Adsorption of Oligonucleotides onto Polyisohexylcyanoacrylate Nanoparticles Protects Them Against Nucleases and Increases Their Cellular Uptake

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Oligonucleotides can be adsorbed on polyisohexylcyanoacrylate nanoparticles in the presence of hydrophobic quartenary ammonium salts. Oligonucleotides bound to nanoparticles are protected from nuclease attack both in buffer and in cell culture media. Cellular uptake of oligonucleotides is increased when they are adsorbed onto nanoparticles as a result of the capture of nanoparticles by an endocytic/phagocytic pathway. Intracellular stability towards nucleolytic degradation is increased in the presence of nanoparticles. These results show that nanoparticles can be considered as convenient carriers for the protection and delivery of oligonucleotides to cells.

KEY WORDS: oligodeoxynucleotides; nanoparticles; polyalkylcyanoacrylate; cellular uptake; oligonucleotide hydrolysis.

INTRODUCTION

Regulation of gene expression by antisense oligonucleotides has been described *in vitro* and in cellular systems (for a review see reference 1). However many problems are faced when oligonucleotides are used in cell culture systems or *in vivo*. Oligonucleotides are rapidly degraded in biological fluids (2–3) and in cells (4–5) by exo- or endonucleases which hydrolyse the phosphodiester linkages. The half-life of oligonucleotides in blood is very short, of the order of a few minutes (3). Degradation of oligonucleotides in the plasma of various mammalian species was found to be mainly mediated by 3'-exonucleases (2). Such an activity was also found in media containing foetal calf-serum (5). Investigations of the mechanism of cellular uptake of oligonucleotides have suggested that receptor-mediated endocytosis might be involved (6). As visualised by fluorescence microscopy, internalised oligonucleotides accumulate in intracellular vesicles (endosomes) where they could be degraded (6). When directly microinjected into the cytosol of cells, fluorescent oligonucleotides accumulate rapidly in the nucleus (7). These results and the biological efficacy of oligonucleotides towards cellular targets which are localised either in the cytoplasm or in the nucleus indicate that a small but sufficient amount of oligonucleotides escapes from endosomes/lysosomes to reach the cellular compartments where they exert their activity. In order to observe the desired biological effect, concentrations of oligonucleotides in the micromolar range are needed in cell culture media.

Different strategies have been developed to protect oligonucleotides from degradation and to increase their transport into target cells. These include the synthesis of chemically-modified oligonucleotides (8) or, the association of oligonucleotides with drug carriers such as liposomes (9–10) or polymers such as poly-L-lysine (11). The most widely used modifications involve the replacement of one of the oxygen atoms of the phosphodiester group by a sulfur atom (phosphorothioates) or by a methyl group (methylphosphonates). Oligonucleotides can also be synthesized with the α-anomers of the nucleosides instead of the natural β-anomers. Such modifications have greatly increased the resistance of oligonucleotides toward digestion by nucleases (8). Phosphorothioates have been reported to competitively block the cellular uptake of natural oligomers and thus may enter cells by the same mechanism (12). It was reported that uptake of methylphosphonates involved a fluid phase endocytosis, distinct from the uptake pathway of phosphodiesters (13). α-oligonucleotides are taken up by cells and are localized in endocytic vesicles as previously observed with natural β-oligonucleotides (unpublished observations from our laboratory).

Oligonucleotides covalently linked at their 5' and/or 3' extremities with polylysine are more resistant toward nucleases and have demonstrated greater cellular uptake (11). Cellular delivery of oligonucleotides with liposomes has greatly increased their biological efficacy (9–10). More recently, oligonucleotides covalently linked to cholesterol were associated with low density lipoproteins. This association increased the half-life of oligonucleotides in plasma of injected rats and diminished their biological excretion (14).

We have recently demonstrated that oligonucleotides can be associated with polymeric biodegradable drug carriers, such as polycyanoacrylate nanoparticles (15). These particles developed earlier by Couvreur *et al.* (16) are produced by emulsion polymerization of cyanoacrylic monomers in acidic medium. These carriers have been shown to enhance the *in vivo* activity of some antitumor drugs (17). Association of oligonucleotides with preformed nanoparticles was achieved by formation of ion pairs between negatively charged oligonucleotides and hydrophobic cations such as quaternary ammonium salts (15).

In this report, we compare the half-life of oligonucleotides free or adsorbed to nanoparticles of polyisohexylcyanoacrylate (PIHCA)[5] in a medium containing snake

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[5] Abbreviations used: PIHCA, polyisohexylcyanoacrylate; PACA, polyalkylcyanoacrylate; CTAB, cetyltrimethylammonium bromide; PDE, phosphodiesterase; $d(T)_{16}$, hexadecathymidylate; TPA, 12-0 tetradecanoylphorbol-13-acetate; 15mer, pentadecamer; PBS, phosphate buffer saline; LDH, lactate deshydrogenase; FCS, foetal calf serum; AMP, adenosine monophosphate.

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venom phosphodiesterase (a 3'-exonuclease) and in cell culture medium. We also compare the uptake of free oligonucleotides to oligonucleotides adsorbed to PIHCA nanoparticles using a macrophage-like cell line U937. Polycyanoacrylate nanoparticles might be useful in promoting cellular uptake via endocytosis or phagocytosis, as demonstrated in a number of cell lines (18,19). Our results show that adsorption of oligonucleotides onto PIHCA nanoparticles increases both their resistance to extracellular and intracellular nucleases and their intracellular uptake.

