequence: 5′-ACGTTCCTCCTGCGGGAAG-3′ (M_r 5805). Cellular penetration was investigated with the 5′-rhodamine-labeled antisense oligonucleotide (FODN).

2.3. Preparation of nanoparticles

MMAEMA copolymer nanoparticles composed of 70% (w/w) methylmethacrylate (MMA) and 30% (w/w) *N*-monomethylaminoethylmethacrylate were produced by free radical polymerization as described previously [13,14]. Briefly, methylmethacrylate and the basic aminoalkylmethacrylate monomer were dissolved in a preheated mixture of acetone (10% w/w) and demineralized water at 78°C. At this temperature ammonium persulfate (0.03% w/v) was added as polymerization initiator. During the preparation the reaction mixture was stirred on a hot well plate (H + P Labortechnik GmbH, München) in closed beakers of 100 ml at 400 rpm. After 24 h, when polymerization was complete, acetone was evaporated for 1 h. The formed nanoparticles were concentrated to a polymer content of approximately 20% (w/v) using an ultrafiltration unit (model 402, Amicon, Witten, Germany) equipped with a Diaflo YC05 filtration membrane (Amicon) to obtain stable stock suspensions for storage. Prior to their use, the stock suspensions were purified by dialysis through a semipermeable membrane with an exclusion size of 12 000–14 000 Da (Dialysis Tubing-Visking, Medicell Ltd., London, UK).

2.4. Oligonucleotide loading

Oligonucleotides were incubated with MMAEMA nanoparticles for 2 h at 20–25°C using a 10 mM phosphate-buffered solution (pH 5.5) isotonized by addition of mannitol as adsorption medium. Rhodamine-labeled oligonucleotides (FODNs) used for cellular uptake studies, antisense oligonucleotides for the inhibition of ecto-5′-nucleotidase expression as well as control oligonucleotides (200 $\mu g\ ml^{-1}$) were adsorbed to MMAEMA nanoparticles at a ratio of 1:10 (w/w). The loading ratio was assessed after the separation of the non-adsorbed oligonucleotides from the particles by ultracentrifugation at $100\,000 \times g$ (Ultracentrifuge Optima L-80, Beckmann, Germany). Quantification of the oligonucleotides in the supernatant was performed by SAX-HPLC [11,15]. The amount of ODN bound to nanoparticles was calculated by the difference between the amount of ODN added for incubation and the ODN content in the supernatant after centrifugation.

2.5. Cell cultures

PC12 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal bovine serum, penicillin, streptomycin, and glutamine in a cell-culture incubator in 5% CO_2 at 37°C (BB6220, Haereus, Hanau, Germany) as previously described [16].

2.6. Inhibition of ecto-5′-nucleotidase expression

2.6.1. PC12 cell incubation with oligonucleotide formulations

Eight-well tissue culture chamber slides made of glass (Falcon, Becton Dickinson, NY) were precoated with a sterile aqueous solution of poly-D-lysine (5 $\mu g\ cm^{-2}$) for 30 min. Polylysine not adsorbed to the surface was removed after 30 min. The slides were washed three times using

sterile demineralized water and finally dried at room temperature. PC12 cells were seeded in these tissue culture chamber slides at a density of approximately 10 000 cells cm^{-2}. After incubation for 24 h with DMEM the oligonucleotide/nanoparticle formulations as well as unbound control oligonucleotides were added at a final oligonucleotide concentration of 1 μM in DMEM supplemented with nerve growth factor (NGF-β, 5 ng ml^{-1}). Again, cell incubation was continued for 24 h.

