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Research Article

Polyalkylcyanoacrylate Nanoparticles as Polymeric Carriers for Antisense Oligonucleotides

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Adsorption of oligothymidylates on polyisobutyl- or polyisohexylcyanoacrylate nanoparticles was achieved in the presence of hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts. Results suggested that oligonucleotide adsorption on the nanoparticles was mediated by the formation of ion pairs between the negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. The adsorption efficiency of oligonucleotide–cation complexes on nanoparticles was found to be highly dependent upon several parameters: oligonucleotide chain length, nature of the cyanoacrylic monomer, hydrophobicity of cations used as ion-pairing agents, and ionic concentration of the medium. Carrier capacity of polyisohexylcyanoacrylate nanoparticles for oligothymidylates (16 nucleotides) complexed with cetyltrimetylammonium bromide in the presence of 0.15 *M* NaCl was determined to be 5 μmol/g polymer. The *in vitro* protection of oligothymidylates adsorbed to nanoparticles against degradation by a 3′-exonuclease (snake venom phosphodiesterase) was also demonstrated. These results showed that nanoparticles can be considered as convenient carriers for the protection and delivery of oligonucleotides to cells in culture and for future applications *in vivo*.

KEY WORDS: oligonucleotides; nanoparticles; polyalkylcyanoacrylate; ion pairs; oligonucleotide hydrolysis.

INTRODUCTION

Antisense oligonucleotides are synthetic fragments of ribo- or deoxyribonucleic acids that recognize and bind specifically to the complementary sequence of a gene or its messenger RNA. As a consequence, transcription or translation of selected genes may be altered. Regulation of gene expression by antisense oligonucleotides has been described mostly *in vitro* or in cellular systems. The investigated target genes comprised endogeneous genes such as protooncogenes or exogeneous genes from viruses or parasites (for recent reviews see Refs. 1 and 2).

However, many problems are encountered when oligonucleotides are used in cellular systems and *in vivo*. In fact, oligonucleotides are rapidly degraded in biological fluids and in cells by exo- or endonucleases which hydrolyze the phosphodiester linkage (3–5). These molecules poorly diffuse across the cell membrane, because of their ionic character and are taken up by cells in a temperature-dependent and saturable manner compatible with receptor mediated endocytosis (6). The mechanism by which they are released to the cytosol remains unknown. The majority is probably degraded in the lysosomes, with a small portion escaping to the cytosol. Therefore, in order to circumvent these problems, relatively high concentrations of oligonucleotides are needed to observe the desired biological effect.

Different strategies have been developed to give protection and to increase transport of oligonucleotides to the target cell (1,2,7). Linking intercalating agents (acridine), hydrophobic groups (cholesterol), or polycations (polylysine) to oligonucleotides resulted in a higher resistance toward exonucleases and increased their penetration into cells (4,8–10). On the other hand, various chemical modifications have been made in the phosphodiester backbone of oligonucleotides. Oxygen atoms of the phosphate group can be substituted by one or two sulfur atoms, giving phosphorothioate or phosphorodithioate oligonucleotides. Substitution of one of the oxygen atoms by a methyl group leads to methylphosphonates. These modifications have greatly increased the resistance of oligonucleotides to digestion by nucleases (11,12). Oligonucleotides have been synthesized possessing the α-anomeric form of the nucleosides rather than the β-anomeric (natural) form. These molecules were found to be more resistant to both exo- and endonucleases (4,13).

Another strategy recently developed consists of encapsulating oligonucleotides in liposomes. By this method, delivery of C-myc antisense oligonucleotides to cells was

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achieved by lipofusion, as reported by Loke *et al.* (14). Similarly, Leonetti *et al.* have demonstrated the efficiency of antibody-targeted liposomes for the intracellular delivery of oligonucleotides complementary to the 5′ end of the N protein messenger RNA of vesicular stomatitis virus (VSV) (15). They have obtained by this method efficient and selective inhibition of VSV replication in L929-infected cells.

In the search for drug delivery systems exhibiting greater stability and cellular uptake for oligonucleotides, one might investigate other forms of biodegradable carriers. Alkylcyanoacrylate nanoparticles are obtained by emulsion polymerisation of various alkylcyanoacrylate monomers in acidic medium (16). Because of their polymeric nature, these small particles (diameter, about 200 nm) are more stable than liposomes in biological fluids and during storage. Moreover, polyalkylcyanoacrylate nanoparticles could be useful in promoting cellular uptake via endocytosis, as demonstrated in a number of cells such as fibroblasts and macrophages (17,18). These carriers were found to enhance the *in vivo* activity of some antitumor drugs or antibiotics (19–21).

Association of drugs to polycyanoacrylate nanoparticles is achieved by hydrophobic Van der Waals interactions between drugs and the polymer. Hydrophilic molecules do not form stable complexes with this type of carriers. Nevertheless, Guise *et al.* (22) have reported the incorporation of nucleosides such as vidarabine into nanoparticles, in the presence of dioctylsulfosuccinate (DOSS). The incorporation of vidarabine was achieved owing to the formation of ion pairs between DOSS and the charged nucleobase at a low pH.

In this paper we describe a new method of loading cyanoacrylate polymers with other hydrophilic compounds, *viz.*, antisense oligonucleotides. It consists of the formation of ion pairs between oligonucleotides and hydrophobic cations that bind to the polymeric carrier. The different parameters that govern the efficient association of oligonucleotides complexed with hydrophobic cations to nanoparticles were studied. Moreover, we have demonstrated that oligonucleotides adsorbed to a polymeric carrier by this method were protected *in vitro* against degradation by a 3′-exonuclease (snake venom phosphodiesterase).