MATERIALS AND METHODS

Oligonucleotide Labelling

5'-dephosphorylated hexadecathymidylates (Pharmacia) and a pentadecamer of sequence 5'CATTTTGATTACTGT3' (Eurogentec) which is complementary to vesicular stomatitis virus N protein mRNA initiation site (9) were used in this work. They were labelled at their 5'-end using T4 polynucleotide kinase (Boehringer Mannheim) and ^{32}P-ATP (Amersham). The internal radiolabelling of a pentadecamer of sequence 5'CATTTTGATTACTGT3' was achieved under the following conditions: an 8-mer of sequence 5'ATTACTGT3' (20 picomoles) was 5' end-labelled and then ligated to an unlabelled 7-mer of sequence 5'CATTTTG3' (20 picomoles) by incubating overnight at 10°C with 40 picomoles of a complementary 20-mer (5' GCACAGTAATCAAAATGCTA3') and 2 u/μl of T4 DNA ligase (Promega). The 15-mer was then purified on a denaturing gel (20% polyacrylamide-7M urea).

Nanoparticle Preparation

Polyisohexylcyanoacrylate (PIHCA) nanoparticles (10 mg/ml) were obtained by adding isohexylcyanoacrylate monomer (SOPAR) to a solution containing hydrochloric acid (10 mM, pH 2) and a dispersant agent, dextran 70 (1% w/v), in distilled water. The polymerisation of isohexylcyanoacrylate monomers was achieved as previously described (15). It was complete after 6 hours.

Adsorption of Oligonucleotides to PIHCA Nanoparticles

The PIHCA suspension was neutralized to pH 7 with NaOH and diluted in 10 mM Tris-HCl buffer containing 1% (w/v) dextran 70 (Fluka) and 0.4% (w/v) poloxamer 188 (Calbiochem). Oligodeoxynucleotide adsorption onto nanoparticles was achieved, as previously described (15), by adding oligomers to the polymeric suspension in the presence of cetyltrimethylammonium bromide (CTAB), as ion-pairing agent. In the conditions used, oligonucleotides were totally adsorbed to PIHCA nanoparticles, with 1 μmole of oligomer adsorbed per gram of polymer.

Stability of Oligonucleotides in the Presence of Phosphodiesterase (Snake Venom) and in Cell Culture Medium

5'-end-labelled oligothymidylates were mixed with sufficient unlabelled oligomers to give a final concentration of 0.5 μM. Degradation experiments were carried out at 37°C in PDE buffer (0.1 M Tris-HCl, pH 7.4, 10 mM $MgCl_2$) containing various concentrations of snake venom phosphodiesterase (Boehringer Mannheim) or in RPMI 1640 medium (GIBCO) supplemented with 5% foetal calf serum (Seromed). To the medium was added $d(T)_{16}$ alone, $d(T)_{16}$ and CTAB (150 μM) or $d(T)_{16}$ adsorbed onto PIHCA nanoparticles (0.5 mg/ml) in the presence of CTAB (150 μM). At the desired time, degradation was stopped by heating at 80°C for five minutes. For those samples containing nanoparticles it was necessary, prior to gel analysis, to degrade the polymeric carrier by adjusting the medium to pH 12 with NaOH and incubating for two hours at 37°C, as previously described (15). Oligonucleotides recovered by phenol extraction and ethanol precipitation were then analysed on a 20% polyacrylamide-7 M urea sequencing gel. The gel was autoradiographed at −70°C using Fuji (X-ray) films, the bands excised and analysed quantitatively by measuring the radioactivity by scintillation counting.

Cell Cultures

The human histiocytic lymphoma cell line U937 was previously characterised by Sundström and Nilsson (20). The cells were cultured in RPMI 1640 supplemented with 10% of heat denatured foetal calf serum (60°C, 30 min) and antibiotics (100 UI/ml penicillin, 100 μg/ml streptomycine, Flow) at 37°C in 5% CO_2 air/atmosphere humidified incubator. These cells were able to differentiate into macrophage-like cells by addition to the medium of phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA).

Cytotoxicity of Empty Nanoparticles or Nanoparticles Loaded with Oligonucleotides

U937 cells were seeded (1.5×10^6 cells) into 35 mm plastic petri dish with coverslip containing 2 ml of medium supplemented with 50 nM TPA (Sigma). After a 48 hour incubation of cells with TPA, these tumor cells became adherent and multiplication was abolished (21). As determined by protein assay (Biorad), an average of 70% of cells became adherent after this treatment. After removal of the medium, and washing of the cells with PBS, 1 ml of medium was added with various volumes (0, 5, 10, 20, 40 and 80 μl) of nanoparticle suspension (1 mg/ml), empty or loaded with a 15-mer oligonucleotide (1 μmole/g polymer), in the presence of CTAB (600 μM). After 24 hours of incubation at 37°C, cell cytotoxicity was evaluated by determining the leakage in cell medium of lactate dehydrogenase (LDH), a cytosolic enzyme. LDH leakage was expressed as a ratio of LDH activity in medium compared to total LDH activity (medium + cells) as reported by Kante *et al* (22). LDH activity was determined by a colorimetric method (Sigma) based on the fact that LDH catalyses the following reaction: Pyruvic acid + NADH⇒Lactic acid + NAD. The amount of pyruvate remaining after incubation with cell extract or medium was quantitated after its reaction with 2,4 dinitrophenylhydrazine to form an intensly colored "hydrazone" which has a peak of absorbance at 400–500 nm. This amount is inversely proportionnal to LDH activity. Aliquots of cell extract or medium were incubated with 0.75 mM sodium pyruvate and 1.28 mM NADH in a total volume of 1 ml. After 30 minutes of incubation at 37°C, 1 ml of 2,4-dinitrophenylhydrazine (0.2 g/l) in 1N HCl was added for 20 minutes at

25°C. Then 10 ml of 0.4 N NaOH was added and absorbance was measured at 464 nm. Quantification of sodium pyruvate was done by adding various known amount of this compound to 2,4-dinitrophenylhydrazine.