2.6.2. Quantification of 5′-nucleotidase expression by immunocytochemistry

For immunocytochemistry, PC12 cells were fixed with methanol followed by application of a polyclonal antibody prepared against 5′-nucleotidase isolated from the electric organ of the electric ray *Torpedo marmorata* (AS3) and a FITC-labeled secondary antibody (anti-rabbit-IgG, Propper Ltd., Smethwick, UK) as previously described [16,17]. The immunofluorescence was visualized using a confocal laser scanning microscope. All cells (approx. 60–100) in six regions of about 250 × 250 μm were scanned per well. Form each picture stack (60 slices) the layer with the highest fluorescence intensity was taken for quantification with an image processing software. A mean value was calculated from all six stacks. Untreated PC12 cells were taken as 100% value. Each experiment was repeated twice. Mean values and standard deviations were calculated from all three experiments. (Image Tool IT Version 1.27, Department of Dental Diagnostic Science, San Antonio, TX).

2.7. Cell penetration studies

A FODN/nanoparticle formulation and unbound FODNs were diluted with cell-culture medium to provide a 1 μM oligonucleotide concentration. PC12 cells were grown as described above and were incubated with these preparations in 16-well tissue culture chamber slides (Lab-Tek, Nunc, Naperville, IL). After 4 h of incubation the cells were washed three times with cold phosphate-buffered saline (PBS) (pH 7.4) to remove residual FODN-preparations and cell-culture medium. Cells were co-stained by incubation with FITC-concanavalin A (50 μg ml^{-1} PBS) for 1 min. Subsequently, the cells were rinsed three times with cold PBS (pH 7.4) and fixed with a 5% (w/w) paraformaldehyde solution in PBS for 10 min. After removing the cell-culture chamber, the slides were washed with PBS and the cells were embedded in Kaiser's glycerin–gelatin solution. Cellular distribution of FODNs was analyzed with a Leitz microscope (Leitz DM IRB, Wetzlar, Germany) and a TCS True Confocal Scanner (TCS 4D, Leica, Heidelberg, Germany) equipped with a krypton–argon laser. All optical sections were recorded with the same laser and detector settings. Images were processed using Scanware TCS version 5.1 and Imaris software (Bitplane AG, Zurich, Switzerland).

3. Results and discussion

3.1. Preparation of MMAEMA nanoparticles

MMAEMA copolymer nanoparticles were prepared from *N*-monomethylaminoethylmethacrylate hydrochloride (30% w/w, referred to the free base) and methylmethacrylate by a free radical emulsion polymerization in the presence of ammonium persulfate (APS) as an initiating reagent according to a previously described method [13]. A milky white homogeneous particle suspension was obtained with a yield above 95% of polymerized material as determined by gravimetric measurements. The nanoparticle suspension was purified by dialysis in order to remove residual monomers, initiator molecules, short-chain water-soluble polymers, and the hydrochloric acid which originated from the monomer hydrochloride. During the preparation process more hydrophilic monomer MMAEMA concentrated at the interface between the aqueous polymerization medium and the organic polymer solution. Consequently, monomethylaminoethyl groups were located at the surface of the nanoparticles while the hydrophobic methacrylic polymer backbone was entangled within the particle matrix. The secondary surface amino groups were protonated in aqueous solution between pH 3 and 10 due to their alkaline character and, thus, led to a positive particle surface charge (zeta potential in deionized water: +45.7 mV; 150 mM phosphate buffer (pH 5.5): +39.9 mV; PBS (pH 7.4): +22.7 mV) [14].

3.2. Loading of oligonucleotides

The antisense oligonucleotide used in our study was directed against a sequence of 20 bases of mature rat ecto-5′-nucleotidase [16]. A sequence complementary to the 3′ acceptor splice junction of herpes simplex virus (HSV 1) pre mRNA 4 which does not occur in PC12 cells was chosen as control oligonucleotide [2,3]. Loading of oligonucleotides to MMAEMA nanoparticles was achieved by the formation of ion-pairs between the positively charged secondary amino groups at the particle surface and the anionic phosphodiester and phosphorothioate backbones of the oligonucleotides. In a previous study it was shown that the loading efficiency of these carriers strongly depends on the pH and the ionic strength of the medium used [14]. The adsorption medium used in the present study was a 10 mM phosphate buffer (pH 5.5) isotonicated by the addition of mannitol because the preparations should be employed in the subsequent cell-culture studies. The loading ratios are presented in Table 1. The antisense oligonucleotides as well as the control oligonucleotides were adsorbed to MMAEMA nanoparticles to nearly the same extent. Compared to the unmodified phosphodiester oligonucleotides, a higher amount of phosphorothioate-modified oligonucleotides was bound to the nanoparticles. Presumably, adsorption of these more lipophilic oligonucleotide derivatives was facilitated by simple adsorption to hydrophobic particle domains