MATERIALS

Dephosphorylated oligothymidylates of structure $d(T)_n$ and 5′ phosphorylated forms, $pd(T)_n$, with $n = 4$, 8, and 16, were purchased from Pharmacia—France. Dodecyl-, tetradecyl-, and cetyltrimethylalkylammonium bromide, (DTAB, TTAB, and CTAB, respectively) and dihydrosphingosine or DL-1,3-dihydroxy-2-aminooctadecane (SP) as well as isobutylcyanoacrylate monomer (IBCA) were obtained from Sigma. Isohexylcyanoacrylate monomer (IHCA) was a gift of SOPAR—Belgium. Tetraphenylphosphonium chloride (TPP) and tetraphenylboron sodium (TPB) were acquired from Aldrich. Poloxamer 188 (polyoxyethylene–polyoxypropylene block copolymer) was supplied by ICI, and dextran 70 by Fluka. Tetra[^{14}C]phenylphosphonium bromide (sp act, 31.4 mCi/mmol) and ^{32}P-ATP (sp act, 3000 Ci/mmol) were purchased from Amersham. Phosphodiesterase (snake venom) and T4 polynucleotide kinase were obtained from Boehringer Mannheim.

METHODS

Nanoparticle Preparation

Nanoparticle suspensions were prepared by adding isobutylcyanoacrylate or isohexylcyanoacrylate monomer (10 mg/ml) to a solution containing hydrochloric acid (1 m*M*, pH 3) and a dispersant, dextran 70 (1%, w/v), in distilled water. The polymerization of cyanoacrylate monomers occurs spontaneously at room temperature (20°C) with stirring. It was complete after 2 hr for IBCA and 6 hr for IHCA. The polymeric suspensions were stored at 4°C, until their use for drug adsorption experiments.

Particle Size Analysis

The size of the resulting particles was determined by laser light-scattering measurements (Nanosizer Coultronics, Andilly, France) of a diluted suspension.

Adsorption of Oligonucleotide–Hydrophobic Cation Complexes to Polyalkylcyanoacrylate Nanoparticles

The nanoparticle suspension (10 mg/ml) was neutralized to pH 7 with phosphate buffer (10 m*M* final). Oligomers were then added to the nanoparticle suspension, at a final concentration of 2 μ*M*, without ions or complexed with various concentrations of tetraphenylphosphonium salts (TPP). The suspension was stirred continuously for 2 hr, at room temperature. As a control, adsorption experiments of oligomers were carried out in the presence of tetraphenylboron (TPB), a negatively charged analogue of TPP.

Alkyltrimethylammonium salts (DTAB, TTAB, CTAB), as well as dihydrosphingosine, were also used to form ion pairs with oligonucleotides. In these experiments the nanoparticle suspension (PIHCA) was neutralized to pH 7 and diluted to a final concentration of 1 mg/ml, in 10 m*M* Tris–HCl buffer with 1% (w/v) dextran 70 and 0.4% (w/v) Poloxamer 188. Various concentrations of ion-pairing agents and $pd(T)_{16}$ (final concentration, 1 μ*M*) were added to nanoparticles in the presence of 150 m*M* NaCl. The suspension was stirred for 2 hr, as described previously.

Influence of Ionic Concentration on the Adsorption onto Nanoparticles of $p(dT)_{16}$ Complexed with CTAB

$pd(T)_{16}$ (1 μ*M*) complexed with various concentrations of CTAB was incubated with nanoparticles (1 mg/ml), without NaCl or in the presence of 75 or 120 m*M* NaCl.

Influence of Poloxamer 188 on the Adsorption of Ion Pairs to Nanoparticles

$pd(T)_{16}$ (1 μ*M*) was adsorbed to nanoparticles (1 mg/ml), stabilized or not stabilized with Poloxamer 188 (0.4%, w/v), in the presence of 50 μ*M* TPP, DTAB, TTAB, or CTAB.

Adsorption Isotherm

The adsorption isotherm for $pd(T)_{16}$ complexed with CTAB was determined at 150 m*M* NaCl. The CTAB concentration was fixed at 300 μ*M*, while the $pd(T)_{16}$ concentration varied in the range of 1 to 10 μ*M*.

Determination of the Adsorption Yield

Dephosphorylated oligothymidylates (20–40 pmol) were labeled at their 5′ end using ATP $\gamma^{32}P$ and T4 polynucleotide kinase. They were purified on a 20% polyacrylamide–7 *M* urea sequencing gel followed by ethanol precipitation. The labeled oligonucleotides were then diluted with concentrated solutions of nonlabeled oligonucleotides.

In order to determine the adsorption of TPP, tetra[^{14}C]-phenylphosphonium bromide was used as a tracer and mixed with nonlabeled TPP. The unbound adsorbent was separated from the nanoparticle suspension by centrifugation at 45,000 rpm for 1 hr, using a TGA 65.38 Kontron ultracentrifuge. Radioactivity for both supernatant and resuspended pellet was counted by liquid scintillation. For scintillation counting of ^{14}C- and ^{32}P-labeled materials, in a nanoparticle suspension or in aqueous medium, we have verified that there was no quenching, by adding a known amount of radioactivity to a control sample. Percentage of adsorption was determined by dividing radioactivity of the pellet by total radioactivity (pellet and supernatant). In most cases, the percentage of oligomer recovery was higher than 95% of the added oligonucleotide. In a few cases, the recovery was lower (~90%), probably due to an experimental error. The experiment was then duplicated.