Oligonucleotide Uptake by the Human Macrophage-like Cell Line U937

For oligonucleotide uptake assays, U937 cells (1.5×10^6 cells/dish) were incubated for various times with 5'-end-labelled or internally labelled oligonucleotides at the final concentration of 5 nM (4×10^6 cpm/dish), free or bound to PIHCA nanoparticles (5 μg/ml) in the presence of CTAB (3 μM). At the desired time, the culture medium was removed and the cellular layer was washed several times with 2 ml of PBS until radioactivity in the medium was less than 10% of cell radioactivity (usually 4 to 5 washes were sufficient).

Oligonucleotides were extracted from cell lysates using two different protocols. In the first one, cells were lysed at 4°C with 0.1% SDS (w/v) in RSB buffer (10 mM tris HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$), in the presence of an excess of non-labelled oligonucleotide (1 μM), or were extracted from nuclear and extranuclear fraction as previously described (4). Then centrifugation of cellular lysate was performed at 1300 g to separate the nuclear fraction (pellet) from extranuclear fraction (supernatant) containing endocellular vesicles. Samples were then analysed on a 20% polyacrylamide-7M urea gel and autoradiographed. The effect of temperature on cellular uptake of internally-labelled 15-mer (25 nM) adsorbed on nanoparticles (5 μg/ml) was tested after incubating the cells at 4 or 37°C for 6 hours.

RESULTS AND DISCUSSION

Stability of Oligonucleotides in the Presence of Phosphodiesterase and in Cell Culture Medium

The kinetics of degradation of 5'-γ^{32}P-labelled hexadecathymidylate (0.5 μM) at 37°C was tested at two concentrations (0.1 and 0.5 μg/ml) of snake venom phosphodiesterase (a 3'-exonuclease) and in cell culture medium supplemented with 5% foetal calf serum (FCS). The autoradiograms of oligonucleotides are shown in Figures 1 and 2, respectively. Quantitation of oligonucleotide degradation was carried out as described in materials and methods. The half-lives (min.) of free oligonucleotides, complexed to CTAB, or adsorbed to PIHCA nanoparticles are compared in Table I.

In the presence of 0.1 μg/ml phosphodiesterase, free oligonucleotide was rapidly degraded (Figure 1, Lane A). Its half-life was determined to be about two minutes (Table I). After increasing enzyme concentration five fold, degradation of free oligonucleotide was too rapid to be determined accurately (Table I). As previously reported (15), oligonucleotides were protected from degradation by phosphodiesterase (0.1 or 0.5 μg/ml) when complexed to CTAB (Figure 1, Part B; Table I). Protection could be due to the formation of ions pairs between the negatively charged phosphate groups of oligonucleotides and the hydrophobic cations. Inactivation of the enzyme due to its interaction with hydrophobic cations cannot be excluded. Interaction of cationic detergents (such as CTAB) with proteins (such as bovine serum albumin) has been reported previously (23). When adsorbed onto PIHCA nanoparticles, oligonucleotides were totally protected against enzymatic degradation even after a five hour incubation with the enzyme (Figure 1, Lane C and Table I). In addition, we determined that about 90% of the oligonucleotide still remained intact after overnight incubation with the enzyme (0.1 μg/ml). This result suggests that phosphodiester linkages or the 3'-end of oligonucleotides were not accessible to enzymatic attack.

We then compared the stability of oligonucleotides either free or adsorbed to nanoparticles in cell culture medium whose serum nucleases were inactivated or not by heating

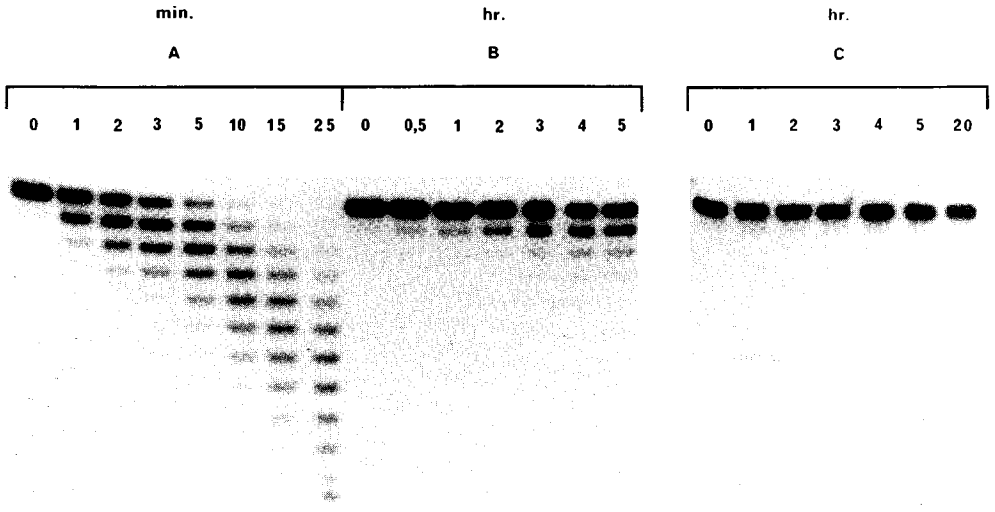

Fig. 1. Kinetics of oligonucleotide degradation by snake venom phosphodiesterase. $d(T)_{16}$ (0.5 μM) free (part A), complexed with 150 μM CTAB (part B) or adsorbed to PIHCA nanoparticles (0.5 mg/ml) in the presence of 150 μM CTAB (part C) was incubated at 37°C with 0.1 μg/ml phosphodiesterase. After various incubation times, indicated at the top of the gel, enzymatic reaction was stopped and degradation products were analysed on a 20% polyacrylamide-7M urea sequencing gel. The gel was then autoradiographed.