Table 1
Oligonucleotide loading to the surface of MMAEMA nanoparticles[a]

Sequence	Unmodified phosphodiester oligonucleotide		Phosphorothioate-modified oligonucleotide	
	Loading (%)	SD (%)	Loading (%)	SD (%)
Antisense	69.2	2.3	82.0	4.4
Control	61.0	2.2	76.4	2.2

[a] Two hundred micrograms per milliliter oligonucleotides were incubated with 2000 μg ml^{-1} using isotonic phosphate buffer (pH 5.5) as adsorption medium (mean ± SD, $n = 5$).

in addition to the above-described ionic interactions between the differently charged groups on the nanoparticles and the oligonucleotides.

The nanoparticles were neither further purified nor washed since most of the oligonucleotides were adsorbed to the particles and the free oligonucleotides showed no effect (see Section 3.4). Also, ultrasonication, which is frequently applied for homogenization after washing, cannot be used for oligonucleotide drugs because the DNA is degraded rapidly [18].

In comparison to unloaded MMAEMA nanoparticles, a negative surface charge (zeta potential in deionized water: −22.6 mV) was determined after oligonucleotide loading. These particles were further characterized in cell-culture medium yielding also negatively charged surfaces (zeta potential in DMEM: −14.6 mV).

3.3. Cell penetration

PC12 cells were chosen as a test model for the cell penetration investigation as well as for the ecto-5′-nucleotidase antisense assay. This cell line has already been employed in a previous study to determine the efficacy of oligonucleotides [16]. The cells were incubated with either 5′-rhodamine-labeled oligonucleotides (FODNs) and FODNs adsorbed on nanoparticles at a concentration of 1 μM for 4 h. Cells were co-stained by the FITC conjugated plant lectin concanavalin A to localize the cellular distribution of the FODNs. Images of 60 subsequent confocal sections were recorded and rearranged to a section view visualizing the horizontal and vertical distribution of fluorescent oligonucleotides inside the cells. Fig. 1C shows the fluorescence image for FODNs adsorbed to MMAEMA nanoparticles. The cells exhibited substantial intracellular fluorescence mostly in the form of dots. The fluorescence was located inside the cytoplasm. Some of the fluorescent dots were close to the plasma membrane. A more diffuse distribution was observed in the nucleus (Fig. 1C,D). As shown before, this cellular distribution of the oligonucleotide/nanoparticle complex appears to result from a phagocytotic uptake [14]. Although in earlier studies some evidence for a receptor-mediated endocytotic pathway of unmodified ODNs was reported [19,20], in our study cellular uptake of unbound oligonucleotides was found to be negligible (Fig. 1B). The pattern of fluorescence corresponds to that of untreated control cells (Fig. 1A). This phenomenon confirms that cellular uptake is a major problem in developing pharmaceutical application of oligonucleotides. However, MMAEMA nanoparticles significantly increased the intracellular level of oligonucleotides.

3.4. Inhibition of the ecto-5′-nucleotidase expression

In addition to the cell penetration studies, the antisense effect of oligonucleotides bound to nanoparticles was investigated. The inhibition of ecto-5′-nucleotidase was chosen as a model system because protein expression can be visualized easily, as described before [17]. Adenosine triphosphate (ATP) plays an important role as an extracellular signaling substance in the central and peripheral nervous system [21]. Growing neuronal cells have to rely on the reuse of the ATP metabolite adenosine. Therefore, extracellular ATP is hydrolyzed to adenosine by a surface-located enzyme cascade. The metabolic reaction from AMP to adenosine is catalyzed by ecto-5′-nucleotidase, an enzyme expressed in cultured neural cells. Ecto-5′-nucleotidase is also located at the surface of PC12 cells [16] which belong to a single-cell clonal line derived from a transplantable rat adrenal pheochromocytoma [22]. PC12 cells respond reversibly to NGF with the outgrowth of neurites and, therefore, may be used as a model system for the study of the physiological function of 5′-nucleotidase in neural cells [17]. Expression of ecto-5′-nucleotidase is essential for the survival of neural cells. It was shown that inhibition of this enzyme prevented neuritic differentiation and could cause cell death [16,23].