In Vitro Protection of Oligonucleotides Adsorbed to Nanoparticles Against Degradation by Nucleases

The 5′ end-labeled oligonucleotides were mixed with sufficient unlabeled oligomers to give a final concentration of 0.5 μ*M*. Degradation experiments were carried out in 0.1 *M* Tris–HCl buffer, pH 7.4 containing 10 m*M* $MgCl_2$. To the medium was added $d(T)_{16}$ alone, $d(T)_{16}$, and CTAB (150 μ*M*) or PIHCA nanoparticles (0.5 mg/ml) containing $d(T)_{16}$ and CTAB (150 μ*M*). Digestion was performed using phosphodiesterase (0.01 to 5 μg/ml) at 37°C for 15 min and then stopped with the addition of EDTA (20 mM) to chelate Mg^{2+} ions and by heating at 80°C for 2 min. For those samples containing nanoparticles it was necessary, prior to gel analysis, to adjust the medium to pH 12 with NaOH and incubate for 2 hr at 37°C in order to degrade the polymeric carrier. After neutralization of the samples oligonucleotides were recovered by phenol extraction and ethanol precipitation. Samples were then analyzed on a 20% polyacrylamide–7 *M* urea sequencing gel. The gel was autoradiographed at −70°C using Fuji (X-ray) films, and the bands were excised and analyzed quantitatively by measuring the radioactivity by scintillation counting.

RESULTS

Mechanism for Oligonucleotide Adsorption to Polycyanoacrylate Nanoparticles

As shown in Fig. 1, only 1–2% of $pd(T)_{16}$ (2 μ*M*) dissolved in the incubation medium in the absence of any added cations were adsorbed to PIHCA nanoparticles (10 mg/ml). This value was not influenced by the nature of the monomer used for the preparation of nanoparticles (IBCA or IHCA). However, the adsorption efficiency of $pd(T)_{16}$ on PIHCA or PIBCA nanoparticles was increased with increasing concentrations of tetraphenylphosphonium salts (TPP) in the medium (Fig. 1). Complete adsorption of $pd(T)_{16}$ on PIHCA nanoparticles was attained under these conditions for a TPP concentration of approximately 80 μ*M*. Conversely, tetraphenylboron (TPB), a structurally related compound but bearing a negative charge, was inefficient at increasing the yield of oligonucleotide adsorption, even at concentrations as high as 150 μ*M*.

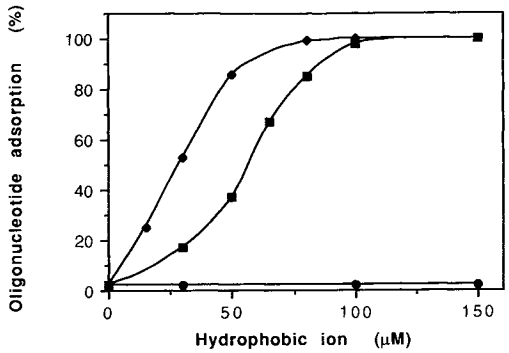

Fig. 1. Adsorption of $pd(T)_{16}$ to polyalkylcyanoacrylate nanoparticles. $pd(T)_{16}$ (2 μ*M*) complexed with increasing concentrations of tetraphenylphosphonium chloride (TPP) was added to PIBCA (■) or PIHCA (◆) nanoparticles (10 mg/ml) and stirred for 2 hr as described under Methods. Adsorption of oligothymidylates to PIHCA nanoparticles in the presence of tetraphenylboron sodium (●) was measured under the same experimental conditions.

For TPP concentrations up to 100 μ*M*, $pd(T)_{16}$ adsorption was obtained with a higher efficiency on PIHCA than on PIBCA nanoparticles (Fig. 1). Concentrations of cations needed to obtain 50% oligonucleotide adsorption (referred to as C_{50}) were 60 and 30 μ*M* for PIBCA and PIHCA nanoparticles, respectively.

By using radiolabeled TPP, adsorption of TPP onto PIBCA and PIHCA nanoparticles, in the presence of $pd(T)_{16}$ (2 μ*M*), was also studied. In order to take into account the differences in diameter between PIBCA and PIHCA nanoparticles (200 ± 30 and 170 ± 15 nm, respectively), the adsorption efficiency was expressed as picomoles of adsorbed cations per square centimeter of polymeric surface area. As shown in Table I, the amount of TPP adsorbed onto nano-

Table I. Adsorption of TPP and $pd(T)_{16}$ to Polyalkylcyanoacrylate Nanoparticles[a]

	Adsorbed to PIBCA nanoparticles		Adsorbed to PIHCA nanoparticles	
TPP (μ*M*)	adsorbed TPP (pmol/cm^2)	adsorbed $pd(T)_{16}$ (fmol/cm^2)	TPP (pmol/cm^2)	adsorbed $pd(T)_{16}$ (fmol/cm^2)
0	0	6.7	0	5.7
15	—	—	4.2	144.8
31.5	9.1	115.8	8.7	305
53.7	14.7	247.1	14.3	491
83	21.2	574.4	21.7	568
106	25.4	656.6	26.5	568
158	32.7	673.4	35.6	568

[a] Experimental conditions were as in Fig. 1.

particles correlated with an increase in TPP concentration in the medium. Moreover, the affinity of cations for the polymeric surface was not dependent on the nature of the cyanoacrylic monomer (IBCA or IHCA). The amount of $pd(T)_{16}$ adsorbed to polycyanoacrylate nanoparticles was found to correlate with the amount of TPP adsorbed on the polymeric surface. For values of TPP below 21 pmol/cm^2, a larger amount of adsorbed oligonucleotide was observed on PIHCA than on PIBCA nanoparticles. For a greater amount of TPP adsorbed, the carrier capacity for oligonucleotides reached a maximum value which corresponded to complete adsorption of $pd(T)_{16}$ on nanoparticles (Fig. 1).