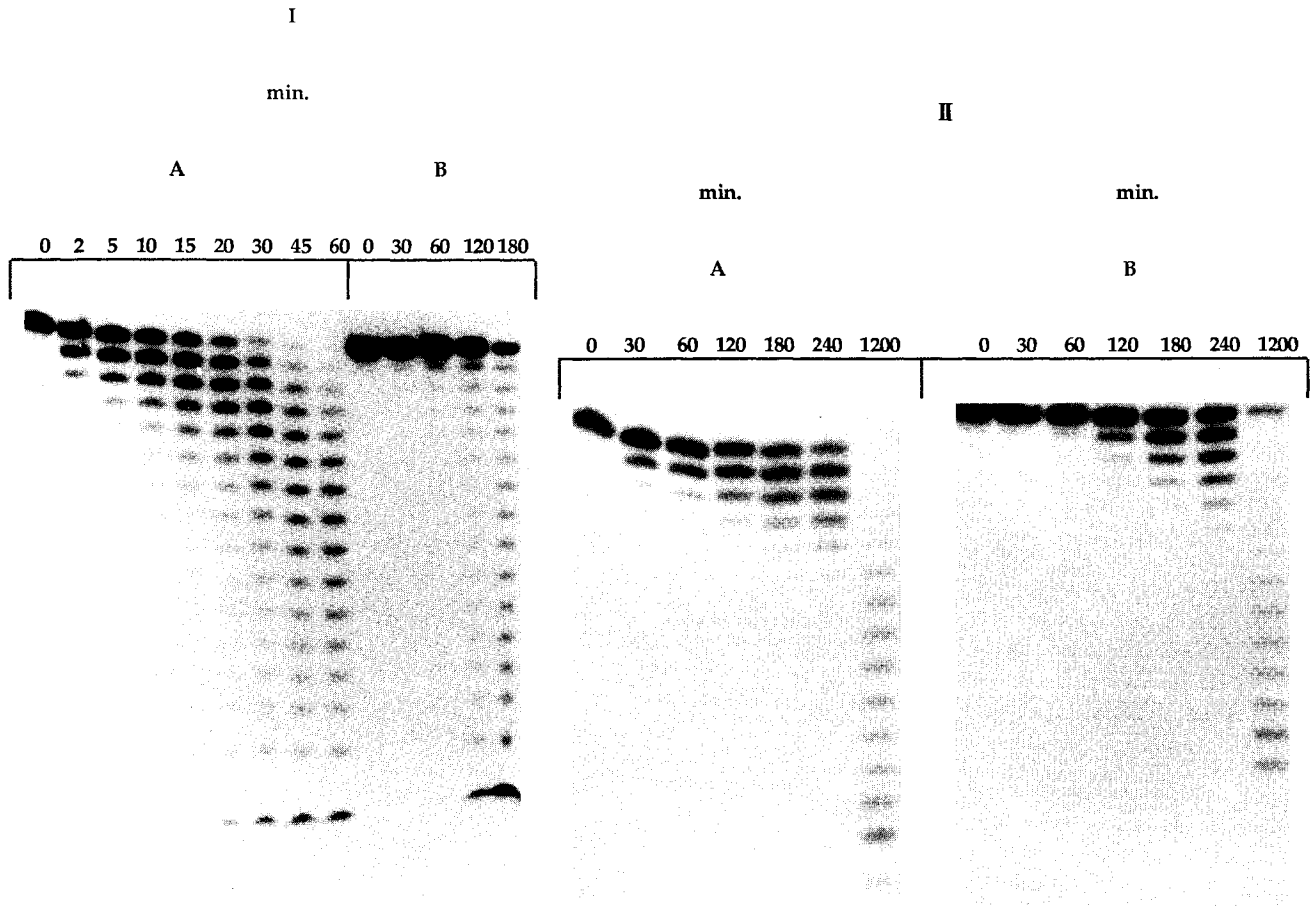

Fig. 2. Stability of $d(T)_{16}$ in RPMI supplemented with 5% foetal calf serum treated by heat at 60°C for 30 minutes (II) or not treated (I). $d(T)_{16}$ (0.5 μM) complexed with 150 μM CTAB (part A) or adsorbed to PIHCA nanoparticles (0.5 mg/ml) in the presence of 150 μM CTAB (part B) was incubated in the culture medium during various periods of time (minutes) as indicated on top of the gel. Gel analysis was carried out as in figure 1.

(60°C, 30 min). The pattern of free oligonucleotide degradation in these two types of medium (Figure 2, IA and IIA) was similar to the one observed with snake venom phosphodiesterase (Figure 1, Part A) and the final reaction products were 5'-mononucleotides. These observations indicated the presence of a 3'-exonuclease activity in the cell culture medium. The half-lives of oligonucleotides in medium of high and low nuclease activity were relatively short (4 and 90 min., respectively) and remained similar when oligonucleotides were complexed with CTAB (Table I), in contrast to the results observed in buffer containing snake venom phosphodiesterase (Figure 1, Part A and B). This could be due to a competition between serum proteins or nucleases and oligonucleotides for interaction with CTAB. The half-lives of oligonucleotides adsorbed onto nanoparticles were longer, 130 and 195 minutes in medium with high and low nuclease activity, respectively (Table I). These observations showed that the rate of oligonucleotide degradation was not markedly modified by a 20-fold increase in the enzymatic activity of the medium. In the medium of high nuclease activity, we observed a different pattern of oligonucleotide degradation depending on whether it was free or adsorbed onto nanoparticles (Figure 2, Part IA and B). On the autoradiogram, degradation of oligonucleotides adsorbed onto nanoparticles was characterized by a decrease in the radioactivity of the band corresponding to intact oligonucleotide and by an increase in the radioactivity corresponding to the final degradation product (5'-^{32}P-mononucleotide). In this medium (high nuclease activity), only a few bands corresponding to intermediate degradation products were observed in contrast to what was observed for degradation of free oligonucleotides. These observations suggest that oligonucleotides ad-

Table I. Half life (minutes) of $d(T)_{16}$ free or adsorbed to PIHCA nanoparticles in the presence of snake venom phosphodiesterase and in cell culture medium[a].