In a previous study the expression of ecto-5′-nucleotidase was inhibited by a phosphorothioate-modified antisense oligonucleotide probe to explore the physiological role of this enzyme in neural differentiation [16]. The oligonucleotide was directed against a sequence of 20 bases partially overlapping the signal peptide and the N-terminus of mature rat ecto-5′-nucleotidase. The differentiation of PC12 cells was subjected to sequence-specific inhibition at a level of 75% by the addition of 1 μM phosphorothioate. In the present study, MMAEMA nanoparticles were employed as colloidal carrier system to enhance the specific antisense effect of these oligonucleotides. Unmodified phosphodiester oligonucleotides as well as phosphorothioate oligonucleotides were adsorbed to the surface of MMAEMA nanoparticles and subsequently tested in this cell-culture system. Although it was not the purpose of our study to investigate the sequence-specific action of the antisense drug, we used an antiviral sequence against HSV I as control to exclude possible side effects of the oligonucleotide/nanoparticle complex. Ecto-5′-nucleotidase was detected by immunocytochemistry using a FITC-labeled antibody and was quantified by CLSM measurements. Fig. 2 shows the CLSM images of PC12 cells incubated with antisense oligonucleo-

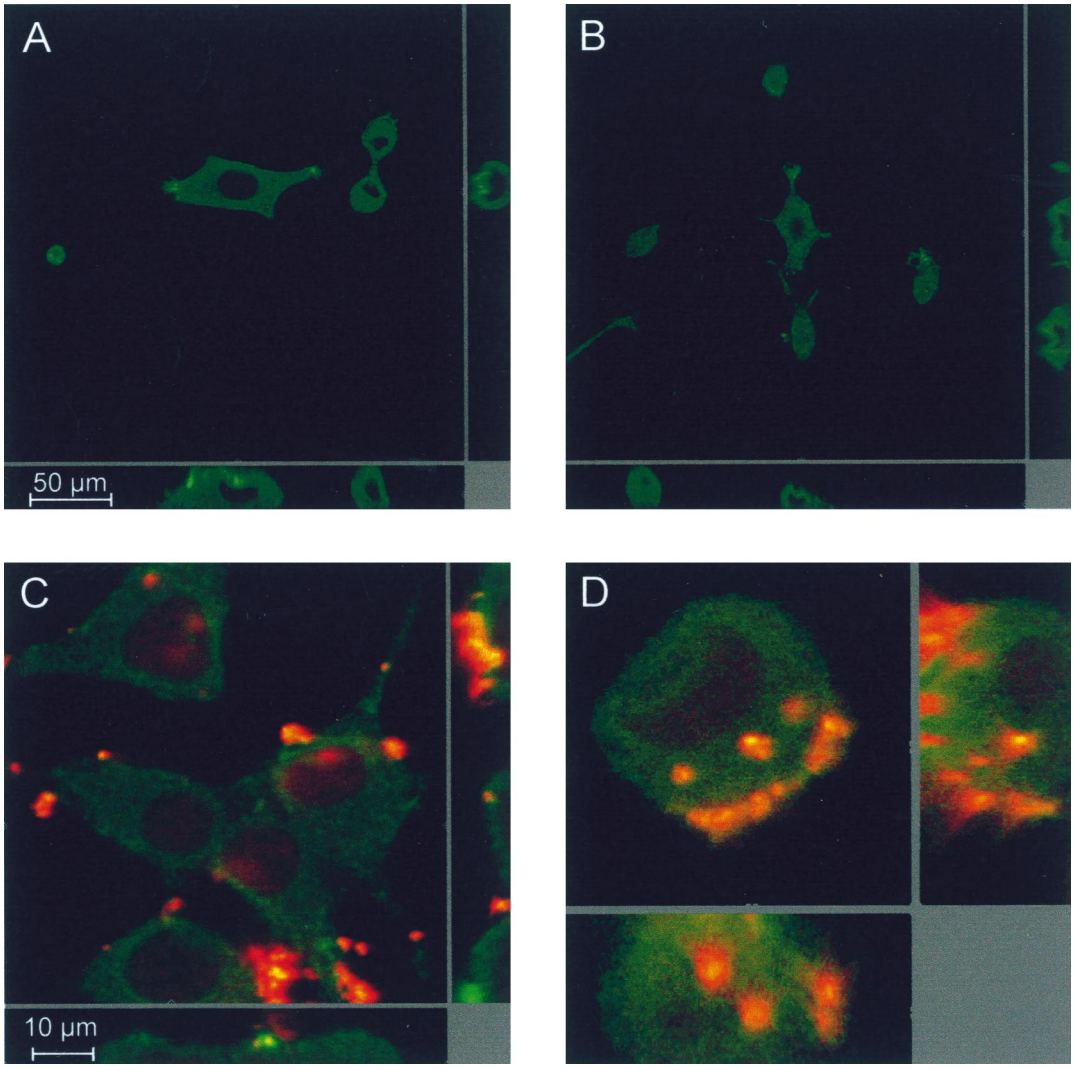

Fig. 1. Penetration of fluorescence-labeled oligonucleotide formulations into PC12 cells at 37°C and 5% (v/v) CO_2. (A) Untreated cells. (B) Incubation with 1 μM rhodamine labeled oligonucleotide without nanoparticles for 4 h. (C) Incubation performed with 6.15 μg ml^{-1} (1μM) rhodamine labeled oligonucleotide adsorbed to 61.5 μg