Adsorption isotherm for TPP on PIBCA nanoparticles, at 25°C in 10 m*M* phosphate buffer, is shown in Fig. 2. The similar results obtained when TPP was adsorbed on nanoparticles during 2 hr or overnight showed that equilibrium conditions were reached (results not shown). The data of TPP adsorption were plotted according to the Langmuir isotherm equation, which is widely used to characterize the adsorption of solutes from acqueous solution. The Langmuir equation (23) is $x/m = NKC/1 + KC$, where x is the amount of solute adsorbed by a weight m of adsorbent, C is the solution concentration of solute at equilibrium, and N and K are constant terms, with N representing the maximum carrier capacity and K the affinity of solute for adsorbent. This model characterizes the adsorption of solutes in monolayer and implicates the uniformity of adsorption sites. A plot of $(x/m)/C$ versus x/m (Scatchard plot) gives no straight line, showing that the adsorption of TPP on nanoparticles does not fit the classic Langmuir isotherm.

Influence of the Oligonucleotide Chain Length

In the presence of TPP, it was found that the effectiveness of oligomer adsorption increased with the oligomer chain length. This is illustrated on Fig. 3. The values of C_{50} were determined for adsorption to PIBCA nanoparticles of a series of oligothymidylates with various chain lengths: $pd(T)_n$ (n = 4, 8, 16). When complexed with TPP, the C_{50} values obtained were 460, 130, and 60 μ*M* for n = 4, 8, and 16, respectively.

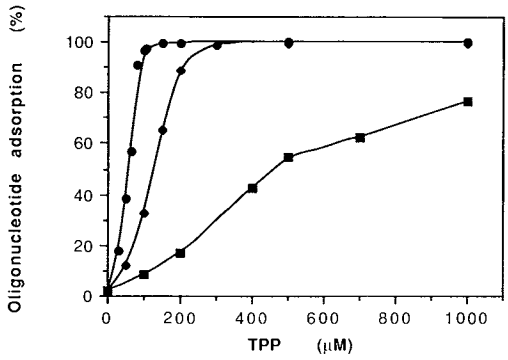

Fig. 3. Influence of oligomer chain length on the adsorption of oligothymidylates onto PIBCA nanoparticles. Adsorption of three oligothymidylates, $pd(T)_4$ (■), $pd(T)_8$ (◆), and $pd(T)_{16}$ (●), onto PIBCA nanoparticles versus increasing concentrations of TPP. Experimental conditions were as in Fig. 1.

Influence of the Hydrophobic Character of Cations

We have studied the adsorption efficiency of oligonucleotides to PIHCA nanoparticles, in the presence of a variety of cations, three of which are quaternary ammonium salts (DTAB, TTAB, CTAB) and the fourth a primary amine (SP) which is protonated at the pH under our experimental conditions. Quaternary ammonium salts differ by the number of carbon atoms of their alkyl chain C (C = 12, 14, 16). They can be classified, in term of increasing hydrophobicity, in the following manner: DTAB < TTAB < CTAB. Dihydrosphingosine is a fatty amine whose alkyl chain length is comparable to that of CTAB. Conditions for $pd(T)_{16}$ adsorption to PIHCA nanoparticles with these lipophilic cations are described under Methods. It should be noted that a nonionic surfactant (Poloxamer 188) was required in the presence of these cations, at a final concentration of 0.4% (w/v), in order to stabilize the polymeric suspension. Moreover, phosphate buffer was replaced by Tris–HCl buffer because it was observed that some cations, such as dihydrosphingosine, interacted with phosphate ions leading to the formation of aggregates.

As reported in Fig. 4, among the three alkylammonium

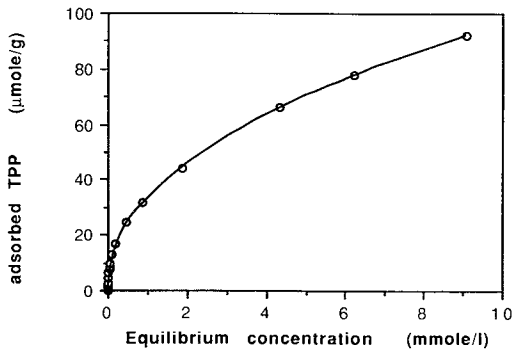

Fig. 2. Adsorption isotherm for TPP to PIBCA nanoparticles. Various concentrations of TPP were added to PIBCA nanoparticles (10 mg/ml), suspended in 10 m*M* phosphate buffer, pH 7, and stirred for 2 hr at 25°C. The amount of TPP adsorbed on nanoparticles is reported against the concentration of TPP in solution (equilibrium concentration).

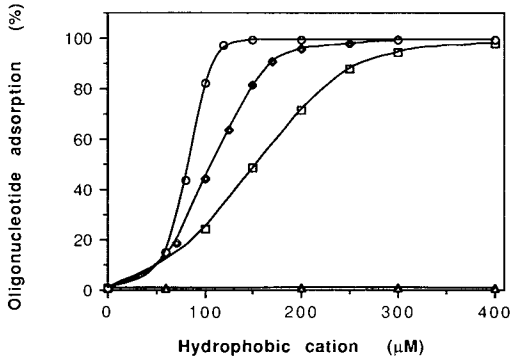

Fig. 4. Adsorption of $pd(T)_{16}$ complexed with cations bearing long alkyl chains onto PIHCA nanoparticles. $pd(T)_{16}$ (1 μ*M*) was complexed with various concentrations of hydrophobic cations (DTAB, △; TTAB, □; CTAB, ◇; SP, ○). Lipophilic complexes were adsorbed onto PIHCA nanoparticles (1 mg/ml) stabilized with Poloxamer 188, as described under Methods.

salts tested, only those having a long alkyl chain (C >12) were efficient to promote adsorption of $p(dT)_{16}$ to PIHCA nanoparticles. One does in fact observe a threshold in the alkyl chain length (C >12) for which such cations are efficient at promoting oligonucleotide adsorption onto nanoparticles. When DTAB was replaced by TPP, oligonucleotides adsorption was not observed (results not shown). Values of C_{50} were determined to be 150, 110, and 85 μM for TTAB, CTAB, and SP, respectively. Sphingosine, having an alkyl chain length comparable to that of CTAB, was found to be more efficient than the latter for binding the oligomer to the polymeric nanoparticles.