	Phosphodiesterase		RPMI + 5% SVF	
	0.1 μg/ml	0.5 μg/ml	(−)	(60°C, 30 min.)
$d(T)_{16}$	2	—	4	90
$d(T)_{16}$ + CTAB	275	38	4	90
$d(T)_{16}$-NP	>1200	>1200	130	195

[a] Quantitative analysis of oligonucleotide cleavage was achieved after cutting of bands on the polyacrylamide gel and counting for radioactivity as described in Methods.

sorbed onto nanoparticles are at least partly protected from nucleases. The digested oligonucleotides could correspond to oligonucleotides which are slowly released from the nanoparticles during their incubation in the medium and then rapidly degraded by nucleases. We previously reported that ion pairs adsorbed onto nanoparticles were destabilised by increasing the ionic concentration of the medium (15). The release of oligonucleotides from nanoparticles could also be explained by a competitive adsorption of serum proteins (such as bovine serum albumine) at the surface of nanoparticles (24) or could be the consequence of the enzymatic degradation of nanoparticles in the presence of serum carboxyesterase (25) (PIHCA nanoparticles are disrupted by hydrolysis of lateral carboxyesters of cyanoacrylic oligomers, as described by Lenaerts *et al.* (26)). The release of drugs initially adsorbed or incorporated into polyalkylcyanoacrylate (PACA) nanoparticles has been correlated with the kinetics of polymer degradation (26). In addition, it was observed that the stabilization of oligonucleotide adsorbed onto nanoparticles was greater in the medium with a high nuclease activity (Table I). This result shows that the rate of oligonucleotide release from nanoparticles is slower than its rate of degradation in this medium ($t_{1/2}$ = 4 min.).

Cytotoxicity of Oligonucleotides Adsorbed onto PIHCA Nanoparticles

Cell toxicity of PACA nanoparticles was previously studied in several human and animal cell lines (27–28). It was reported that nanoparticle toxicity was dependent on the rate of polymer degradation (28). In the present study, PIHCA was chosen for oligonucleotide targeting since this polymer was found to be sufficiently slowly degraded in cell culture media (25). This was not the case with other cyanoacrylic derivatives. In addition, PIHCA was found to display the lowest cytotoxicity (28). Finally, ion pairs formed between oligonucleotides and CTAB were found to be adsorbed with a better efficacy onto PIHCA nanoparticles, in comparison with other tested cyanoacrylic polymers (15).

The cytotoxicity of PIHCA nanoparticles towards U937 cells was evaluated by measuring the release into the medium of lactate dehydrogenase (LDH), a cytosolic enzyme. LDH activity in cell culture medium was determined after incubation of adherent U937 cells during 24 hours with various concentrations of empty (not supplemented with 15mer and CTAB) nanoparticles or 15mer- CTAB-loaded nanoparticles (Figure 3a). No cytotoxicity was observed after 24 hour incubation of U937 cells with 5 μg/ml of empty PIHCA nanoparticles (Figure 3a). At higher concentrations of nanoparticles (10–80 μg/ml), cell toxicity increased. These results are similar to those previously observed with L 929 mouse fibroblasts (28) or various human normal or malignant mesenchymal cells (27). No difference in toxicity was observed between empty and loaded nanoparticles until a 20 μg/ml concentration of polymer was reached. However, above this value, the cytoxicity of nanoparticles was increased when they were loaded with oligonucleotides in the presence of CTAB. The concentration of the polymer needed to induce a 50% LDH release (CT_{50}) was calculated, taking into account the basal LDH activity of control cells (not incubated with nanoparticles). After 24 hour incubation,

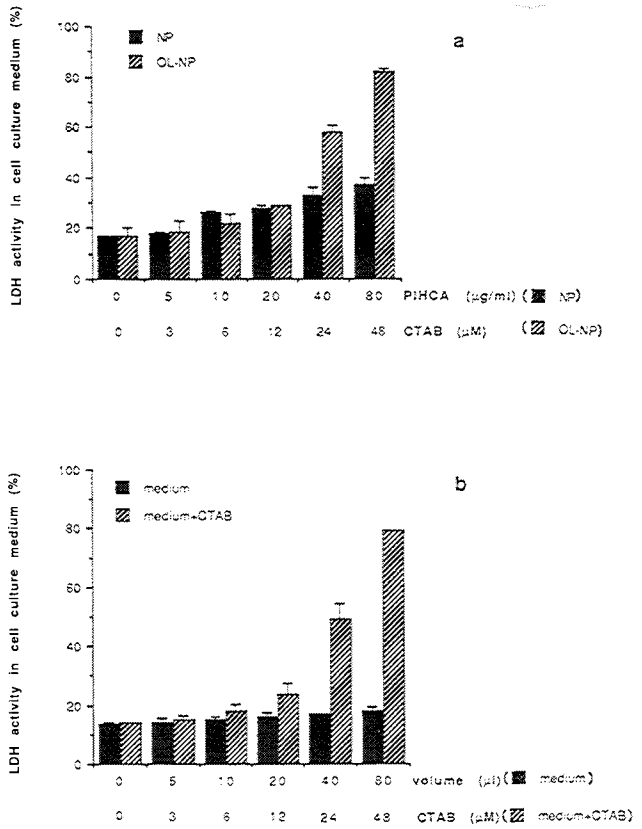

Fig. 3. Lactate dehydrogenase activity in cell culture medium after 24 hr. incubation of U937 cells: a- with various concentrations of PIHCA nanoparticles not supplemented with CTAB and oligonucleotide (NP), or with nanoparticles loaded with 15mer in the presence of CTAB (OL-NP) b- with corresponding volumes of nanoparticles suspension medium (medium) supplemented with CTAB alone (medium + CTAB). Experiments were carried out in duplicates. Bars represent the range.