ml^{-1} nanoparticles for 4 h. (D) Cellular distribution of oligonucleotides with nanoparticles (zoom factor 2.5×) All pictures are combined CLSM sections of 60 slices showing horizontal and vertical distribution of the fluorescence as section view. Cells were co-stained with FITC-labeled concanavalin A.

tides adsorbed to MMAEMA nanoparticles and corresponding controls. Images were recorded in the optical section exhibiting the brightest fluorescence. Incubation with antisense oligonucleotides adsorbed to nanoparticles led to a reduction in enzyme expression which resulted in a significant decrease in fluorescence intensity (Fig. 2B) in comparison to untreated control cells (Fig. 2A) and cells treated with unbound oligonucleotides (Fig. 2D). The incubation with anti-HSV I oligonucleotides bound to nanoparticles resulted only in a mild reduction of immunofluorescence (Fig. 2E).

In order to discuss the downregulation of a protein it is important to know the half-life of the protein. The ecto-5′-nucleotidase reaches the cell surface within 20–30 min after protein expression and its half-life is on the order of about 30 h. This has been shown previously in rat hepatoma cells as well as in PC12 cells [24]. Therefore, ecto-5′-nucleotidase can be blocked with antisense oligonucleotides during a 24–96 h incubation [16]. However, a prolonged incubation (>48 h) of PC12 cells with the antisense oligonucleotide led to an increased detachment from the culture slide due to the involvement of ecto-5′-nucleotidase in cell/matrix interactions [21]. For that reason, in our study we followed the onset of the antisense oligonucleotide effect within the first 24 h to prevent cell detachment.