Influence of Poloxamer 188 on the Adsorption to Nanoparticles of $p(dT)_{16}$ Complexed with Hydrophobic Cations

We wanted to know if the surprisingly absence of adsorption onto nanoparticles of oligonucleotides complexed with DTAB or TPP could be attributed to the addition of poloxamer 188. As shown in Fig. 5, when oligonucleotides were complexed with TTAB or CTAB, adsorption of $pd(T)_{16}$ on PIHCA nanoparticles was not significantly modified by the addition of Poloxamer 188 (Fig. 5). In contrast, adsorption of oligonucleotides complexed with the less hydrophobic cations, i.e., TPP and DTAB, was completely inhibited in the presence of Poloxamer 188. Nonionic detergents such as poloxamers (polyoxyethylene–polyoxypropylene surfactants) are known to be strongly adsorbed to the surface of colloidal particles (24) and could thereby compete with the binding of the less hydrophobic cation–oligonucleotide complexes.

Influence of Ionic Concentration on the Adsorption of Oligonucleotides Complexed with CTAB to PIHCA Nanoparticles

The effect of varying the concentration of NaCl on the yield of $pd(T)_{16}$ association onto PIHCA nanoparticles in the presence of CTAB was addressed. As shown in Fig. 6, the efficiency of oligonucleotide adsorption was inversely proportional to the ionic concentration of the medium. In the absence of NaCl, 50% adsorption was obtained with 28 μM CTAB, whereas in the presence of NaCl, C_{50} values of 55, 75, and 110 μM were observed for concentrations of 75, 120, and 150 mM NaCl, respectively.

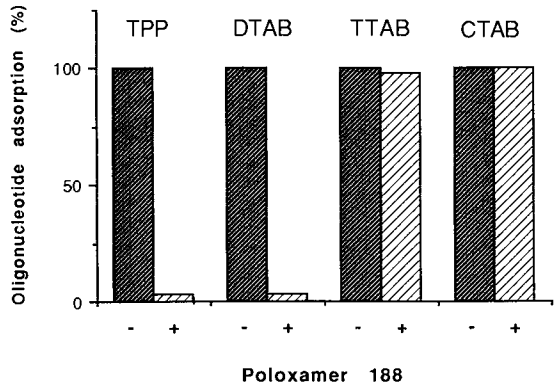

Fig. 5. Influence of Poloxamer 188 on the adsorption onto nanoparticles of $pd(T)_{16}$ complexed with hydrophobic cations. $pd(T)_{16}$ (1 μM) complexed with various cations (50 μM) was adsorbed onto PIHCA nanoparticles (1 mg/ml) stabilized (+) or not stabilized (−) with 0.4% (w/v) Poloxamer 188.

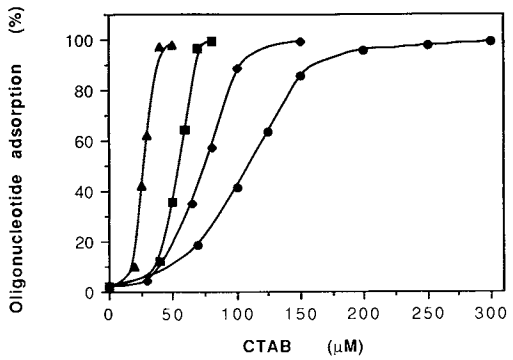

Fig. 6. Influence of ionic concentration on the adsorption of $pd(T)_{16}$ complexed with CTAB to PIHCA nanoparticles. Adsorption of $pd(T)_{16}$ (1 μM) complexed with CTAB at various concentrations onto PIHCA nanoparticles (1 mg/ml): without NaCl (▲) or in the presence of 75 mM (■), 120 mM (♦), or 150 mM (●) NaCl.

Adsorption Isotherm for $pd(T)_{16}$ on PIHCA Nanoparticles

The adsorption isotherm of $pd(T)_{16}$ complexed with CTAB (300 μM) on PIHCA nanoparticles (1 mg/ml) was determined in the presence of 150 mM NaCl (Fig. 7). A Scatchard plot of the data gives no straight line, showing that the isotherm is not of the Langmuir type. However, the data fitted well with a model of adsorption on two classes of independent sites whose equation is given by $x/m = [(N_1 \cdot C)/(K_1 + C)] + [N_2 \cdot C)/(K_2 + C)]$, where x/m is the amount of solute bound, C is the equilibrium concentration of solute, and (N_1, N_2) and (K_1, K_2) are, respectively, the maximum amount of solutes bound and dissociation constants for each type of sites. Values for (N_1, N_2) and (K_1, K_2) obtained by analysis of data according to a nonlinear regression (iterative method) were, respectively, 2.697 ± 0.225, 2.431 ± 0.232 μmol/g and 0.014 ± 0.0032, 1.222 ± 0.516 μM).