CT_{50} of nanoparticles loaded with 15-mer was 60 μg/ml. For empty nanoparticles, CT_{50} was not reached for a concentration of 80 μg/ml. The medium used for suspending the nanoparticles was not toxic at all for the cells whatever the volume used (Figure 3b). The 15mer was also not toxic for cells when added to the medium at the highest concentration used in this experiment (80 nM) (data not shown). This observation excludes the possibility that the cellular toxicity observed with oligonucleotide loaded nanoparticles was due to non specific effects of the oligonucleotide. When the suspension medium was supplemented with CTAB, no cell toxicity was seen until a concentration of 6 μM in CTAB was reached. For higher concentrations (12–48 μM), cell toxicity increased and CT_{50} for CTAB was evaluated to be close to 35 μM. The concentrations of polymer (40 and 80 μg/ml) for which cell toxicity of oligonucleotide-loaded nanoparticles was higher than empty nanoparticles corresponded to toxic concentrations of CTAB. For polymer concentration lower than 40 μg/ml, no toxicity was observed with oligonucleotide nanoparticles (OL-NP). For the higher concentrations (40 and 80 μg/ml), it was calculated that the observed cytotoxicity was due to the presence of CTAB (24 μM or 48 μM) in the preparation. Toxicity of quaternary ammonium salts such

as CTAB was previously observed with L929 mouse fibroblasts (29).

Intracellular Distribution and Stability of 15mer Free or Adsorbed to PIHCA Nanoparticles

Cell uptake studies of 15mer adsorbed onto nanoparticles was performed in non toxic conditions (PIHCA = 5 μg/ml, CTAB = 3 μM, 15mer = 5 nM) as reported in Figure 3. It was observed that the uptake of oligonucleotide was dramatically increased when associated with nanoparticles. Thus, after 24 hour incubation, uptake of oligonucleotide was 8 times higher when adsorbed to nanoparticles (Figure 4). Similar results were obtained in three independent experiments. The addition in cell culture medium of CTAB cation alone (3 μM) or empty nanoparticles (5 μg/ml) did not increase the uptake of free 15mer (data not shown). Moreover, after similar dilution of the 15mer—CTAB—loaded nanoparticles in culture medium (15mer = 8 nM, CTAB = 5 μM, PIHCA = 8 μg/ml), 52 percent of the labelled 15mer was still found to be associated with the polymer after two hours (data not shown). All these observations confirm that intracellular uptake of 15mer was achieved via its adsorption onto nanoparticles.

The cellular uptake of the 15-mer adsorbed onto PIHCA nanoparticles was analysed at 4°C and 37°C (Figure 5). Results showed that cellular uptake of oligonucleotide was markedly reduced (95%) at 4°C as compared to 37°C. These results show that oligonucleotides adsorbed onto nanoparticles were internalized in U937 cells by an endocytotic/phagocytic process rather than simply adsorbed at the membrane surface. The polymeric structure of PIHCA nanoparticles exclude their cellular uptake by fusion. After internalisation, nanoparticles accumulate in phagosomes. Such a process of intracellular localisation for nanoparticles was previously reported (18–19,30). Using electron microscopy, Astier *et al.* (30) have shown that polymethacrylate nanoparticles were localized within endocytotic vesicles in U937 cells. When the nanoparticle concentration in the medium was doubled in order to obtain a final oligonucleotide concentration of 50 nM, the amount of internalized oligonucleotide only increased by 1.5 times (Figure 5). This result suggests that a saturation process of uptake was reached at this nanoparticle concentration.

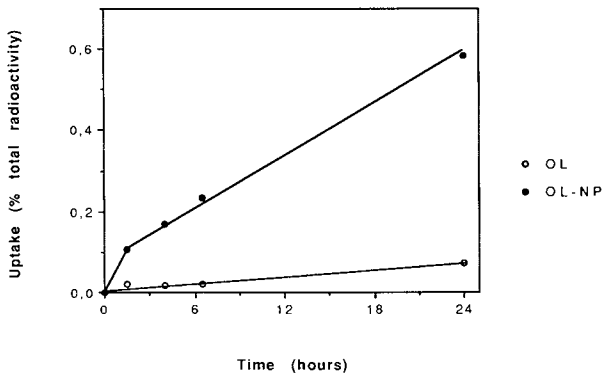

Fig. 4. Uptake by U937 cells of pentadecamer free or adsorbed to PIHCA nanoparticles. U937 cells were incubated at 37°C with 5 nM internally labelled pentadecamer free (OL) or adsorbed to PIHCA nanoparticles in the presence of CTAB (OL-NP), as reported in material and methods; The concentration of PIHCA nanoparticles and CTAB were respectively 5 μg/ml and 3 μM. Cell uptake was monitored as the ratio of radioactivity in cell lysate against total radioactivity (cell + medium).

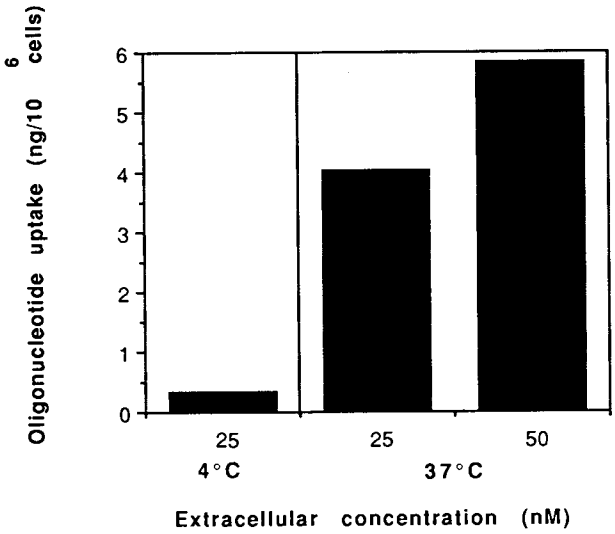

Fig. 5. Effect of temperature on the cellular uptake of 15 mer adsorbed on PIHCA nanoparticles. Internally labelled 15mer adsorbed onto PIHCA nanoparticles (1 μmole/g) was incubated with U937 cells during 6 hours at 4 or 37°C, as described in Materials and Methods. Two concentrations of oligonucleotide (25 and 50 nM) were used at 37°C.