Surprisingly, the fluorescence intensities of cells incubated with control nanoparticles without oligonucleotides were above the values observed for the untreated cell controls. Additionally, our study revealed a positive effect of nanoparticles on the differentiation of PC12 cells (formation of neurites, Fig. 2C). The positive effect on cell differentiation may be due to an enhanced transport of growth

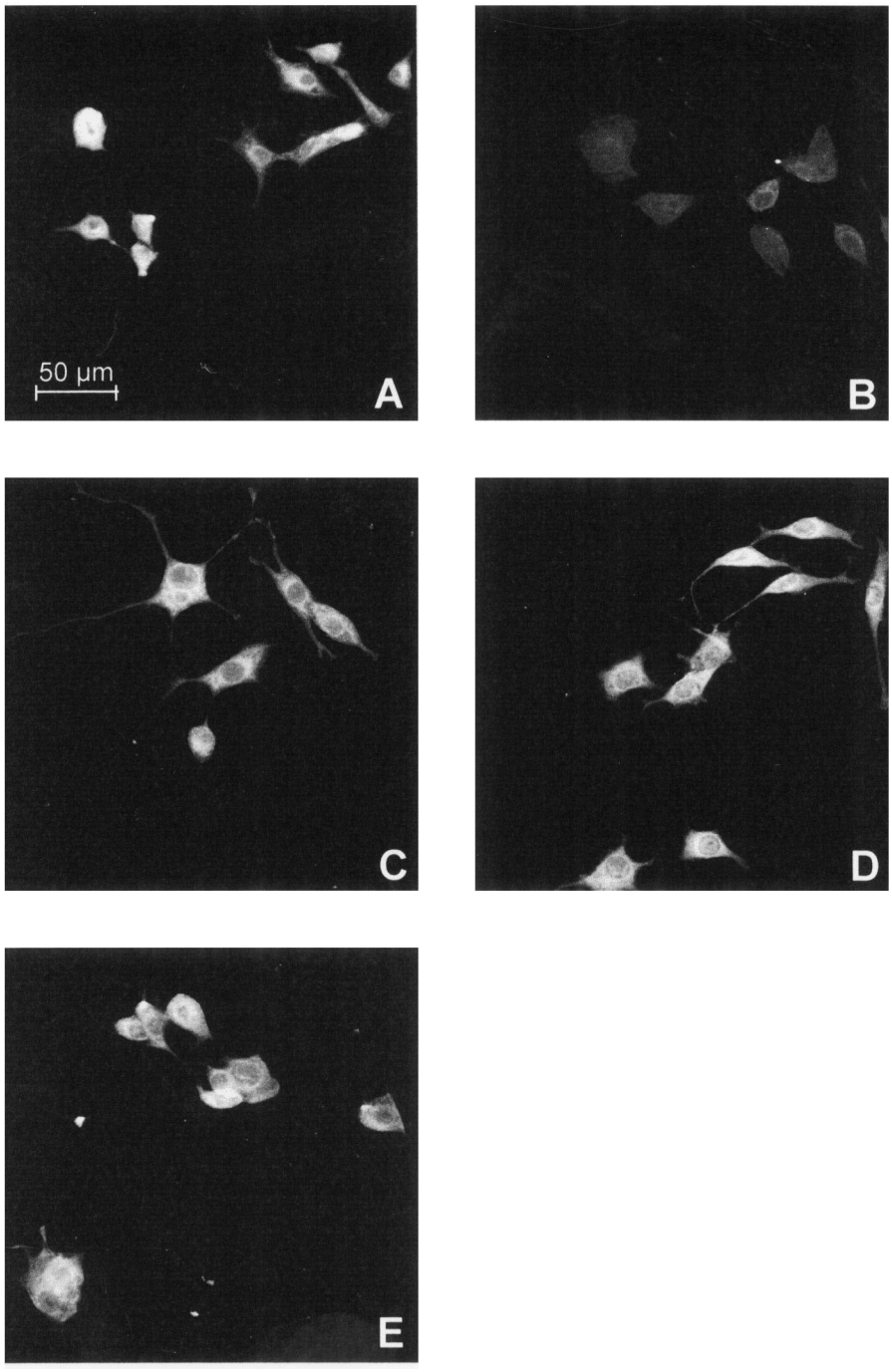

Fig. 2. Inhibition of ecto-5′-nucleotidase expression in PC12 cells. Detection of the nucleotidase was performed after 24 h by immunocytochemistry using a FITC-labeled secondary antibody. CLSM images were recorded in the optical section exhibiting the brightest fluorescence. (A) Cell control. (B) One micromolar antisense oligonucleotide adsorbed to MMAEMA nanoparticles at a ratio of 1:10. (C) MMAEMA nanoparticle control. (D) Incubation of PC12 cells with 1μM antisense oligonucleotide without nanoparticles. (E) 1 μM control oligonucleotide/MMAEMA nanoparticles. Bar = 50 μm (A–E).