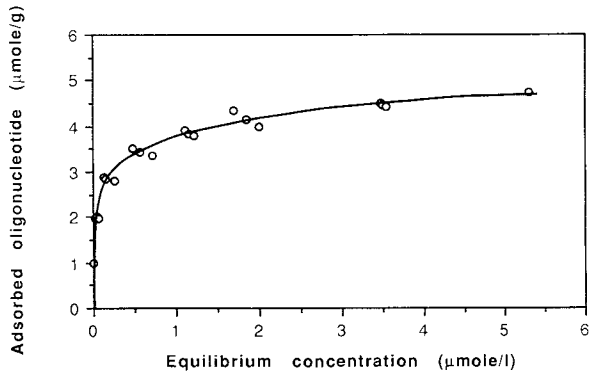

Fig. 7. Adsorption isotherm for $pd(T)_{16}$ complexed with CTAB (300 μM) onto PIHCA nanoparticles (1 mg/ml) in the presence of 150 mM NaCl.

The size of PIHCA nanoparticles was 124 ± 38 nm in this experiment. The difference in size and size distribution of PIHCA particles, compared with their usual value (170 ± 15 nm), was the result of the presence of a lower content of polymerization inhibitor in this batch of monomer. To slow down the polymerization process, it was carried out at pH 2 instead of pH 3. A decrease in the pH of polymerization medium was previously demonstrated to decrease the size and to increase the size distribution of polycyanoacrylate nanoparticles (25). Laser light-scattering measurements showed that the size of PIHCA nanoparticles coated with CTAB and/or with poloxamer 188 was significantly larger (Table II). The size of PIHCA nanoparticles loaded with $pd(T)_{16}$ was not significantly different from that of unloaded nanoparticles (Table II). A mean value of 160 ± 50 nm was determined.

In Vitro Protection of Oligonucleotides Adsorbed to Nanoparticles Against Degradation by Nucleases

The potentiality of polycyanoacrylate nanoparticles to protect oligonucleotides against degradation by snake venom phosphodiesterase was tested. This enzyme (3′-exonuclease) hydrolyzes the phosphate linkage of oligonucleotides from their 3′ end. Since oligonucleotides were ^{32}P-labeled at their 5′ end, degradation products migrate as individual bands on polyacrylamide gel and can be visualized by autoradiography (Fig. 8, I). Quantification of oligonucleotide degradation was carried out as described under Methods. Results are plotted in Fig. 8, II. It can be seen that 50% of $d(T)_{16}$ (0.5 μ*M*) was degraded in 15 min at a phosphodiesterase concentration of 0.03 μg/ml. No difference was observed in the enzymatic digestion of oligonucleotides when experiments were performed in the presence of unloaded polycyanoacrylate nanoparticles (0.5 mg/ml) (results not shown). An increase in the enzyme concentration by a factor of 20 (0.6 μg/ml) was necessary to degrade 50% of $d(T)_{16}$ when the latter were incubated in the presence of CTAB (150 μ*M*) (Fig. 8, II). Finally, oligonucleotides adsorbed on PIHCA nanoparticles (0.5 mg/ml) in the presence of CTAB (150 μ*M*) were completely protected, even at enzyme concentrations up to 5 μg/ml. At this enzyme concentration $d(T)_{16}$ complexed with CTAB alone was completely digested (Fig. 8, II).

Table II. Size of PIHCA Nanoparticles, Stabilized with Poloxamer 188, Empty or Loaded with $p(d)T_{16}$ in the Presence of CTAB (300 μ*M*)[a]

Conditions		
Cation	Adsorbed $Pd(T)_{16}$ (μmol/g)	Size (nm ± SD)
—	—	149 ± 45
CTAB	—	161 ± 51
CTAB	2.7	158 ± 50
CTAB	3.9	159 ± 51

[a] Conditions were as in Fig. 7.

DISCUSSION AND CONCLUSION

We have demonstrated that oligonucleotide association to polycyanoacrylate nanoparticles was possible only in the presence of hydrophobic cations, such as TPP (Fig. 1) or quaternary ammonium salts. The poor yield of oligonucleotide association without cations could be explained by the hydrophilic character of nucleic acid chains that are known to be highly soluble in water. It should be noted that the pK_a of the phosphate group, in the phosphodiester linkage, is close to 1, and consequently, all the phosphate groups of the nucleic acid chain are negatively charged at pH 7.

The inefficiency of tetraphenylboron, an anionic analogue of TPP, to achieve significant adsorption of $pd(T)_{16}$ to PIHCA nanoparticles (Fig. 1) suggested that oligonucleotide adsorption takes place via the formation of ions pairs. Ions pairs are formed by electrostatic interactions between hydrophobic cations and the negatively charged phosphate groups of the nucleic acid chain. Because of their lipophilic character, the ions pairs could adsorb onto the nanoparticle surface by hydrophobic interactions between the hydrophobic moiety of the cations (phenyl group of TPP) and the lateral alkyl chains of polycyanoacrylic polymers.

A similar mechanism of drug loading, based on the formation of ion pairs, was recently reported by Guise *et al.* (22). This work dealt with the incorporation of vidarabine during the polymerization process of PIHCA nanoparticles, in the presence of dioctylsulfosuccinate (DOSS). The authors also reported that covalent linkage of vidarabine, a nucleoside, to the PIHCA polymer occurred as a result of the alkylation of the base (adenine) during the polymerization process (22).

In our method, the adsorption of oligonucleotides to the preformed polymer allowed us to avoid chemical modifications of oligonucleotides, by reactions of the nucleic bases with the monomer during the polymerization process at low pH.