The intracellular and extracellular stability of internally labelled 15mer (5 nM) free or adsorbed to PIHCA nanoparticles was tested at various incubation times with U937 cells. Results are shown in Figure 6. The stability of 15mer adsorbed to PIHCA nanoparticles after dilution in cell culture medium, which leads to low and non toxic concentrations of cations and polymer (CTAB = 3 μM, PIHCA = 5 μg/ml), remains greater than that of free oligonucleotides (Figure 6, Part IA). After 1.5 hour incubation with U937 cells, only 20% of free oligonucleotide was intact, as determined by scintillation counting of radioactive bands in the gel. Under the same conditions, 59% of oligonucleotide adsorbed to nanoparticles remained intact. After four hours, non degraded oligonucleotides were detected on the autoradiogram only when they were incubated as nanoparticles. Using a ^{32}P-label in the internal sequence of the 15mer (5′CATTTTG*ATTACTGT3′), the smaller degradation products detected on autoradiography were an 8mer and a 5′-mononucleotide (AMP). This last product was only observed with longer exposure of the gel. This particular pattern of 15mer degradation confirmed the presence in the medium of a 3′-exonuclease activity. No intact oligonucleotide was detected in cell lysate after 1.5 hour incubation with free oligonucleotide (Figure 6, Part IA). On the contrary, the 15mer adsorbed onto PIHCA nanoparticles remained completely intact after 6.5 hours of incubation (Figure 6, Part IB). At 24 hours, some degradation products appeared but the fraction of intact oligonucleotides remained important. Some radioactivity was reincorporated in cellular nucleic acids as seen by bands of low mobility on the gels.

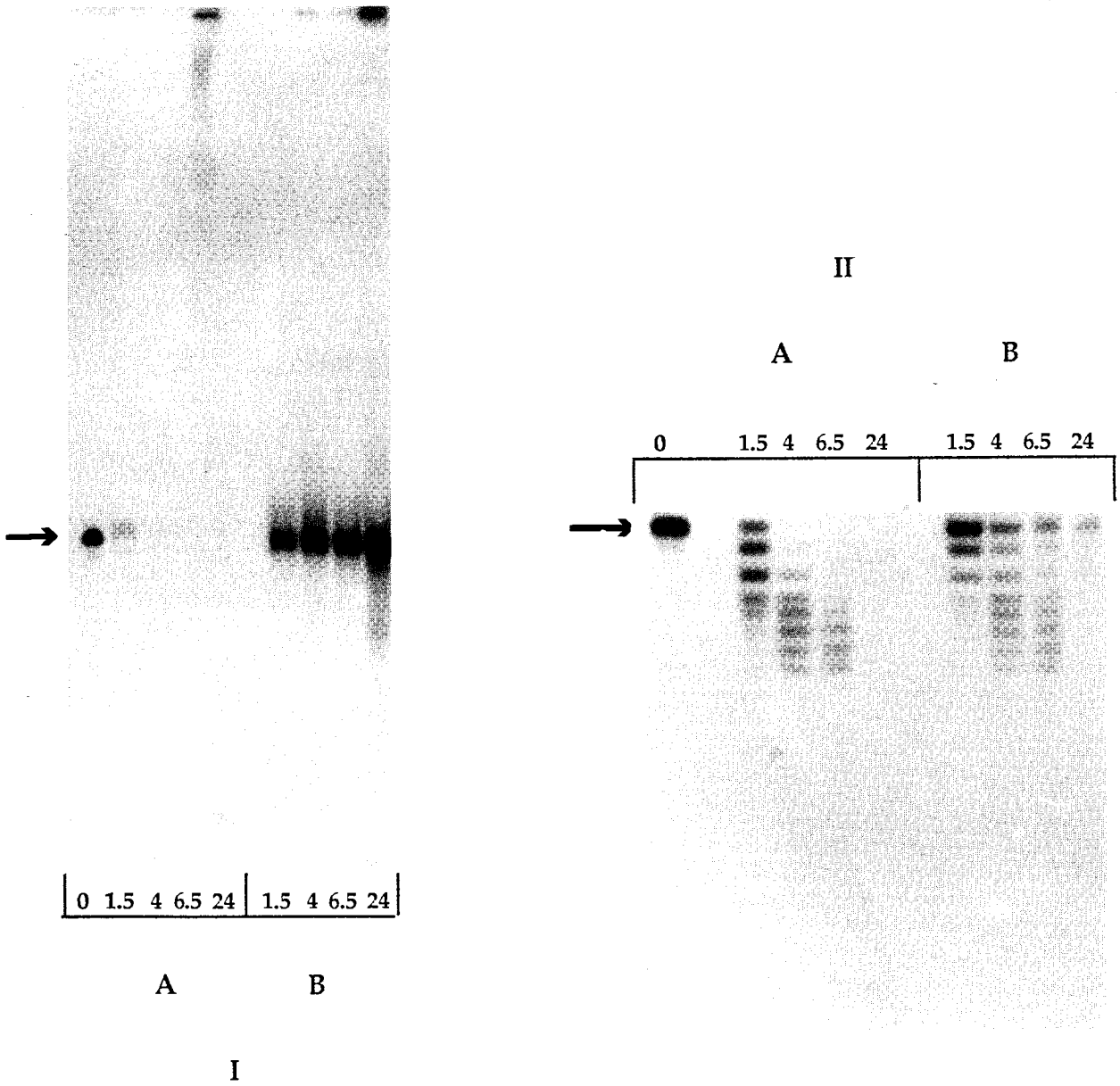

Fig. 6. Stability of 15mer free or adsorbed to nanoparticles in cell lysate (I) and in cell culture medium (II). Study of the stability of internally labelled 15mer free (lanes A) or adsorbed to PIHCA nanoparticles (lanes B) was carried at 37°C in cell culture medium (II) and in cell extracts (I) as described in Materials and Methods. Incubation times in hours are indicated below (I) or above (II) each gel. Arrows in part I and II indicate the position of intact oligonucleotide.