factors with nanoparticles into PC12 cells. Growth factors are present in the cell-culture medium and can be adsorbed to nanoparticles like other proteins. In a previous study it was already shown that a broad range of blood serum proteins are adsorbed onto the surface of nanoparticles after their application [25].

The effect of different oligonucleotide formulations on the expression of ecto-5′-nucleotidase is further demonstrated in Fig. 3. The method is based on a semi-quantitative analysis of microscopic pictures. Although the results are more qualitative rather than quantitative, they support the pure microscopic visualization of the ecto-5′-nucleotidase by immunocytochemistry. In agreement with the data presented in Fig. 2, no significant antisense effects were

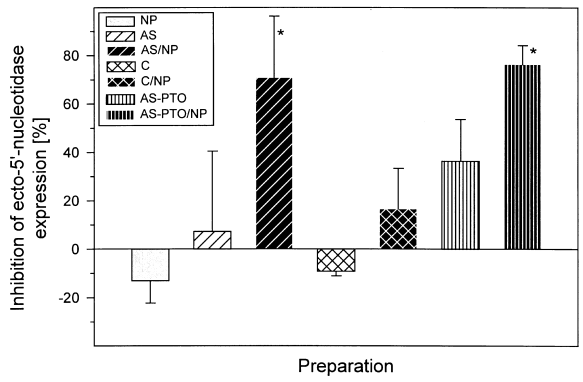

Fig. 3. Inhibition of ecto-5′-nucleotidase expression in PC12 cells. NP, MMAEMA nanoparticle control; AS, antisense oligonucleotide; AS/NP, antisense oligonucleotide adsorbed to MMAEMA nanoparticles; C, control oligonucleotide; C/NP, Control oligonucleotide adsorbed to MMAEMA nanoparticles; AS-PTO, antisense phosphorothioate; AS-PTO/NP, antisense phosphorothioate adsorbed to MMAEMA nanoparticles. Statistics: mean values, $n = 3$; *Significantly different from unbound oligonucleotides, t-test, $P = 0.05$).

observed for unmodified phosphodiester antisense oligonucleotides (7.3% inhibition). Phosphorothioates showed moderate inhibition (36.4%) but antisense effects were significantly enhanced by the adsorption to MMAEMA nanoparticles for both phosphodiester (70.9%) and phosphorothioate antisense oligonucleotides (76.5%). The inhibition of ecto-5′-nucleotidase expression could be attributed to an antisense mechanism because comparable preparations of the control oligonucleotide revealed only low effects (Figs. 2E and 3), and our results confirmed data from an earlier study which demonstrated the sequence specificity of these oligonucleotides [16].

Toxic effects were not observed with PC12 cells. In general, the toxicity of MMAEMA nanoparticles was investigated in vitro in one of our earlier studies with an MTT assay and a hemolysis assay. In comparison to other positively charged TMAEMA (trimethylaminoethylmethacrylate) nanoparticles the MMAEMA derivative showed no significant cellular toxicity [14].

4. Conclusions

MMAEMA copolymer nanoparticles exhibited a positive surface charge. This surface charge facilitated the adsorption of negatively charged oligonucleotides by ion-pair interactions. Adsorption to MMAEMA nanoparticles enhanced the cellular uptake of oligonucleotides. Once internalized into the cells, both unmodified and phosphorothioate-modified oligonucleotides exhibited significantly enhanced antisense effects in comparison to unbound oligonucleotides. In conclusion, our results of the cell penetration and the antisense assay demonstrated that MMAEMA nanoparticles are potential carriers for oligonucleotide administration which should be included in further in vitro and in vivo antisense studies.

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