The adsorption of oligothymidylates–TPP complexes onto PIBCA and PIHCA nanoparticles was compared. As shown in Fig. 1 and Table I, a higher yield of oligothymidylate adsorption was achieved with the more hydrophobic polymer (PIHCA). Similarly, the yield of oligonucleotide adsorption to nanoparticles was correlated with the hydrophobicity of cations used to form ion pairs (Fig. 4). The low capacity of oligonucleotide adsorption to PIHCA nanoparticles obtained with the less hydrophobic cations (DTAB, TPP) could be explained by the coating of nanoparticles with poloxamer 188, needed to stabilize the polymeric suspension and which compete with the binding of the less hydrophobic oligonucleotide–cation complexes (Fig. 5). Moreover, when adsorbed to nanoparticles, the hydrophobic polyoxypropylene group of poloxamer 188 was anchored to the nanoparticle surface, whereas the hydrophilic polyoxyethylene groups remained free in the aqueous environment (24), providing a hydrophilic surface which could also decreased the affinity of oligonucleotide–cation complexes for nanoparticles surface.

The TPP affinity for PIBCA or PIHCA nanoparticles was not found to be dependent on the hydrophobic character of polymeric surfaces (Table I). Consequently, the greater

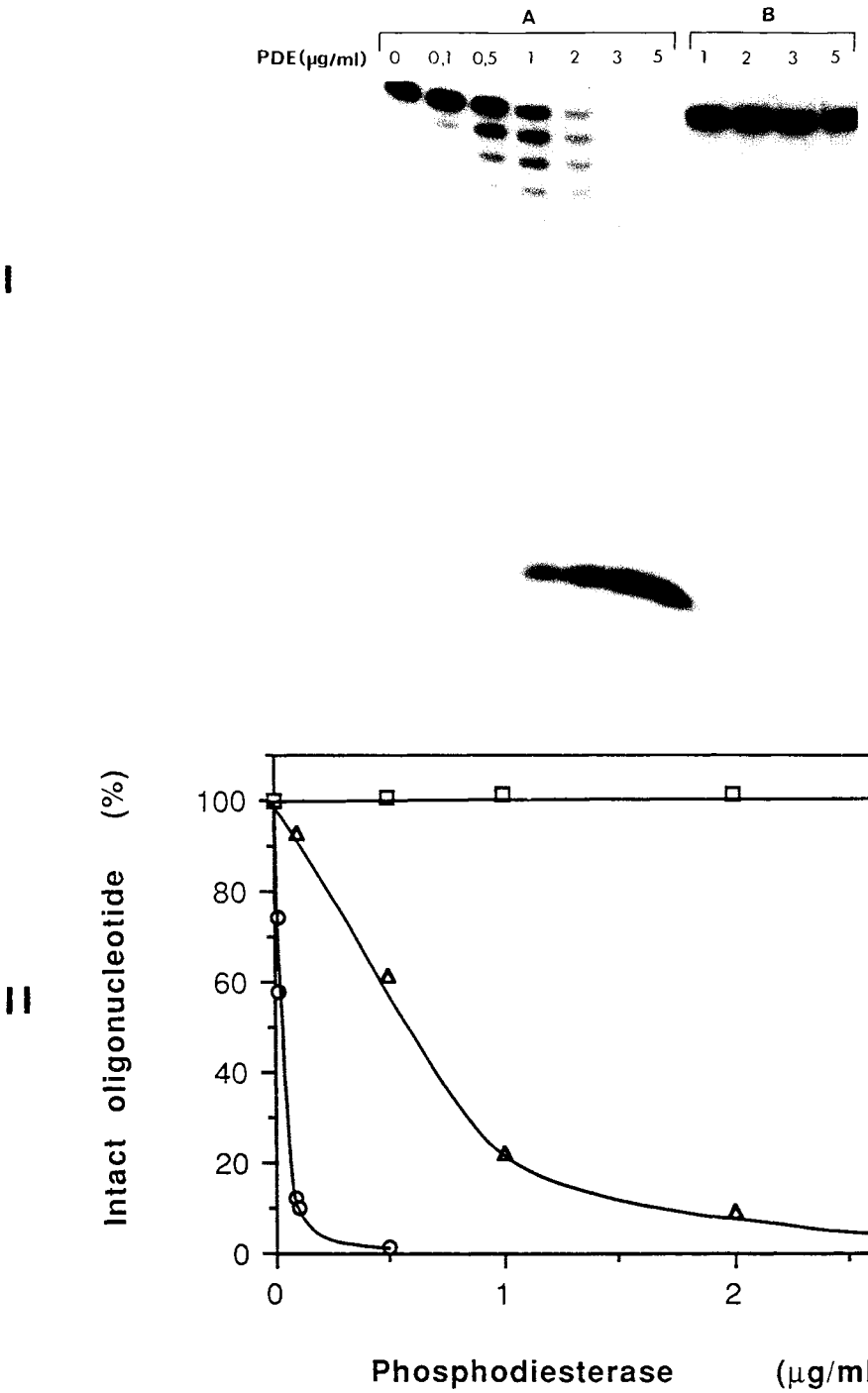

Fig. 8. *In vitro* protection of oligonucleotides adsorbed on nanoparticles against degradation by exonucleases. (I) Gel-purified, 5'-^{32}P-labeled $d(T)_{16}$ was mixed with unlabeled oligomer (0.5 μ*M*) and added to 150 μ*M* CTAB (A) or adsorbed to PIHCA nanoparticles (NP, 0.5 mg/ml; CTAB, 150 μ*M*) (B) at various concentrations of phosphodiesterase (0.01–5 μg/ml). After 15 min at 37°C, the enzymatic reaction was stopped and degradation products were analyzed on 20% polyacrylamide–7 *M* urea sequencing gel. The gels were then autoradiographed. The number above each lane corresponds to the enzyme concentration (μg/ml). (II) Quantitative analysis of oligonucleotide cleavage after cutting of bands and counting for radioactivity. Oligonucleotide $d(T)_{16}$ incubated in the absence of added cations and nanoparticles (○), in the presence of 150 μ*M* CTAB (△), and adsorbed to PIHCA nanoparticles in the presence of CTAB (□), under the conditions described for I.