The intracellular distribution of oligonucleotides (5'-^{32}P-labelled oligomer) was measured after cell lysis in presence of nonidet-P40, a nonionic detergent which protects the nuclear structure (Figure 7). It has to be noted that the cytoplasmic fraction contains also endocellular vesicles like lysosomes and/or phagosomes. About 20% of radioactivity corresponding to oligonucleotides free or adsorbed to PIHCA nanoparticles were found in the nuclear fraction. The autoradiogram (Figure 7, Part A) shows that intact oligonucleotide was found only in the nuclear but not in the extranuclear fraction after 6 hours of incubation with the free oligonucleotide. This could be explained by the presence in the cytoplasmic fraction of lysosomes and/or phagosomes which are the expected major site of oligonucleotide degradation. On the contrary, when adsorbed onto nanoparticles, intact oligonucleotides were detected in both nuclear and extranuclear fractions even after 24 hours of incubation (Figure 7, Part B). However, some degradation products appeared in the extranuclear fractions at 6 and 24 hours. The increased stability of 15mer adsorbed onto nanoparticles which is observed in the extranuclear fraction could be explained by the fact that nanoparticles protect them against digestion by lysosomial enzymes. Another possibility would be that 15mer-loaded nanoparticles escape from this compartment by unknown mechanism.

CONCLUSION

Increasing the stability of oligonucleotides towards cel-

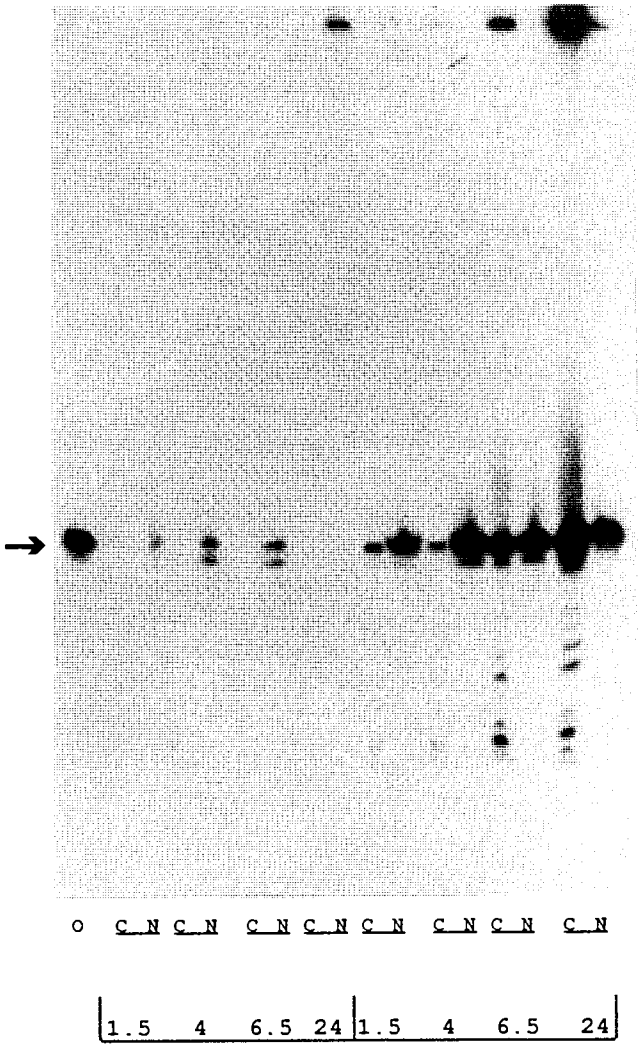

Fig. 7. Intracellular distribution and stability of 15mer free or adsorbed to PIHCA nanoparticles. U937 cells stimulated by TPA (50 nM, 48 hr) were incubated at 37°C with 5 nM of 5'-end-labelled 15mer free (part A) or adsorbed to 5 μg/ml PIHCA nanoparticles in the presence of CTAB (part B) during various incubation times (indicated in hours at the bottom of the gel). Oligonucleotides were recovered from nuclear (N) and extranuclear (C) fractions as described in Materials and Methods. Arrow indicates the position of intact oligonucleotide.

lular nucleases is a real challenge to improve the biological efficacy of these compounds and to make them credible candidates as new therapeutic agents. Our results have shown that PIHCA nanoparticles greatly improve both the extracellular and the intracellular stability of oligonucleotides. In addition the cellular uptake of oligonucleotides in the U937 cell line was increased by their adsorption onto PIHCA nanoparticles, as a result of the capture of nanoparticles by an endocytotic/phagocytic process. The presence of intact oligonucleotide in the cell nuclear fraction suggests that part of internalized oligonucleotides escape from the endosomal/lysosomal compartment. The mechanism by which oligonucleotides escape from endocytic compartments is not known yet. Nanoparticles might be enzymatically degraded in lysosomes slowly releasing intact oligonucleotide molecules. The intracellular localization of fluorescent oligonucleotides adsorbed onto nanoparticles is presently under investigation. In addition, work is in progress to compare the biological activity of oligonucleotides free or adsorbed onto nanoparticles in different cellular systems.

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