affinity of oligothymidylates complexed with TPP for PIHCA nanoparticles cannot be attributed to a greater amount of TPP adsorbed on this polymer (Table I). These results suggest that oligothymidylate adsorption on nanoparticles was achieved by hydrophobic interactions between ions pairs and the polymeric surface. Nevertheless, electrostatic interactions of phosphate groups of the nucleic acid chains with cations adsorbed at the polymeric surface could be envisaged as well by the fact that TPP was able to adsorb on nanoparticles even in the absence of $pd(T)_{16}$ and with the same efficiency (result not shown). As supported by the non-Langmuirian behavior of the adsorption isotherm, a multilayer mode of TPP adsorption on nanoparticles could be envisaged. PIBCA and PIHCA nanoparticles were found to possess a similar negative surface charge (26), therefore adsorption of TPP to nanoparticles could be achieved by electrostatic interactions between cations and the negatively charged polymeric surface. A second layer could be obtained, when all the surface charges were neutralized, by intermolecular interactions between the hydrophobic moiety of TPP. Connor *et al.* (27) have reported a similar mechanism for the adsorption of cationic surface active agents on polystyrene latex particles.

Adsorption on nanoparticles of oligothymidylates complexed with hydrophobic cations depends on several parameters. The process was found to be highly dependent on the oligonucleotide chain length (Fig. 3). The sigmoïdal shape of the binding curves suggests that a minimal amount of adsorbed cations is required for the oligonucleotide to bind. This threshold value depends on the oligomer chain length and is directly correlated with the cooperative character of the adsorption process.

The yield of oligonucleotide adsorption to nanoparticles, when complexed with hydrophobic cations, was highly dependent on the ionic concentration of the medium (Fig. 6). Results showed that lipophilic ions pairs were dissociated by NaCl. This observation can be understood as a result of competition between Na^+ ions and hydrophobic cations for interaction with phosphates groups of the oligonucleotidic chain. Therefore, in order to form stable ion pairs in a medium of high ionic strength, higher concentrations of hydrophobic cations were needed.

Adsorption onto nanoparticles of oligonucleotides complexed with hydrophobic cations was found to fit with a model of adsorption on two classes of independent sites. This result was in agreement with the possibility for oligonucleotides to be bound onto nanoparticles by hydrophobic interactions between ion pairs formed in solution and the polymer and by electrostatic interactions with cations adsorbed at the polymeric surface, as previously suggested by results with TPP. Maximum adsorption of $pd(T)_{16}$ complexed with CTAB (300 μM) on PIHCA nanoparticles was found to be 5 μmol/g in the presence of 150 mM NaCl. Assuming an average particle size of 160 nm and a polymer density of 1.01 g/cm^3, the nm/g of polymer is calculated to be $4.6 \cdot 10^{14}$, which translates to an average of 6500 molecules of $pd(T)_{16}$ adsorbed at the polymeric surface of one nanoparticle.

Using unilamellar liposomes as drug carriers, an oligonucleotide of similar oligomer chain length (15 nucleotides) was encapsulated at the concentration of 2.5 mM (15). The carrier capacity of these liposomes of 200-nm size was found to be comparable to that of nanoparticles; approximatively 5600 molecules of oligonucleotides can be entrapped in the internal aqueous volume of these liposomes, which was calculated to be $3.7 \cdot 10^{-18}$ liter. Nevertheless, it should be noted that in order to obtain such a carrier capacity with liposomes, high concentrations of oligonucleotides were required in the bulk aqueous volume, thus leading to an encapsulation efficiency of only 3% of the aqueous phase.

Concerning the use of conventional nonmodified oligonucleotides, it was of interest to investigate their protection against degradation by nucleases when the oligonucleotides are adsorbed onto nanoparticles. We have shown that oligonucleotides could be protected against enzymatic hydrolysis when they were complexed with CTAB (Fig. 8, II). This result suggests that the phosphate linkage can be protected from enzyme attack by electrostatic interactions between hydrophobic cations and phosphate groups of the oligonucleotide chain. In addition, the inactivation of enzymes by interaction with hydrophobic cations can likewise be envisaged. At phosphodiesterase concentrations where oligonucleotides complexed with CTAB were completely digested, efficient protection of oligonucleotides adsorbed onto nanoparticles was achieved (Fig. 8, II). Because they escape the enzyme degradation, one might hypothesize that oligonucleotide chains are entirely adsorbed at the polymeric surface with internal phosphate linkages or the 3′ end being masked by hydrophobic cations. These results show that nanoparticles can be considered as convenient carriers for the protection and delivery of oligonucleotides in cell culture. Experiments are in progress in our laboratory to test the capacity of these carriers to promote the penetration of oligonucleotides into cells.

A number of clinically important viruses can be inhibited in culture by antisense oligonucleotides and various oncogenes have been suppressed with promising effects on cell growth and differenciation (1,2). The *in vivo* efficacy of modified oligonucleotides against viral infection in mice was demonstrated (2). Nevertheless, the therapeutic potential of oligonucleotides *in vivo* remained limited because of their distribution and clearance following administration to whole animals. Nonmodified oligonucleotides were rapidly cleared from the blood and degraded after intravenous injection into rabbits (29). Adsorption of oligonucleotides to nanoparticles could be used to protect them against nucleases present in the blood, slow down their clearance from blood, and increase their bioavailability.

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